Altered serotonergic neurotransmission as a main player in the pathophysiology of Alzheimer’s disease: structural and ultrastructural studies in a triple transgenic mouse model of the disease.

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Abstract

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative pathology that deteriorates cognitive function including learning and memory. AD is characterised neuropathologically by the presence of neuritic plaques (Aβ), neurofibrillary tangles (NFTs), synaptic loss and neuronal death. AD affects specific brain regions involved in mnestic function such as the neocortex and the hippocampus. The dorsal (DR) and the median raphe (MR) nuclei give rise to serotonergic (5-HT) projections that innervate multiple brain regions including the cortex and the hippocampus, playing an important role in learning and memory processes. For a long time the degeneration of cholinergic (ACh) system was considered as the main neurochemical changes in AD brains, however, more recent studies highlight the involvement of other neurotransmitter systems including 5-HT. This thesis entitled “Altered serotonergic neurotransmission as a main player in the pathophysiology of Alzheimer’s disease: structural and ultrastructural studies in a triple transgenic mouse model of the disease” demonstrates that there exist specific alterations in the serotonergic projections of the hippocampus during the progression of AD using the triple transgenic (3xTg-AD) mouse model of the disease, which closely resemble human AD. Mr. Harun N. Noristani is submitting this thesis to the University of Manchester for the degree of PhD in the Faculty of Life Science.

The results obtained in this thesis show for the first time a biphasic increase in serotonergic fibre sprouting in the 3xTg-AD mouse model of AD that occurs in parallel with evident intraneuronal/extracellular Aβ deposition in the hippocampus (Chapter 3). In addition, serotonergic fibre sprouting correlated with reduced perforated synapses in the hippocampus, suggesting a structural remodeling process to maintain hippocampal connectivity (Chapter 4). Increased 5-HT neurotransmission (via high dietary intake of tryptophan, 5-HT precursor) reduced intraneuronal Aβ accumulation in the hippocampus, suggesting a direct role of 5-HT neurotransmission in modifying AD neuropathology (Chapter 5). Given the protective role of increased 5-HT neurotransmission, treatment with 5-HT enhancing drugs may be beneficial in reducing the underlying pathology as well as improving the behavioural and cognitive abnormalities associated with AD. Nevertheless, the role of specific 5-HT receptors responsible for such neuroprotective effect of 5-HT in AD awaits further research.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or quantification of this or any other university or other institute of learning.

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Research work of
Harun Najib Noristani

Supervised by:
Prof. José Julio Rodríguez Arellano
Dr. Owen T. Jones (co-supervisor)

2012

For the degree of PhD in Neuroscience
in the
Faculty of Life Science
The University of Manchester
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<td>192IgG-saporin</td>
<td>Immunoglobuline G-saporine</td>
</tr>
<tr>
<td>[Ca(^{2+})]_i</td>
<td>Cytosolic calcium</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td>Triple transgenic mouse model of AD</td>
</tr>
<tr>
<td>5,7-DHT</td>
<td>5,7-dihydroxytryptamine</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>5-HT-IR</td>
<td>5-HT immunoreactive</td>
</tr>
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<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>5-HTPDC</td>
<td>5-hydroxytryptophan decarboxylase</td>
</tr>
<tr>
<td>5-HTR</td>
<td>5-hydroxytryptamine receptor (serotonin receptor)</td>
</tr>
<tr>
<td>5xTg</td>
<td>Quintuple-transgenic</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino)-tertralin</td>
</tr>
<tr>
<td>A</td>
<td>Amygdala</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AD-B</td>
<td>Alzheimer’s disease patients with behavioural symptoms (irritability, apathy and delusion but without depression)</td>
</tr>
<tr>
<td>AD-D</td>
<td>Alzheimer’s disease patients with depression</td>
</tr>
<tr>
<td>ADL</td>
<td>Activity of daily living</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>aMCI</td>
<td>Amnesic mild cognitive impairment</td>
</tr>
<tr>
<td>AMPA</td>
<td>Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AnC</td>
<td>Anterior singulate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aq</td>
<td>Aqueduct</td>
</tr>
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<td>AT</td>
<td>Axon terminal</td>
</tr>
<tr>
<td>Au</td>
<td>Autoradiography</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta (beta amyloid)</td>
</tr>
<tr>
<td>Aβ*56</td>
<td>Soluble Aβ aggregate</td>
</tr>
<tr>
<td>Aβ1-40</td>
<td>Non-amyloidogenic amyloid beta</td>
</tr>
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<td>Aβ1-42</td>
<td>Amyloidogenic amyloid beta</td>
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<td>Raphe pallidus</td>
</tr>
<tr>
<td>B2</td>
<td>Raphe obscurus</td>
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<td>B3</td>
<td>Nucleus raphe magnus</td>
</tr>
<tr>
<td>B4</td>
<td>Nucleus pontins central oralis</td>
</tr>
<tr>
<td>B5</td>
<td>Median raphe nucleus</td>
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<tr>
<td>B6-B7</td>
<td>Dorsal raphe nucleus</td>
</tr>
<tr>
<td>B8</td>
<td>Caudal linear nucleus</td>
</tr>
<tr>
<td>B9</td>
<td>Medial lemniscus</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BF</td>
<td>Beaded fibres</td>
</tr>
<tr>
<td>bFGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>BM</td>
<td>Barnes maze test</td>
</tr>
<tr>
<td>Bmax</td>
<td>Density</td>
</tr>
<tr>
<td>BP</td>
<td>Binding potential</td>
</tr>
<tr>
<td>BS</td>
<td>Binding study</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cortex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CA</td>
<td>Cornu ammonis (hippocampus)</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornus amonis 1</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornus amonis 2</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornus amonis 3</td>
</tr>
<tr>
<td>CA4</td>
<td>Cornus amonis 4 (hilus)</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>Cerebellum,</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>Cdk5</td>
<td>Cyclin dependent kinase 5</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>cLH</td>
<td>Congenital learned helplessness rat</td>
</tr>
<tr>
<td>CN</td>
<td>Caudate nucleus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>Caudate putamen</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin releasing factor</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSN</td>
<td>Central superior raphe nucleus</td>
</tr>
<tr>
<td>DA</td>
<td>Dopaminergic</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’diaminobenzidine</td>
</tr>
<tr>
<td>DCVs</td>
<td>Dense core vesicle</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DR</td>
<td>Dorsal raphe nucleus</td>
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<tr>
<td>DRD</td>
<td>Dorsal raphe dorsal</td>
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<tr>
<td>DRI</td>
<td>Dorsal raphe interfascicular part</td>
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<tr>
<td>DRV</td>
<td>Dorsal raphe ventral</td>
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<tr>
<td>DRVL</td>
<td>Dorsal raphe ventrolateral part</td>
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<td>DS</td>
<td>Down’s syndrome</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
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<td>EC</td>
<td>Entrohinal cortex</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>f</td>
<td>Hippocampal fissure</td>
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<tr>
<td>FAD</td>
<td>Familial form of AD</td>
</tr>
<tr>
<td>FC</td>
<td>Frontal cortex</td>
</tr>
<tr>
<td>FF</td>
<td>Fine fibres</td>
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<td>FSL</td>
<td>Flinders sensitive line rat</td>
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<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>FTDP-17</td>
<td>Frontotemporal dementia and parkinsonism linked to chromosome 17</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GDNF</td>
<td>Glia derived neurotrophic factors</td>
</tr>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>G/G_o protein</td>
<td>Guanine nucleotide-binding proteins</td>
</tr>
<tr>
<td>GP</td>
<td>Globus pallidus</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>G protein</td>
<td>Stimulatory guanine nucleotide-binding proteins</td>
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<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
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<td>H</td>
<td>Hippocampus</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<td>HF</td>
<td>Hippocampal formation</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HTrP</td>
<td>High TrP diet</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>ST</td>
<td>Striatum</td>
</tr>
<tr>
<td>STN</td>
<td>Sensory trigeminal nuclei</td>
</tr>
<tr>
<td>Su</td>
<td>Subiculum</td>
</tr>
<tr>
<td>SuCo</td>
<td>Superior colliculus</td>
</tr>
<tr>
<td>Ss</td>
<td>Area density</td>
</tr>
<tr>
<td>SVs</td>
<td>Synaptic vesicles</td>
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<td>SYN-IR</td>
<td>Synaptophysin immunoreactivity</td>
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<tr>
<td>T</td>
<td>Thalamus</td>
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<tr>
<td>tau</td>
<td>Tubulin and ubiquitin</td>
</tr>
<tr>
<td>TC</td>
<td>Temporal cortex</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>TMN</td>
<td>Tuberomammillary nucleus</td>
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<tr>
<td>TPH-1</td>
<td>Tryptophan hydroxylase-1</td>
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<td>TPH-2</td>
<td>Tryptophan hydroxylase-2</td>
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<td>TrP</td>
<td>L-tryptophan</td>
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<tr>
<td>TS</td>
<td>Trizma base saline</td>
</tr>
<tr>
<td>UA</td>
<td>Unlabelled axon</td>
</tr>
<tr>
<td>UD</td>
<td>Unlabelled dendrite</td>
</tr>
<tr>
<td>usp</td>
<td>Unlabelled spine</td>
</tr>
<tr>
<td>UT</td>
<td>Unlabelled axon terminal</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular dementia</td>
</tr>
<tr>
<td>VAL</td>
<td>Ventral anterior lateral nucleus of the thalamus</td>
</tr>
<tr>
<td>vDBB</td>
<td>Ventricular limb of the diagonal band of Broca</td>
</tr>
<tr>
<td>VGCCs</td>
<td>Voltage-gated Ca(^{2+}) channels</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
</tr>
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<td>VN</td>
<td>Vagal nucleus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WB</td>
<td>Western blots</td>
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</table>
Preface

Harun N. Noristani studied BSc (Hons) in Pharmacology at Portsmouth University, UK (2003-2006). He developed a specific interest for Neurosciences since the Second year of his Bachelor degree (2005), when he had received a Medical Research Council (MRC) Summer Student Grant to work on “Animal model of depression: The effect of chronic administration of selective serotonin re-uptake inhibitor (SSRI; sertraline) on 8-OH-DPAT-induced hypophagia in rats”.

Following his undergraduate studies, Mr. Noristani decided to pursue a scientific career, which brought him to the completion of a M.Sc. in Neuroscience at the Institute of Psychiatry at Kings College University, London, UK. During his M.Sc. project Mr. Noristani was involved in a study investigating the role of Neuronatin gene in differentiation of embryonic stem cell using small inhibitory RNA (siRNA). These findings have been recently published in journal of Stem cells (2010).

In September 2007 Mr. Noristani started his Ph.D. in Neurodegenerative disease in particular Alzheimer’s disease, at the University of Manchester, UK under the supervision of Prof. José. J. Rodríguez Arellano. His Ph.D. project had primarily focused on deciphering the role of serotonin (5-HT) neurotransmission in Alzheimer’s disease (AD) during its progression using a triple transgenic (3xTg-AD) model of the disease that closely mimics the spatio-temporal evolution of the disease. His studies have included age-related changes in serotonergic innervations of the hippocampus (both at light and electron microscopic level) as a region with critical importance for mnesic functions, which is also one of the first brain regions affected in AD, after the entorhinal cortex; area in which he is also interested and collaborating with other members of his group.

Results obtained from the Mr. Noristan’s project led to 2 publications as a first author (see list below) in the European Journal of Neuroscience (2010) and Cell Death and Disease (2011). In parallel, Mr. Noristani, as middle author, also has importantly contributed to writing of a review that is currently under revision in journal of Progress in Neurobiology, covering in depth the role and relevance of 5-HT in cognition process and its alterations throughout progression of AD. More recent studies from his project involved uncovering the role of altered 5-HT neurotransmission (through dietary intake of 5-HT precursor L-tryptophan) underlying AD neuropathology in order to positively modulate 5-HT transmission and its role in cognitive processes (manuscript in preparation).

Throughout his Ph.D. project, Mr. Noristani has also actively participated in other related studies, which have resulted in additional published manuscripts (see list below) including: (i) the effect of environmental manipulation such as voluntary wheel running and enrichment on hippocampal neurogenesis in 3xTg-AD animals, and (ii) astrogial cytoskeleton and functional alterations in Alzheimer’s disease and (iii) microglial alterations in 3xTg-AD throughout the progression of disease.


Note: Publications in red are part of this PhD research work
Chapter 1

General Introduction
1.1. INTRODUCTION

1.1.1. Alzheimer’s disease

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative disease that constitutes the most common cause of dementia in the elderly population (Braak & Braak 1991). The disease was first described in 1906 and published in 1907 by the German psychiatrist and neuropathologist Alois Alzheimer (14 June 1864 – 19 December 1915) (Alzheimer 1907). Alois Alzheimer observed the disease in a 51 year old woman named Auguste D (presented to him on 1901) who was suffering from memory deficits, paranoia and having difficulty in speaking and understanding what was said to her (Alzheimer 1907) (see also Fig. 1.1). Initially, Alois Alzheimer described the disease as dementia praecox, which was several years later named as Alzheimer’s disease by his colleague and friend Emil Kraepelin (Kraepelin 1910). Following the death of Auguste D (8 April 1906, aged 55), Alois Alzheimer carried out detailed histo-pathological examination of her brain and observed what he described as “miliari foci” spread around the cortex and degenerative neurones containing bundles of “fibrils” (Alzheimer 1907). Nowadays, these neuropathological hallmarks are named senile plaques (also called neuritic plaques) and neurofibrillary tangles (NFTs), respectively. The neuritic plaques are formed by the abnormal processing and accumulation of the intraneuronal beta amyloid (Aβ) protein and its subsequent release and deposition into neuropil (Masters et al. 1985). Aβ plaques accumulate in the brain parenchyma as well as the wall of blood vessels (causing what is known as congophilic angiopathy). In fact, the cerebral congophilic angiopathy may support AD diagnosis (Thal et al. 2008). NFTs are intraneuronal inclusions and appear following anomalous hyperphosphorylation of the tau protein (a fundamental microtubule associated protein (MAP) that constitutes the neuronal cytoskeleton) (Duyckaerts et al. 2009), which are primarily found in neuronal somata, apical dendrites and long axons (Gotz et al. 2004a).

In the brain, AD-related alterations begin with an initial degeneration of acetylcholine (ACh)-ergic neurones in the nucleus basalis of Meynert (nbM) and the septum. This degenerative process continues with the accumulation of
intraneuronal Aβ followed by the formation of extracellular Aβ plaques and intracellular NFTs as well as synaptic loss and eventual neuronal death (Mesulam et al. 2004). Synaptic malfunction and loss of synapses occur prior to the development of Aβ plaques and NFTs, which are directly related with a major deterioration in synaptic strength and synaptic plasticity including long-term potentiation (LTP) (Selkoe 2002; Scheff et al. 2006). AD-associated alterations are particularly evident in brain regions involved in cognition including the entorhinal cortex (EC), the hippocampus, the basal forebrain, the amygdala and the frontal cortex (Yankner 1996).

Figure 1.1. Clinical and pathological description of AD. Extracts from Alois Alzheimer’s record on Auguste D, written on the 26th of November 1901 (A). Actual photomicrograph of a neurofibrillary tangle taken from Alzheimer’s pathological preparation from Auguste D’s brain, first described by Alois Alzheimer on 1906 and published on 1907 (B). Original drawings of Alois Alzheimer’s illustrating the presence of neurofibrillary tangles in Auguste D’s brain (C). A and C adapted from (Maurer et al. 1997). B adapted from (Zilka & Novak 2006).
Epidemiological studies have described two forms of AD depending on the genetic link and the disease age onset (Blennow et al. 2006). The late-onset (also called sporadic AD, SAD) accounts for the majority of AD cases (95%), which usually affects people above 65 years of age (Kern & Behl 2009). The exact triggering cause of SAD is not known, although it is suggested to result from a combination of both biological and environmental factors (Ferri et al. 2005). The most well-known risk factor associated with SAD is old age; however, low level of formal education and reduced physical activity may also contribute as risk factors (Wang et al. 2002; Benedetti et al. 2008). In addition, the inheritance of one or two ε4 allele of the apolipoprotein E (APOE), a protein involved in Aβ clearance, is also directly linked with increased risk of the late-onset SAD (Saunders et al. 1993; Poirier 1996; Roses 1996; Martins et al. 2006).

The early-onset familial form of AD (FAD) accounts for the minority (< 5%) of AD cases. The FAD is associated with mutations in three different genes namely: the amyloid precursor protein (APP) on chromosome 21 (Goate et al. 1991), the presenilin-1 (PS-1) on chromosome 14 (Sherrington et al. 1995) and the presenilin-2 (PS-2) on chromosome 1 (Levy-Lahad et al. 1995). A common feature between these three genetic mutations is that they all alter the proteolytic processing of the APP, which ultimately leads to an increased production of either total Aβ or of a slightly longer hydrophobic and highly toxic peptide (Aβ1-42, also known as fibrillogenic peptide) (Shastry & Giblin 1999). The FAD usually affects people between 40 – 65 years of age and is characterised with a rapid progression of the disease (Shastry & Giblin 1999). Genetic studies in the FAD have been of great importance in understanding the molecular pathology associated with AD, for review see (Bird 2008).

More than 20 different APP mutations have been identified in FAD cases including the Swedish (KM670/671NL), London (V717I), Dutch (E693Q), Flemish (A692G) and Italian (E693K) mutations (Janus & Westaway 2001; Bird 2008). Most of the known pathogenic APP mutations are located close to the major APP processing sites (Hardy 1997), either adjacent to the Aβ domain (the β and γ-secretase sites) or within the Aβ domain itself (the α-secretase site) (see Fig. 1.2). Mutations near the β-site, such as the Swedish (KM670/671NL) mutation, increase total levels of both
the Aβ_{1-40} and the Aβ_{1-42} peptides (Citron et al. 1992). Mutations close to the γ-sites, such as the London (V717I) and Florida (I716V) mutations, selectively enhance the production of the fibrillogenic Aβ_{1-42} peptides (Goate et al. 1991). The Dutch (E693Q), Flemish (A692G) and Italian (E693K) mutations are all located within the Aβ sequence of the APP (Fig. 1.2). These mutations are believed to cause Aβ accumulation by augmenting aggregation or protofibril formation (Demeester et al. 2001; Murakami et al. 2002).

The PS-1 and PS-2 proteins form the enzymatic segment of the γ-secretase enzyme, which is involved in the cleavage of the APP and produces two soluble proteins i.e. (i) the p3 and p6 proteins via the non-amyloidogenic pathway; or (ii) the p6 and fibrillogenic Aβ_{1-42} through the amyloidogenic pathway (Esch et al. 1990). More than 100 different mutations have been identified in the PS-1 gene, which account for 50% of all FAD cases (Larner & Doran 2006). In addition, 8 different mutations have been identified in PS-2 genes (Gotz et al. 2004a). These mutations, in both the PS-1 and the PS-2 genes, have been linked with a selective increase in the fibrillogenic Aβ_{1-42} level (Kumar-Singh et al. 2006). The majority of the fibrillar Aβ found in the neuritic plaques are composed of the hydrophobic Aβ_{1-42} deposits (Jarrett et al. 1993). Mutations in the PS-1 gene causes the most aggressive form of AD, in some cases with an onset younger than 29 years of age (Golan et al. 2007). Selective aggregation of the fibrillogenic Aβ_{1-42} and an early disease onset in the PS-1 and the PS-2 mutations trigger a rapid neurodegenerative process in patients with FAD (Golan et al. 2007).

The clinical diagnosis of AD depends on the outcome of several investigations including cognitive testing, neurological investigation, medical history and imaging analysis. The main neuropsychological features associated with AD include cognitive deficits, learning impairment, memory dysfunction and language abnormalities, as was first recorded by Alois Alzheimer (Fig. 1.1) (Alzheimer 1907; Braak & Braak 1991; Braak et al. 1999; Harciarek & Jodzio 2005). Memory encoding and memory storage is impaired in AD as depicted by patients having troubles with both short- and long-term memory processes (Knopman et al. 2003). Another aspect of memory disturbance in AD patients includes the loss of newly learned verbal information (Welsh et al. 1992; Kaltreider et al. 2000). Patients with
AD also exhibit communication difficulties (Harciarek & Jodzio 2005). During AD progression, the deficit in memory function extends to other domains such as language (aphasia), skilled movement (apraxia), recognition (agnosia) and executive function including planning and decision making (Talwalker 1996). Patients with AD also display behavioural disturbances such as agitation, irritability, anxiety, delusion and depression (Lyketsos & Olin 2002).

**Figure 1.2.** The major pathogenic APP mutations found in FAD. Note: Highlighted in red are the mutated amino acid residues. The area highlighted by red line illustrates the Aβ sequence, which encompasses the Dutch (E693Q), Flemish (A692G) and Italian (E693K) mutations. Adapted from (Hardy, 1997).
1.1.1.1. Pathological hallmarks of AD

Despite the presence of neuropsychological symptoms, post-mortem neuropathological examination of the brain is critical for the ultimate confirmation of AD diagnosis (Coll et al. 2003). Macroscopic alterations associated with AD include cerebral atrophy, dilation of the ventricular system and shrinkage of the hippocampus (Chen et al. 2000b) (see also Fig. 1.3).

**Figure 1.3.** Macroscopic alterations of AD brain compared to healthy control brain. Adapted from (http://www.kickoff.net.au/Neurological-Illnesses,-Disorders-&-Disease.html)

Microscopic alterations associated with AD include the presence of the two major histological hallmarks namely: the Aβ neuritic plaques and NFTs as well as angiopathy (Braak & Braak 1991). Aβ plaques initially become evident in the neocortical area of the temporal lobe and orbitofrontal cortex, which then progressively spread towards the parietal cortex and finally throughout the entire neocortex (Braak & Braak 1991). However, NFTs first appear in the medial
temporal lobe including the EC and the hippocampus, which then progressively spread to the limbic area and the primary cortex (Braak & Braak 1991).

More specifically, the Braak-staging is the most commonly used method for describing the evolution of Aβ plaques and NFTs in AD brains (Braak & Braak 1991). The evolution of Aβ plaques in AD brains is divided into three stages (stage A – C) (Braak & Braak 1991) (Fig. 1.4B). Stage A is characterised by the low density of Aβ aggregates in the basal portion of the frontal cortex, the temporal cortex and the occipital cortex but not in the hippocampus. During stage B, there is a progressive increase in Aβ accumulation in the mentioned cortical area as well as the hippocampus (Braak & Braak 1991). Stage C is characterised by a widespread and densely packed Aβ plaque deposits throughout the cortex and other sub-cortical structures including the striatum, the thalamus, the hypothalamus and the hippocampus (Braak & Braak 1991) (Fig. 1.4B).

The evolution of NFTs in AD brains is divided into six Braak stages (Braak stage I – VI) (Braak & Braak 1991) (Fig. 1.4C). Braak stages I and II are characterised by mild to severe build-up of NFTs in the trans-entorhinal region, which is located between the entorhinal region and the adjoining temporal cortex. There is a modest density of NFTs in the hippocampus but not in the cortex during Braak stage II (Braak & Braak 1991). At Braak stages III and IV, there is a progressive build-up of NFTs in the EC, trans-entorhinal regions as well as the hippocampus. Only low densities of NFTs become evident in the cortex during Braak stage III and IV (Braak & Braak 1991). Braak stages V and VI are characterised by a pronounced spread of NFTs in the hippocampus and the cortex (Braak & Braak 1991) (Fig. 1.4C). In addition, there are severe losses of cortical and hippocampal neurones during Braak stage V and VI (Braak & Braak 1991). Although the evolution of Aβ plaques varies between different AD brains, NFT formation displays a more uniform distribution pattern in all AD brains throughout different stages of disease progression (Braak & Braak 1991).
Figure 1.4. The evolution of Aβ plaques and NFTs in AD brains. Computer model of the human brain showing some of the cortical and sub-cortical structures (A). Evolution of Aβ plaques (B) and NFTs (C) at different Braak stages in AD brains. Increasing density of shading indicates progressive increase in Aβ deposits (B) and NFT accumulation (C).
A from (http://www.sciencephoto.com/media/133795/enlarge), B and C from (Braak & Braak 1991).
AD-associated neurodegeneration also triggers glial alterations, which were initially mentioned by Alois Alzheimer (Alzheimer 1907). In the histological preparations of Auguste D’s brain, Alois Alzheimer observed a clear hypertrophy of astrocytes (astrogliosis) surrounding Aβ plaques, which displayed a large number of gliofilaments and a major microglial activation (Alzheimer 1907) (Fig. 1.5).

**Figure 1.5.** Drawing of Alois Alzheimer illustrating (A) “fibrils” and (B) the glial reaction (astrogliosis and hypertrophy) in a pathological brain containing Aβ plaques. Key: gaz: neurone, glz: glial cell. P₁: central part of the Aβ plaque, P₂: peripheral part of the Aβ plaque. From (Alzheimer 1910).

Other post-mortem studies, have confirmed an AD-associated astroglial hypertrophy (Beach et al. 1989), microglial activation (Shepherd et al. 2000) as well as impaired neurogenesis (Jin et al. 2004; Rodríguez & Verkhratsky 2011a). These findings are in agreement with recent studies in trangenic mouse models of AD, which also displayed multiple reactions of neuroglia from astroglial atrophy and reactive astrogliosis to activation of microglia (Rodríguez et al. 2009b; Heneka et al. 2010b; Olabarria et al. 2010; Rodríguez et al. 2010; Verkhratsky et al. 2010; Rodríguez & Verkhratsky 2011b; Yeh et al. 2011).
Finally, AD brains are also associated with increased inflammatory response (McGeer & McGeer 2001), oxidative stress (Butterfield et al. 2001; Yu et al. 2003) and mitochondrial dysfunction (Baloyannis et al. 2004; Sas et al. 2007). Accumulation of Aβ plaques plays an important role in initiating such widespread degenerative process in AD brains.

1.1.1.2. Amyloid processing and abnormal accumulation in AD

In AD brains, there are predominantly two forms of Aβ peptides with different number of amino acids i.e. the Aβ_{1-40} and the Aβ_{1-42} peptides (Selkoe 2001a; Selkoe 2001b). Although the Aβ_{1-40} peptide is the most abundant form, the Aβ_{1-42} peptide is more hydrophobic and is more likely to oligomerise and from Aβ fibrils (Jarrett et al. 1993; Younkin 1998). In fact, AD-associated neuritic plaques are primarily composed of the Aβ_{1-42} peptides, which are produced by the abnormal processing of the APP (Younkin 1998). The APP is a single transmembrane glycoprotein (consisting of 695 – 770 amino acids) that is highly expressed throughout the body and the brain, with a small cytosolic C-terminal domain and a large extracellular luminal N-terminus (Gotz et al. 2004b) (Fig. 1.2 and 1.6).

The APP undergoes two alternative proteolytic cleavages called the non-amyloidogenic (normal) and the amyloidogenic (pathological) pathways, with the former being the major APP processing path in most cell types (Esch et al. 1990) (see also Fig. 1.6)

In the non-amyloidogenic pathway, the enzymatic cleavage of the APP takes place within the Aβ domain itself (the α-secretase site), which precludes the formation and release of the intact Aβ peptide (Nunan & Small 2000) (Fig. 1.2, see also Fig. 1.6, left). The APP is cleaved by the α-secretase at a position that is 83 amino acids away from the carboxyl C-terminus, which results in the secretion of a soluble extracellular N-terminal fragment of APP (sAPPα) and a membrane-bound 83-amino-acid residue carboxyl C-terminal fragment (C83) (Esch et al. 1990). The C83 fragment undergoes further enzymatic cleavage by the γ-secretase and generates two soluble proteins: the p3 (a short peptide, which is unable to form aggregate) and the p6 proteins (the intracellular domain of the APP, Fig. 1.6, left) (Esch et al. 1990).
Figure 1.6. APP processing in AD. The non-amyloidogenic processing of APP is mediated via α- and γ-secretases (left), which generate two soluble proteins i.e. the p3 and p6 with no intraneuronal Aβ aggregates (A). The amyloidogenic processing of APP via β- and γ-secretases (right) results in neurotoxic accumulation of intraneuronal Aβ (B) that is subsequently released to the extracellular space and may contribute to the build-up of extracellular Aβ deposition and neuritic plaque formation (C). Adapted from (Noristani et al. 2012).

The amyloidogenic processing of APP, which is associated with AD, involves its proteolytic cleavage by the β- and γ-secretase enzymes (Fig. 1.6, right). The APP undergoes cleavage at a position that is located 99 amino acids away from the C-
terminus (β-site) by the β-secretase, which generates a soluble extracellular fragment of the APP (SAPPβ) and a membrane-bound 99-amino-acid C-terminal (C99) fragment (Esler & Wolfe 2001). The C99 fragment undergoes further cleavage by the γ-secretase enzyme, which releases the p6 and the Aβ peptide (38 – 43 amino acids in length) (Fig. 1.6, right). The majority of the Aβ1-42 peptide is generated in the plasma membrane that is then released to the extracellular space and contributes to the build-up of extracellular Aβ plaques (LaFerla et al. 2007).

Intraneuronal Aβ immunoreactivity precedes extracellular Aβ plaque depositions in AD-associated vulnerable brain regions including the EC and the hippocampus (Gouras et al. 2000), suggesting that intraneuronal Aβ deposition is an early event in the pathogenesis of the disease. Studies in different transgenic mouse model of AD have also shown that intraneuronal Aβ deposition appears as an early event before the deposition of extracellular Aβ plaques (Wirths et al. 2001; Oddo et al. 2003a; Oakley et al. 2006; Knobloch et al. 2007). Intraneuronal Aβ deposition triggers the initial pathological progression in AD brains such as disrupting the proteasome and mitochondria function, triggering calcium (Ca^{2+}) influx and synaptic dysfunction (LaFerla et al. 2007; Wirths et al. 2009).

Following the intraneuronal Aβ deposition, the neurone starts to secrete Aβ into the extracellular space, hence contributing to the build up of extracellular Aβ plaques in AD brains (Kang et al. 1987). The extracellular Aβ plaques may originate, at least partially, from the intraneuronal Aβ aggregates (LaFerla et al. 2007). Reduced extracellular Aβ plaques (by Aβ immunotherapy) also cleared intraneuronal Aβ deposition in the triple transgenic (3xTg-AD) mouse model of AD (Oddo et al. 2004). Following cessation of Aβ immunotherapy and re-emergence of Aβ pathology, the reappearance of extracellular Aβ plaques occurred after the intraneuronal accumulation of Aβ (Oddo et al. 2006), suggesting that the AD-associated intraneuronal Aβ aggregates may act as a source for some of the extracellular Aβ plaques. Although the precise mechanism is not clearly defined, excessive intracellular accumulation of Aβ1-42 peptide in the cortical and hippocampal pyramidal neurones have been shown to trigger cell lysis, which resulted in dispersion of the intraneuronal Aβ1-42 aggregates into the surrounding extracellular space (D’Andrea et al. 2001).
The formation of extracellular Aβ plaque is a complex multi-step process where the individual Aβ monomers undergo conformational changes into insoluble β-pleated sheet secondary structures, which in turn render them more prone to form aggregates and mature into large insoluble Aβ fibrils found in neuritic plaques (Jarrett et al. 1993; Younkin 1998; Fryer & Holtzman 2005) (see also Fig. 1.7).

**Figure 1.7.** Amyloid deposition in AD. At early stages of the disease Aβ oligomers are arranged as non-amyloid aggregates that are surrounded by activated microglia (A). At more advanced stages of the disease Aβ aggregates form neuritic plaques consisting of a central core that are composed of Aβ_{1-42} peptides (B – D). Aβ plaque deposition also triggers activation of microglia (B) and astrocytes (C) as well as aberrant axonal sprouting (D). A and B from (Rodríguez et al. 2010), C from (Olabarria et al. 2010), D from (Rodríguez et al. unpublished confocal micrograph).
There are two types of Aβ aggregates found in AD brains i.e. diffuse plaques and senile/neuritic plaques. Diffuse plaques (also known as pre-amyloid plaques) are lower deposits of Aβ peptides that do not have amyloid core structure and may represent an early stage of the extracellular Aβ deposition (Dickson 1997) (see also Fig. 1.7A). In diffuse plaques, individual Aβ monomers are arranged as non-amyloid aggregates but there are no conformational changes into β-pleated sheet secondary structures (Tagliavini et al. 1988). Classical senile/neuritic plaques are spherical structures (between 50 – 200μm in diameter) that have an evident amyloid core enriched with Aβ1-42 peptide aggregates (Iwatsubo et al. 1994) (see also Fig. 1.7B, 1.7C and 1.7D). Extracellular Aβ plaque deposition causes severe homeostasis disruption and triggers neurotoxic damage to the surrounding neuropil. As a result, Aβ neuritic plaques are surrounded by reactive astrocytes and microglia as well as dystrophic neurities (axons and dendrites) (Geddes et al. 1986; Mandybur & Chuirazzi 1990; Shepherd et al. 2000) (see also Fig. 1.7B and 1.7C). Aβ plaque deposition has been also associated with aberrant axonal sprouting (Masliah et al. 1991; Noristani et al. 2010) (see also Fig. 1.7D). It is not clear how long it takes for the development of Aβ plaques in human brains, but it is likely that it is a long process that may take years. However, there is evidence that Aβ plaque formation in the neocortex occurs relatively early during the pathogenesis of AD (Morris & Price 2001; Ingelsson et al. 2004).

1.1.1.3. The Amyloid cascade hypothesis of AD
As mentioned previously, AD is a multi-factorial disease involving both environmental and genetic factors. Over the years many theories have been proposed to describe the pathogenesis of AD (Delacourte and Buee, 2000; Gotz et al. 2004a; Thal et al. 2002). The 'amyloid cascade hypothesis' combines histopathological and genetic information, which links Aβ deposition, both inside the neurone and in the form of extracellular Aβ plaque, with the pathological processes associated with AD (Wirths et al. 2004). The 'amyloid cascade hypothesis' highlights that it is the gradual aggregation of Aβ that triggers the degenerative cascades of events in AD brains including synaptic impairment and neuronal loss as well as inflammation and glial activation (Fig. 1.8). Such degenerative events cause severe atrophy of affected brain regions leading to cognitive failure in AD patients (Wirths et al. 2004).
The Amyloid cascade Hypothesis of AD

Risk factors: ageing, Trisomy 21, APP-, PS1-, PS2-mutations

Increased levels of intraneuronal $A\beta_{40/42}$

*Figure 1.8. Schematic diagram displaying pathogenic progression in AD. Multiple factors including ageing, Down syndrome as well as mutations in the APP, PS-1 and PS-2 genes contribute to the increase of intraneuronal $A\beta_{40}$ and $A\beta_{42}$ levels, leading to synaptic and neuronal dysfunction as well as glial alteration and degeneration in specific brain regions involved in cognitive functions. In parallel, increased secretion and deposition of $A\beta_{42}$ contributes to the extracellular $A\beta$ plaque formation and deposition as well as astrogial hypertrophy and microglial activation. Based on (Wirths et al. 2004).*
There are several lines of evidence that support the link between Aβ deposition and degenerative events associated with AD.

- Autosomal dominant mutations in the APP, PS-1 and PS-2 genes linked with the FAD cases increase Aβ production by favouring the enzymatic cleavage of the APP through the amyloidogenic pathway (Gotz et al. 2004a; Gotz et al. 2004b).
- The gene for the APP is located in chromosome 21, which is triplicated in individuals with Down’s syndrome, therefore leading to over-production of Aβ and the development of “AD-like” neuropathology (Motte & Williams 1989).
- Transgenic mice with increased Aβ production display deficits in memory and cognitive functions as seen in AD patients (Oddo et al. 2003a; Oddo et al. 2003b).
- Application of Aβ peptide induces a toxic effect on cortical neurones (Pike et al. 1991; Busciglio et al. 1992; Hartley et al. 1999).

Although the 'amyloid cascade hypothesis' suggest that Aβ is the triggering event in AD-associated degenerative processes, the underlying mechanisms responsible for Aβ-mediated toxicity are not conclusively clear (Karran et al. 2011). Shankar and colleagues (2008) have shown that soluble Aβ oligomers, extracted from the cortex of AD brains, potently impaired LTP and reduced dendritic spine density in normal mice hippocampus as well as disrupted memory of a learned behaviour in normal rats (Shankar et al. 2008). Interestingly, the insoluble Aβ plaque cores did not impair LTP unless they were first dissolved to release the Aβ dimmers (Shankar et al. 2008). Others also have reported that intraventricular injection of a 56-kDa soluble Aβ aggregate (Aβ*56) impaired spatial memory (measured using Morris water maze, MWM) in cognitively normal rats (Lesne et al. 2006). Taken together, these data suggest that Aβ plaque cores are largely inactive but may sequester Aβ dimmers that are synaptotoxic and may contribute to cognitive deficits associated with AD.
Whilst early studies supported the 'amyloid cascade hypothesis' of AD, more recent studies suggest that there is no direct correlation between Aβ deposition in form of neuritic plaques and dementia in AD patients (Price et al. 2009). In addition, the 'amyloid cascade hypothesis' does not explain why many apparently healthy individuals start to accumulate Aβ in the brain upon normal ageing (Funato et al. 1998). Furthermore, several therapeutic strategies to reduce Aβ production or aggregation have failed in their clinical outcomes to reverse the cognitive decline associated with AD (Hardy 2009; Saxena 2010).

1.1.1.4. Tau protein alteration and hyperphosphorylation in AD

The morphology of a neurone is maintained by its cytoskeleton protein organisation. Microtubules are the main cytoskeleton proteins that maintain neuronal morphology and function as tracks for axonal transport of synaptic vesicles (Fig. 1.9). Tau is a microtubule-associated protein that is exclusively found in higher eukaryotes (Goedert et al. 1989). Tau protein is primarily expressed in neurones and it is involved in microtubule assembly and stabilisation of neuronal cytoskeleton (Goedert et al. 1988; Novak et al. 1991) (see also Fig. 1.9). Within neurones, tau protein is mainly located on the microtubule surface, where it binds to microtubules and stabilises microtubule assembly; hence regulating numerous cellular processes including (i) axonal structure and growth, (ii) transport of cellular organelles and synaptic vesicles via axons as well as nervous (iii) signal propagation along the nerve network formed by microtubules (Avila et al. 2004).

Tau protein is encoded by a single gene located in chromosome 17 in humans, which gives rise to 6 neurone-specific splice variants (Neve et al. 1986). Tau protein consists of numerous serine, threonine and proline resides that are phosphorylated by different kinase enzymes (Gotz et al. 2004a). Under physiological conditions, tau protein undergoes constant phosphorylation (by different kinase enzymes) and dephosphorylation (by different phosphatase enzymes), which regulates tau binding to microtubules, stabilises microtubule polymers and provides a mechanism for controlling cytoskeleton stability (Avila et al. 2004) (Fig. 1.9). Physiological phosphorylation of tau protein decreases tau's affinity for microtubules, which is critical for microtubule polymerisation during development and axonal growth (Geschwind 2003).
In AD brains, there is an abnormal hyperphosphorylation of tau protein leading to the formation of intraneuronal aggregate called NFTs (Burghaus et al. 2000) (Fig. 1.9). Hyperphosphorylation of tau protein occurs at its microtubule-binding domain that is mediated by multiple kinase enzymes including the kinase cyclin dependent kinase 5 (Cdk5) and the glycogen synthase kinase 3 (GSK-3) (Drewes 2004). Hyperphosphorylation of tau protein dissociates it from microtubule surface and prevents its binding to microtubules, which in turn disintegrates the microtubules and triggers the collapse of the neuronal cytoskeleton (Drewes et al. 1995; Sengupta et al. 1998). Disintegration of neuronal cytoskeleton is detrimental for neuronal survival as it halts multiple tau-dependent cellular functions including the propagation of nerve signal along the axon, the transport of the vesicles and organelles along axon as well as axonal growth (Gendron & Petrucelli 2009). The dissociated hyperphosphorylated tau protein undergoes conformational changes that ultimately leads to NFT formation and neuronal death (Geschwind 2003) (Fig. 1.9).

Figure 1.9. Tau protein hyperphosphorylation and NFT formation in AD. Tau is normally located on the surface of microtubules and regulates microtubule assembly. In AD brains, tau protein undergoes hyperphosphorylation by GSK3 and Cdk5 kinases, which triggers its dissociation from the surface of microtubules leading to the destabilisation of microtubules and the collapse of axonal transport system. Unbound hyperphosphorylated tau protein form intraneuronal aggregates that accumulate into NFTs and eventually lead to neuronal death. Key: GSK-3: glycogen synthase kinase-3, Cdk5: cyclin-dependent kinase 5, NFT: neurofibrillary tangle. Modified from (Drewes 2004).

NFTs are mainly composed of filaments arranged in paired helices (also referred to as paired helical filament, PHF) (Braak et al. 1999). Hyperphosphorylated tau
protein, purified from AD brains, sequesters normal tau protein, microtubule associated protein 1 (MAP1), microtubule associated protein 2 (MAP2) and inhibits microtubule assembly (Iqbal et al. 2003). In addition, hyperphosphorylation of tau protein triggers degeneration of neuronal axons and dendrites leading to the loss of affected neurones (Braak et al. 1999). Increased NFTs in the cortex is associated with greater pre-mortem cognitive decline (measured using mini-mental state examination, MMSE scores) in AD patients (Nelson et al. 2007a).

The presence of NFTs have been linked with neurodegenerative processes associated with tangle-only dementia (in the absence of Aβ) that are generally referred to as ‘tauopathies’ (Lee et al. 2001). These ‘tauopathies’ dementia are due to mutations in tau gene and include sporadic corticobasal degeneration, progressive supranuclear palsy and Pick's disease as well as hereditary frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Lee et al. 2001). Studies in tau transgenic mice over-expressing the cdk5 activator (p25) with severe tangle pathology have revealed that tau-induced toxicity is evident at early stages during aberrant tau phosphorylation before NFT formation (Cruz et al. 2003; Geschwind 2003).

Taken together, the data undoubtedly support that pathologically modified tau protein causes a toxic effect on axonal transport leading to neuronal death. This is particularly important in AD where the interaction between Aβ and tau proteins may accelerate the underlying neurodegenerative processes associated with the disease.

1.1.1.5. Interaction between Aβ and tangle pathology

More recent studies support an interactive role between Aβ and tau proteins (Seyb et al. 2008). In vitro studies have shown that neuronal exposure to Aβ triggered a toxic effect by irreversible opening of a Ca²⁺ channel, increased extracellular Ca²⁺ entry into the affected neurones and elevated cytosolic calcium [Ca²⁺]ᵢ (Mattson et al. 1992; Brorson et al. 1995; Blanchard et al. 1997). The Aβ-induced increase in [Ca²⁺]ᵢ also stimulates tau phosphorylation, suggesting an interaction between the two pathological hallmarks of AD (Seyb et al. 2008).
Injection of the synthetic Aβ into the brains of tau transgenic mice accelerated tau hyperphosphorylation and increased NFT formation (Gotz et al. 2001). Crossing transgenic mutant APP mice with mutant tau mice also increased tangle pathology in the limbic system and the olfactory cortex, suggesting that the increased Aβ pathology amplifies tangle formation (Lewis et al. 2001). Studies in 3xTg-AD mice, which display both Aβ plaques and NFTs, have shown that accumulation of Aβ peptides can trigger or accelerate a retrograde signalling cascade that leads to hyperphosphorylation of tau protein leading to tau aggregation and neurotoxicity (Oddo et al. 2003a; Oddo et al. 2003b).

Concomitant expression of both Aβ plaques and NFTs trigger pronounced synaptic and neuronal loss leading to AD (Wirths et al. 2004). Such severe loss of neurones exerts a significant effect on the neurochemistry of the AD brains.

1.1.1.6. Neurochemical alterations in AD
Traditionally, the primary role in AD pathology was assigned to degeneration of cholinergic (ACh) neurones and an overall decrease in the activity of choline acetyltransferase (ChAT, the enzyme responsible for ACh synthesis) (Bowen et al. 1976; Fibiger 1991; Birks & Melzer 2000; Eglen et al. 2001; Caccamo et al. 2006). Other neurotransmitter systems, however, are also involved in cognitive processes and undergo pathological remodelling in AD brains (Nazarali & Reynolds 1992; Dringenberg 2000; Lai et al. 2002; Garcia-Alloza et al. 2005; Grudzien et al. 2007). More recent studies have shown that AD is a complex neurodegenerative disease involving multiple neurotransmitter systems such as the glutamatergic (Palmer & Gershon 1990), noradrenergic (Lyness et al. 2003), dopaminergic (Kumar & Patel 2007) and serotonergic (5-HT) systems (see section 1.5.).

1.1.1.6.1. Cholinergic alterations in AD
The nucleus basalis of Meynert (nbM) encompasses the majority of ACh neurones that provide ACh projections to the cerebral cortex in humans (Mesulam 2004). Classically, AD-related neuropathology has been closely linked with the severe damage of the ACh system with selective loss of ACh neurones in the nbM of AD brains (Whitehouse et al. 1981; Whitehouse et al. 1982; Kasa et al. 1997). Specifically, ACh neurones that project to the cortical areas with severe Aβ plaques
presence undergo neurodegeneration, suggesting that the accumulation of Aβ plaques in their projection sites contribute to the loss of ACh neurones in the nbM of AD patients (Arendt et al. 1985). The ACh neuronal loss is paralleled by a decrease in the cortical ACh fibres (Geula & Mesulam 1996) and a reduction in the activity of ChAT in AD brains (Bowen et al. 1976; Davies & Maloney 1976; Perry et al. 1977). Loss of ChAT activity was reported in multiple regions of AD brains including the cortex, the amygdala and the hippocampus (Bowen et al. 1976; Davies & Maloney 1976; Perry et al. 1977), which may be due to AD-associated loss of ACh neurones in the nbM (Whitehouse et al. 1981; Whitehouse et al. 1982).

Cortical biopsy samples from AD patients also showed reduced ACh synthesis (measured using $^{14}$C-glucose metabolism and its incorporation into ACh [$^{14}$CACH]) in vitro (Sims et al. 1980). Reduced ACh synthesis may explain the decrease in ACh levels in the temporal cortex and the frontal cortex in AD brains (measured using high performance liquid chromatography, HPLC) (Richter et al. 1980; Gil-Bea et al. 2005).

The findings of selective loss of ACh neurones, reduced AChT enzyme activity, decreased ACh synthesis and reduced ACh neurotransmitter levels in AD brains led to the development of the “cholinergic hypothesis of AD”, for review see (Bartus et al. 1985; Collerton 1986; Cummings & Kaufer 1996). Based on the ‘cholinergic hypothesis of AD’, early pharmacological therapies for AD focused primarily on increasing the ACh neurotransmission in the brain (Weinstock 1995).

The ubiquitous enzyme acetylcholinesterase (AChE) is responsible for terminating the synaptic action of ACh neurotransmitter via its catalytic hydrolysis into choline and acetate. Currently four different inhibitors of the enzyme (AChEIs, cholinesterases inhibitors) have been approved for the treatment of mild to moderate AD namely: donepezil hydrochloride (donepezil), galantamine hydrochloride (galantamine), rivastigmine tartrate (rivastigmine) and tacrine hydrochloride (tacrine) (Birks 2006; Hansen et al. 2008). All AChEIs prevent the catalytic hydrolysis of ACh in the synaptic terminals, which in turn increase the synaptic concentration of ACh, thereby enhancing or prolonging the action of the neurotransmitter on their post-synaptic targets (Nordberg & Svensson 1998; Cummings 2003). There are differences in pharmacological properties between
AChEIs, with each drug displaying diverse selectivity, AChE inhibition and bioavailability (Nordberg & Svensson 1998; Cummings 2003). Administrations of AChEIs have been shown to delay the functional decline between 5 months and 2 years in AD patients (Mohs et al. 2001; Bullock et al. 2005). However, it is important to note that AChEIs are efficient merely at the early stages of the disease and they do not attenuate the underlying neurodegeneration associated with AD (Birks & Melzer 2000).

1.1.1.6.2. Cholinergic receptor alterations in AD
There are two different types of ACh receptors: the nicotinic acetylcholine receptors (nAChRs) and the muscarinic acetylcholine receptors (mAChRs), which are further divided into different sub-types (see also Table 1.1). Multiple sub-types of neuronal nAChR can be formed from homomeric or heteromeric combinations of nine α (α2 – α10) and three β (β2 – 4β) subunits, e.g. α4β2 and α7 that are the most commonly expressed nAChR in the brain (Abreu-Villac 2011). Five sub-types of mAChRs (M1 – M5) have been identified (Table 1.1). Neuronal nAChRs are cation selective ligand-gated ion channels that are widely expressed in multiple regions of the human brain including the thalamus, the striatum, the cortex, the cerebellum and the hippocampus (Paterson & Nordberg 2000; Falk et al. 2003). On the other hand, mAChRs are coupled to G proteins that are also highly expressed throughout the human brain including the frontal cortex, the basal ganglia, the thalamus, the cerebellum and the hippocampus (Gremo et al. 1987; Schroder 1992).

Post-mortem binding studies have consistently shown an AD-associated decrease in α3, α4 and α7 nAChRs binding sites especially in the cortex and the hippocampus (measured using the nicotinic agonists [3H]epibatidine, [3H]nicotine and [3H]ACh) (Nordberg & Winblad 1986; Hellstrom-Lindahl et al. 1999; Marutle et al. 1999). Western blot analysis on post-mortem AD brains confirmed reduced α3, α4 and α7 nAChR protein levels in the temporal cortex and the hippocampus (Guan et al. 2000). Positron emission tomography (PET) studies have also shown reduction in all sub-types of the nAChRs (measured using (S)(-)^11C-nicotine binding) in the frontal cortex and the hippocampus of AD patients, suggesting that AD-associated loss of nAChR may be an early event during the course of the disease (Nordberg et al. 1995; Nordberg 2001). Similarly, binding studies also reported AD-associated

High densities of nAChRs are particularly expressed in ACh neurones of the basal forebrain, which project to the brain regions with enhanced Aβ plaque deposition in AD such as the hippocampus and the cortex (Paterson & Nordberg 2000). The Aβ$_{1-42}$ peptide display high binding affinity for the α7 nAChR that in turn facilitates the intraneuronal accumulation of Aβ by endocytosis of the Aβ$_{1-42}$-α7nAChR complex in affected neurones (Wang et al. 2000; Nagele et al. 2002). In fact, intraneuronal Aβ$_{1-42}$ aggregates predominantly occur in the hippocampal and cortical neurones that abundantly express the α7 nAChRs (Nagele et al. 2002). In vitro studies have shown that prolonged exposure to Aβ$_{1-42}$ led to toxicity particularly in neurones that highly expressed the α7 nAChRs (Wang et al. 2000; Nagele et al. 2002). Interestingly, Aβ-induced toxicity was blocked by selective activation of the α7 nAChR with other more specific agonists such as nicotine and epibatidine (Wang et al. 2000), suggesting that interaction between the α7 nAChR and Aβ peptide is a pivotal mechanism involved in the intraneuronal Aβ deposition and subsequent neurodegeneration associated with AD.
Table 1.1. Receptor sub-types of different transmitter systems in the brain.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Characteristic</th>
<th>Sub type</th>
<th>Brain region expressed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholinergic Receptor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic ACh receptors</td>
<td>Cation selective ligand-gated ion</td>
<td>Homomeric or heteromeric</td>
<td>T, C, H</td>
<td>(Paterson &amp; Nordberg 2000)</td>
</tr>
<tr>
<td></td>
<td>channels</td>
<td>combinations of 12 different</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>subunits: (α2 - α10) and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(β2 - β4) e.g. α7, α4β3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscarinic ACh receptors</td>
<td>G protein coupled receptors</td>
<td>M₁-M₅</td>
<td>FC, PC, H</td>
<td>(Abreu-Villaca et al. 2011)</td>
</tr>
<tr>
<td><strong>Glutamatergic Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionotropic receptors</td>
<td>Form the ion channel pore, which is</td>
<td>NMDA, Kainate, AMPA</td>
<td>H, Su, EC, C</td>
<td>(Wang et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>activated following glutamate binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>to the receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabotropic receptors</td>
<td>G protein coupled receptors</td>
<td>3 groups: 1 (mGluR₁, mGluR₃)</td>
<td>H, PFC, FC</td>
<td>(Di Giorgi Gerevini et al. 2004; Ghose et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (mGluR₂, mGluR₃),</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (mGluR₄, mGluR₆,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGluR₇ and mGluR₈)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Noradrenergic Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αAR</td>
<td>G protein coupled receptors</td>
<td>α₁ and α₂</td>
<td>PFC, FC, cb, H</td>
<td>(Gibbs &amp; Summers 2002)</td>
</tr>
<tr>
<td>βAR</td>
<td>G protein coupled receptors</td>
<td>β₁, β₂ and β₃</td>
<td>PFC, FC, cb, H</td>
<td>(Kalaria et al. 1989)</td>
</tr>
<tr>
<td><strong>Dopaminergic Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1 like family</td>
<td>G protein coupled receptors</td>
<td>D₁ and D₅</td>
<td>Pu, cN, H, A</td>
<td>(Grandy &amp; Civelli 1992)</td>
</tr>
<tr>
<td>D2 like family</td>
<td>G protein coupled receptors</td>
<td>D₂, D₃ and D₄</td>
<td>Pu, cN, H, A</td>
<td>(Grandy &amp; Civelli 1992)</td>
</tr>
</tbody>
</table>


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Preclinical studies suggest selective nAChR and mAChR agonists may provide potential therapeutic strategies in AD (Svensson & Nordberg 1999; Fisher et al. 2002). *In vitro* studies have shown that activation of α7 nAChRs suppress Aβ-induced neurotoxicity (Svensson & Nordberg 1999; Kihara et al. 2001). The protective role of other sub-types of nAChR in AD neuropathology is less clear at the current stage. Similarly, activation of the M₁ sub-type of mAChRs has been shown to increase the secretion of soluble αAPP and thereby decrease Aβ production both *in vitro* and *in vivo* (Fisher et al. 2002). Activation of M₁ receptors
in 3xTg-AD mice reduced Aβ and tau pathologies in the hippocampus and the
cortex that was also associated with improved spatial memory functions (Caccamo
et al. 2006). Further studies are required to uncover the role of other mAChR sub-
types in AD.

Taken together, these data suggest that both nAChRs and mAChRs represent
important therapeutic targets in AD. However, the causal clinical symptoms of AD
is due to dysfunction of multiple neurotransmitter systems, therefore, new
therapeutical approaches should also take into account the impairment of other
neurotransmitter systems in AD.

1.1.1.6.3. Glutamatergic alterations in AD

Glutamate is the most abundant excitatory neurotransmitter in the brain and is used
by approximately 70% of all synapses in the neocortex and the hippocampus
(Fonnum 1984). Glutamatergic neurotransmission is implicated in almost all
aspects of cognitive function including leaning and memory processes (Baudry &
Lynch 2001).

Accumulating evidence suggest a malfunction in glutamatergic activity in AD
brains (Francis 2003). In AD, both glutamatergic hypoactivity and hyperactivity
have been reported (Schaeffer & Gattaz 2008). The most convincing evidence on
AD-associated deficits in glutamatergic system came from histopathological studies
(Francis 2003). Glutamate is the proposed neurotransmitter of the cortical and
hippocampal pyramidal neurones, which undergoes severe degeneration in AD
(Braak & Braak 1991; Francis 2003). Post-mortem AD studies have reported severe
atrophy of the temporal and the frontal cortex of AD brains associated with
degeneration of pyramidal neurones and synapses in the EC and the hippocampus
(Najlerahim & Bowen 1988; DeKosky & Scheff 1990; Terry et al. 1991; Gomez-
Isla et al. 1996; Price et al. 2001). These findings were further supported by an
immunohistochemical study that showed a reduced number of glutamate-
immunoreactive neurones in the hippocampus of AD brains (Kowall & Beal 1991).
Hypoactivity of the glutamatergic system is further supported in pre-mortem in vivo
imaging studies, which have shown glucose hypometabolism in several brain
regions including the neocortex, the medial thalamus, the mamillary bodies, the
posterior cingulate and the hippocampus in AD patients (Meguro et al. 2001; Nestor et al. 2003).

It has been more challenging to provide biochemical evidence in support of reduced glutamatergic neurotransmission in AD due to the widespread distribution of the transmitter throughout the brain (Francis et al. 1993). However, few post-mortem studies have reported reduced glutamate neurotransmitter levels in the cortex and the hippocampus of AD brains (Hyman et al. 1987b; Lowe et al. 1990).

Normal glutamatergic neurotransmission is critical for hippocampal function including learning and memory. AD-associated decrease in glutamatergic neurotransmission triggers a fall in the tri-synaptic circuit and stimulates hippocampal dysfunction that may account for the impaired memory function in AD patients. Taken together, these data suggest hypoactive glutamatergic neurotransmission during AD progression prior to excitatory degeneration associated with advanced stage of the disease.

1.1.1.6.4. Glutamatergic receptor alterations in AD

Glutamate exerts its effect by binding and activating two different types of receptors: (i) the ionotropic receptors, (which include the N-methyl-D-aspartate, NMDA and the amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid,AMPA/kainate sub-types) and (ii) the metabotropic glutamate receptors (Tanabe et al. 1992). The ionotropic glutamate receptor sub-types have varying permeability to the Na\(^+\) and Ca\(^{2+}\) ions whilst the metabotropic glutamate receptor sub-types are coupled to the adenylyl cyclase, phospholipase C or ion channels (Tanabe et al. 1992) (see also Table 1.1). The NMDA receptor is a major sub-type of ligand-gated ionotropic glutamate receptors, which is highly expressed throughout the brain including the neocortex and the hippocampus (Wang et al. 2004). Physiological stimulation of the NMDA receptor facilitates learning and memory processes (Monahan et al. 1989) and is involved in LTP, a neuronal phenomenon that is thought to underlie the basis of learning and memory processes (Bliss & Lomo 1973; Bliss & Collingridge 1993; Baudry & Lynch 2001). Inhibition of NMDA receptor activation, using the specific competitive NMDA
receptor antagonist (d(-)-aminophosphonovalerate) blocks LTP induction in the hippocampus (Collingridge et al. 1983; Errington et al. 1987).

Post-mortem studies revealed reduced AMPA and NMDA receptor binding sites in the EC, the hippocampus and the subiculum of AD brains (Jansen et al. 1990; Penney et al. 1990; Ulas et al. 1992; Yasuda et al. 1995; Armstrong & Ikonomovic 1996). These findings are supported by pre-clinical studies that also showed reduced NMDA receptor expression following exposure to the Aβ₁₋₄₂ peptide (Snyder et al. 2005; Lacor et al. 2007). In fact, neuronal cultures derived from APP_swe transgenic mice, with severe Aβ pathology, displayed a decrease in NMDA receptor expression (Snyder et al. 2005). Cortical neurones exposed to Aβ₁₋₄₂ peptide displayed reduced NMDA receptor expression, which was reversed by reducing the Aβ₁₋₄₂ peptide (achieved by treating the neurone culture with γ-secretase inhibitor) (Snyder et al. 2005). In addition, a more recent study in the APP_V717I mice revealed reduced NMDA-receptor expression that was associated with impaired NMDA receptor-dependent LTP as well as impaired fear memory (measured using contextual fear conditioning) (Dewachter et al. 2009). These findings highlight that AD-associated decrease in NMDA receptor may be due to increased deposition of Aβ plaques.

The NMDA receptor is highly permeable to Ca²⁺, over-activation of which initiates glutamate-mediated neurotoxicity (Choi 1995). Increased presence of glutamate in the synaptic micro-environment and persistent Ca²⁺ influx through the NMDA receptor play a critical role in the neurodegenerative processes seen in AD brains (Lipton & Rosenberg 1994; Brown et al. 1997). In vivo Aβ₁₋₄₂ infusion in the nucleus basalis magnocellularis (NBM) of rats, triggered excitotoxic pathway including increased extracellular glutamate neurotransmitter concentration via NMDA receptor activation and intracellular Ca²⁺ overload, which leads to neuronal death (Harkany et al. 2000a). This neurotoxic effect of the Aβ₁₋₄₂ was inhibited by NMDA receptor antagonist MK-801 (Harkany et al. 2000b), suggesting that blocking of MNDA receptor may be beneficial in reducing AD-associated neurodegeneration.
In fact, memantine (1-amino-3,5-dimethyl-adamantane), which is a low-affinity, non-competitive NMDA receptor antagonist blocks NMDA receptor (Parsons et al. 2007). Specifically, memantine binds to NMDA receptor channel pore and inhibits prolonged Ca\textsuperscript{2+} influx, which is the basis of neuronal excitotoxicity (Chen et al. 1992). Memantine was initially approved for the treatment of mild to severe cases of AD (2002 EU, 2003 USA) and is used commonly worldwide nowadays for the treatment of AD patients that are resistant to AChEIs (Parsons et al. 2007). Whilst AChEIs are prescribed to patients with mild to moderate AD, memantine is the only medicine approved for the treatment of moderate to severe cases of AD, which protect neurones against glutamate-induced excitotoxicity without preventing the physiological NMDA receptor activation needed for cognitive function (Wilcock 2003). Memantine inhibits excessive Ca\textsuperscript{2+} influx induced by NMDA receptor over-stimulation and may prevent AD-associated neuronal loss as well as AD-linked memory and cognitive dysfunction (Parsons et al. 2007).

Indeed, pre-clinical studies have shown that treatment with memantine improved spatial learning and memory performance (measured using MWM and object recognition test) in multiple transgenic mouse models of AD including the APP/PS1, APP23 and APP\textsubscript{swe} mice (Minkeviciene et al. 2004; Van Dam & De Deyn 2006; Scholtzova et al. 2008). In addition, treatment with memantine also reduced A\textbeta{} plaque burdens in the neocortex and the hippocampus of the APP/PS1 and APP\textsubscript{swe} mice (Unger et al. 2006; Scholtzova et al. 2008). Furthermore, a recent study in 3xTg-AD mice had reported that chronic (3 months) treatment with memantine not only improved memory performance (measured using MWM, object recognition and passive inhibitory avoidance tasks) but it also lowered the total levels of A\textbeta{} plaques and hyperphosphorylation of tau protein (measured using western blot in homogenates of the whole brain hemisphere) (Martinez-Coria et al. 2010). Taken together, these pre-clinical findings suggest that treatment with memantine may have a disease modifying effect on AD brains.

Randomised double-blind clinical trials have shown that treatment with memantine reduced clinical deterioration and improved the ability to perform activity of daily living in patients with moderate to severe AD (Reisberg et al. 2003). Specifically, memantine reduced the decline in AD patient’s ability to perform both instrumental
and basic activities of daily living such as difficulty in putting on clothing independently, handling the mechanics of bathing and toilet use as well as maintaining continence (Reisberg et al. 2003). Although a single clinical trial also suggested that memantine may be beneficial in patients with mild to moderate AD (Peskind et al. 2006), a more recent study suggested lack of conclusive evidence for benefit of memantine in patients with mild AD (Schneider et al. 2011).

1.1.1.6.5. Noradrenergic alterations in AD
The locus coeruleus (LC) of the brain stem is the major source of noradrenergic (NA) neurones, which in turn sends projections to numerous regions of the brain such as the cortex, the thalamus, the cerebellum and the hippocampus that play important roles in multiple brain functions including attention and memory (Gibbs & Summers 2002; Smith et al. 2006).

Post-mortem studies reported severe degeneration (50 – 70%) of NA neurones in the LC of AD brains (Matthews et al. 2002; Lyness et al. 2003; Zarow et al. 2003). In addition to severe degeneration, the remaining NA neurones in the LC displayed altered morphology characterised by swollen cell bodies and reduced branched dendrites in AD brains (Chan-Palay & Asan 1989). Greater NA neuronal loss was observed particularly in AD patients with longer duration of the disease (Zarow et al. 2003), suggesting that degeneration of LC may be used as a diagnostic marker during the progression of the disease (Haglund et al. 2006). Degeneration of NA neurones is accompanied by reduced NA projections in the LC and the thalamus (Gulyas et al. 2010) as well as low levels of NA in the frontal cortex, the temporal cortex, the putamen and the hippocampus (Palmer et al. 1987b; Reinikainen et al. 1988). In AD, increased degeneration of NA neurones in the LC has been shown to correlate with increased aggression (Matthews et al. 2002), suggesting that the loss of NA activity may trigger the appearance of aggressive behaviours in AD. In addition, reduced NA concentration in the temporal cortex was associated with increased deterioration of cognitive function (measured using MMSE score) in AD patients (Matthews et al. 2002). Given the important role of NA in attention and memory performance, reduced NA neurotransmission in AD has been linked with disruption of cognitive processes involving attention (Matthews et al. 2002).
In addition to its role as a neurotransmitter, NA also exerts an anti-inflammatory effect by inhibiting the expression of pro-inflammatory genes (Feinstein et al. 2002) and modulating microglia activation throughout the brain (Heneka et al. 2010a). Degeneration of NA neurones in rats (achieved by injection of NA selective neurotoxin N-(2-chloroethyl)-N-ethyl-2 bromobenzylamine, DSP4, into the LC) exacerbated Aβ1-42-induced neuro-inflammatory processes including induction of nitric oxide synthase (NOS) as well as increased expression of interleukin (IL)-1β and IL-6 (Heneka et al. 2002). Similarly, degeneration of the LC in multiple transgenic mouse models of AD including the APP23, APPV717F and APP/PS1 mice triggered increased Aβ plaque deposition that was also associated with increased astrocytes/microglia activation, pronounced neurodegeneration and severely impaired memory performance (measured using radial arm maze and social partner recognition tests) (Heneka et al. 2006; Kalinin et al. 2007; Jardanhazi-Kurutz et al. 2011).

Taken together, data from pre-clinical studies suggest that AD-associated loss of NA neurones in the LC and reduced NA in the projection sites may increase inflammation and neuronal degeneration. However, currently there is no clinical therapy directed towards reduced NA neurotransmission in AD.

1.1.1.6.6 Noradrenergic receptor alterations in AD

The action of NA is mediated by its binding to adrenergic receptors that are G protein coupled receptors. There are two types of NA receptors namely: the α and the β, which are further divided into multiple receptor sub-types (Gibbs & Summers 2002) (see also Table 1.1).

Post-mortem binding studies (using α2 receptor agonists [3H]p-aminoclonidine and [3H]bromoxidine as radioligand) reported decreased α2 receptor binding sites in the prefrontal cortex, the frontal cortex, the hypothalamus, the cerebellum and the hippocampus of AD brains (Kalaria & Andorn 1991; Meana et al. 1992; Pascual et al. 1992). On the other hand, Russo-Neustadt and Cotman (1997), using the same radioligand, reported significant increase in α2 receptor binding sites in the cerebellar cortex of AD patients with concomitant aggression, agitation and disruptive behaviours (Russo-Neustadt & Cotman 1997), suggesting that AD-
associated decrease in $a_2$ receptors is restricted to the sub-group of AD patients with no symptoms of aggression or agitation. Similarly, other post-mortem AD studies have reported stable or increased $\beta$ adrenergic receptors in the frontal cortex, the temporal cortex, the prefrontal cortex and the hippocampus (D'Amato et al. 1987; Kalaria et al. 1989), which were more evident in AD patients with depressive behaviours (Russo-Neustadt & Cotman 1997). Altogether, these data highlight the importance of sub-classification of AD subjects, based on the presence of behavioural symptoms, when investigating specific neurochemical changes.

1.1.1.6.7. Dopaminergic alterations in AD

The majority of dopaminergic (DA) neurones are located in the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), which send projections throughout multiple brain regions including the cortex and the hippocampus (Arsenault et al. 1988; Gasbarri et al. 1994a). Two main factors that support DA involvement in AD include the presence of behavioural symptoms in AD and the increased incidence of both AD and Parkinson's disease (PD) with advanced age (Kumar & Patel 2007). In addition, PD patients at more advanced stage of the disease display degeneration of the medial SNc and VTA and the subsequent loss of mesolimbic and mesocortical DA projections, which in turn contribute to the development of AD-type dementia (Ito et al. 2002). Indeed, patients with PD have six-fold greater risk of developing dementia compared to general population of the same age and sex (Pavese 2012).

Only limited post-mortem studies have investigated DA neurotransmitter level in AD brains, with contradictory findings (Reinikainen et al. 1990; Langlais et al. 1993). Whereas Reinikainen and colleagues (1990) reported AD-associated decrease in DA concentrations in the temporal cortex and the hippocampus (Reinikainen et al. 1990), Langlais and co-authors (1993) found stable DA content in the basal ganglia (Langlais et al. 1993), which suggest region-specific alterations of DA neurotransmitter level in AD brains.

1.1.1.6.8. Dopaminergic receptor alterations in AD

The effect of DA is mediated by activation of two classes of metabotropic G-protein coupled receptor families namely: the D1 and D2 families (Grandy & Civelli
Patients with AD showed reduced D1 receptor binding sites in the putamen and the caudate nucleus as shown by *in vivo* PET imaging (Kemppainen *et al.* 2000). The decrease in D1 receptor protein was subsequently confirmed using immunohistochemistry, which showed reduced D1 receptor in the AD frontal cortex (Kumar & Patel 2007).

*In vivo* imaging study using PET also reported decreased D2 receptor binding potentials in the hippocampus, which was associated with impaired verbal memory in AD patients (Kemppainen *et al.* 2003). Others also reported AD-associated decrease in D2 receptor binding sites in the amygdala and the hippocampus that may contribute to the clinical symptoms associated with the disease (Joyce *et al.* 1993). A single photon emission computed tomography (SPECT) study found reduced D2 receptors in the striatum of AD patients without overt extra pyramidal symptoms (such as muscle rigidity, repetitive muscle movement and involuntary movements) (Pizzolato *et al.* 1996), suggesting that reduced D2 receptor may be part of the widespread neurodegenerative processes associated with AD.

In summary, and as depicted in Table 1.2, AD-associated degeneration causes severe losses and alterations in all major neurochemical systems of the brain as well as their different receptor sub-types.
Table 1.2. Summary of the major neurotransmitter system pathology in different regions of AD brains.

<table>
<thead>
<tr>
<th>Neurochemical Studied</th>
<th>Brain Region</th>
<th>Main Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholinergic system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh Neurones</td>
<td>nbM</td>
<td>Lost</td>
<td>(Whitehouse et al. 1981; Whitehouse et al. 1982)</td>
</tr>
<tr>
<td>ACh Concentration</td>
<td>TC, FC</td>
<td>Reduced</td>
<td>(Richter et al. 1980; Gil-Bea et al. 2005)</td>
</tr>
<tr>
<td>ACh Synthesis</td>
<td>C</td>
<td>Reduced</td>
<td>(Geula &amp; Mesulam 1996)</td>
</tr>
<tr>
<td>ACh Projection</td>
<td>C</td>
<td>Reduced</td>
<td>(Bowers et al. 1976; Davies &amp; Maloney 1976; Perry et al. 1977)</td>
</tr>
<tr>
<td>ChAT Activity</td>
<td>C, Am, H</td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td>nACh Receptors</td>
<td>C, H</td>
<td>Reduced α3, α4 and α7</td>
<td>(Hellstrom-Lindahl et al. 1999; Marutle et al. 1999)</td>
</tr>
<tr>
<td>mACh Receptors</td>
<td>PC</td>
<td>Reduced M1, M3, M4 and M5</td>
<td>(Tsang et al. 2008; Potter et al. 2011)</td>
</tr>
<tr>
<td><strong>Glutamatergic system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate Neurones</td>
<td>TC, H, FC</td>
<td>Lost</td>
<td>(Neary et al. 1986; Kowall &amp; Beal 1991)</td>
</tr>
<tr>
<td>Glutamate Synapse</td>
<td>TC, FC</td>
<td>Loss</td>
<td>(DeKosky &amp; Scheff 1990; Terry et al. 1991)</td>
</tr>
<tr>
<td>Glutamate Concentration</td>
<td>H</td>
<td>Reduced</td>
<td>(Jansen et al. 1990; Ulas et al. 1992)</td>
</tr>
<tr>
<td>NMDA Receptors</td>
<td>H, Su</td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td>AMPA Receptors</td>
<td>EC, H</td>
<td>Reduced</td>
<td>(Yasuda et al. 1995)</td>
</tr>
<tr>
<td><strong>Noradrenergic system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA Neurones</td>
<td>LC</td>
<td>Lost</td>
<td>(Lyness et al. 2003; Zarow et al. 2003)</td>
</tr>
<tr>
<td>NA Concentration</td>
<td>FC, TC, P, H</td>
<td>Reduced</td>
<td>(Palmer et al. 1987b; Reinkainen et al. 1988)</td>
</tr>
<tr>
<td>NA Projection</td>
<td>LC, T</td>
<td>Reduced</td>
<td>(Gulyas et al. 2010)</td>
</tr>
<tr>
<td>αNA Receptors</td>
<td>PFC, FC, cb, H</td>
<td>Reduced in α2NA</td>
<td>(Meana et al. 1992; Pascual et al. 1992)</td>
</tr>
<tr>
<td>βNA Receptors</td>
<td>PFC, FC, H</td>
<td>No change in βNA</td>
<td>(D’Amato et al. 1987; Kalaria et al. 1989)</td>
</tr>
<tr>
<td><strong>Dopaminergic system</strong></td>
<td></td>
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<tr>
<td>DA Neurones</td>
<td>nr</td>
<td>nr</td>
<td></td>
</tr>
<tr>
<td>DA Concentration</td>
<td>TC, H</td>
<td>Reduced</td>
<td>(Reinkainen et al. 1990)</td>
</tr>
<tr>
<td>D1 Like Receptors</td>
<td>Pu, eN</td>
<td>Reduced D1</td>
<td>(Kempainen et al. 2000)</td>
</tr>
<tr>
<td>D2 Like Receptors</td>
<td>H</td>
<td>Reduced D2, D3 and D4</td>
<td>(Kempainen et al. 2003; Kumar &amp; Patel 2007)</td>
</tr>
</tbody>
</table>

Key: nbM: nucleus basalis of Meynert, FC: frontal cortex, PC: parietal cortex, TC: temporal cortex, EC: entorhinal cortex, LC: locus coeruleus, Pu: putamen, H: hippocampus, Su: subiculum, PFC: prefrontal cortex, cb: cerebellum, CN: caudate nucleus, nr: not reported. Note: Highlighted in yellow are studies that reported AD-associated deficit in ACh, glutamate, NA and DA system, whilst highlighted in green are two reported studies that showed no AD-associated changes in βNA receptor. AD-associated change in serotonergic neurotransmission, which is the main focus of this thesis, is described in details in section 1.5.

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In addition to alterations in ACh, NA and DA systems, accumulating evidence from both clinical and preclinical studies suggest the involvement of 5-HT system in AD, which is the main focus of this thesis. Although AD-associated alterations of 5-HT
system are described in greater details in the section 1.5 of this introduction, below is a brief summary on 5-HT system changes in AD pathology.

Patients with AD showed reduced 5-HT neurotransmitter levels in the cerebrospinal fluid (CSF) (Tohgi et al. 1992; Tohgi et al. 1995), in the platelets (Mimica et al. 2008; Muck-Seler et al. 2009) and in multiple brain regions including the frontal cortex, the temporal cortex, the putamen and the amygdala (Nazarali & Reynolds 1992; Garcia-Alloza et al. 2005). In vivo PET and MRI studies reported AD-related decrease in 5-HT receptors throughout the brain (Meltzer et al. 1998a; Kepe et al. 2006; Hasselbalch et al. 2008). Post-mortem analysis of AD brains have reported reduced extracellular levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) as well as decreased 5-HT receptor expression in various brain regions including the neocortex and the hippocampus (Palmer et al. 1987a; Nazarali & Reynolds 1992; Chen et al. 1996; Lai et al. 2002; Garcia-Alloza et al. 2005; Lorke et al. 2006; Truchot et al. 2008). Impaired 5-HT neurotransmission in AD is consistent with the described degeneration of 5-HT neurones from the dorsal (DR) and the median (MR) raphe nuclei as well as associated loss of the cortical 5-HT projections (Yamamoto & Hirano 1985; Chen et al. 2000a).

Regarding the clinical symptoms, AD-related deficits in 5-HT neurotransmission is associated with accelerated cognitive decline (determined by using the MMSE score) (Lai et al. 2002) and increased behavioural symptoms including depression, aggression and psychosis (Garcia-Alloza et al. 2005).

Treatment with selective 5-HT re-uptake inhibitors (SSRIs) increased CSF concentration of 5-HT and improved cognitive function in AD patients (Tohgi et al. 1995; Marksteiner et al. 2003; Mowla et al. 2007; Mossello et al. 2008). Drugs acting on specific 5-HT receptors have been suggested as therapeutic agents to improve cognitive function in AD (Terry et al. 2008) (for details see chapter 1, section 1.5).
1.1.2. Anatomy of AD affected regions

The clinical symptoms associated with AD are indisputably related to the degenerative processes in memory related structures such as the nbM, the EC, the hippocampus and the prefrontal cortex (PFC) (Braak & Braak 1991). In addition, the DR and MR raphe nuclei, which encompass the majority of 5-HT neurones, are classically considered to display severe alterations in AD (Chen et al. 2000a; Hendriksen et al. 2004). The work of this thesis is specifically focused on the hippocampus not only due to its central role in the establishment of learning and memory functions, but also due to its rich 5-HT innervations (Lavenex and Amaral, 2000; Nadel, 1995). The hippocampus acts as a storage room for higher-order cortices (Squire & Alvarez 1995) and is one of the first and most vulnerable regions affected in AD neuropathology.

1.1.2.1. Nucleus basalis of Meynert

Using Nissl staining, it was estimated that in total the nbM contained around 200,000 neurones in humans (Arendt et al. 1985). Combined immunohistochemical staining using ChAT antibody followed by counterstaining with Nissl substance showed that 80 – 90% of all neurones in the nbM were cholinergic (Mesulam & Geula 1988) and mainly project to the cerebral cortex in humans (Mesulam 2004). The nbM also encompasses non-ACh neurones including the γ-aminobutyric (GABA)-ergic,peptidergic and tyrosine hydroxylase (TH)-positive neurones (Mesulam 2004). The nbM is sub-divided into six major regions: the anteromedial, anterolateral, anterointermediate, intermediodorsal, intermedioventral and posterior regions, each with specific cortical projection profile (Mesulam & Geula 1988). Specifically, the anteromedial region of the nbM provides ACh projections to the medial cortical areas including the cingulate gyrus; the anterolateral sub-division of the nbM provides ACh input to the frontal and the parietal cortex as well as the amygdala; the anterointermediate part projects to the frontal cortex, the parietal cortex and the temporal cortex, and finally, the posterior region of the nbM sends ACh projections to the superior temporal cortex (Mesulam et al. 1983a; Mesulam et al. 1983b; Mesulam et al. 1986). Although the nbM projects throughout all cortical regions, it also receives input from the cortex (Zaborszky et al. 1997), the
hypothalamus, the septum, the nucleus accumbens and the amygdala (Mesulam & Mufson 1984).

As mentioned in section 1.1.1.6.1 and 1.1.1.6.2, AD-associated changes in ACh system include severe loss of ACh neurones in the nbM, impaired AChT enzyme activity, decreased ACh synthesis, reduced ACh neurotransmitter level and reduced densities of ACh receptors in multiple regions of AD brains (Bartus et al. 1985; Collerton 1986; Cummings & Kaufer 1996). In addition, other functional component of ACh neurones have been also reported to undergo severe alterations in AD brains including the expression of nerve growth factor (NGF) receptor (trkA and p75NTR) that are critical for ACh neuronal survival (Cuello et al. 2007; Schliebs & Arendt 2011).

Although severe loss of cortical ACh innervations is an early event during the progression of AD, ACh neurones in the nbM remain stable in patients with mild cognitive impairments that have higher risk for developing AD (Gilmor et al. 1999; Mufson et al. 2007). However, there was a decline in the expression of trkA and p75NTR-containing ACh neurones in the nbM of patients with mild cognitive impairment and early AD (Mufson et al. 2007), suggesting dysfunction of ACh neurones rather than ACh neurodegeneration at the early stages of the disease.

Other studies have also reported that, in AD brains, NFTs pathology first appear in the nbM before its expression in the EC (Sassin et al. 2000; Geula et al. 2008). Indeed, in the nbM, hyperphosphorylation of tau protein becomes evident in Braak stage I that is then steadily increased through AD progression (Sassin et al. 2000). Initially, the abnormal tau protein is distributed diffusely throughout the soma and neuronal processes in the nbM before its aggregation into NFTs structures (Sassin et al. 2000). The development of tau pathology in the nbM may participate in the early dysfunction of ACh neurones via impaired axonal transport and their subsequent degeneration associated with AD (Whitehouse et al. 1981; Whitehouse et al. 1982).
1.1.2.2. Entorhinal cortex

The entorhinal cortex (EC) is located at the rostral end of the temporal lobe and is deeply involved in cognitive functions by establishing the cortico-hippocampal circuits (Suzuki & Amaral 1994). The EC comprises six layers that are divided into superficial (I – III) and deep layers (IV – VI), which display differential anatomical and functional organisation (Suzuki & Amaral 1994; Witter & Amaral 2004; Witter 2007) (see also Fig. 1.10). The superficial layers receive most of the cortical information and are the main output source of the EC (Witter 2007). The perforant path is the major output of the EC that projects to the dentate gyrus (DG) of the hippocampus (Witter 2007). The axonal projections of the perforant path originate mainly from layers II and III, but also with relatively low contribution from the deeper layers V and VI of the EC (Witter et al. 1989). More specifically, neurones of the layers II/V of the EC project to the middle and outer molecular layer of the DG (Witter et al. 1989; Naber et al. 2001; Baks-Te Bulte et al. 2005). Projections arising from layer II of the EC also send collaterals to the hippocampal CA2 and CA3 subfields (Naber et al. 2001). Neurones from layers III/V of the EC project mainly to the CA1 subfield of the hippocampus and the subiculum; whilst the latter (subiculum) sends projections back to the layer V of the EC (Naber et al. 2001). Due to their origin from either the lateral or medial EC, the prefrontal path can be divided into the lateral and medial pathways (Baks-Te Bulte et al. 2005). Neurones located in the deeper layers of the EC give rise to projection that extends to the cortex and the parahippocampal areas of the brain (Suzuki & Amaral 1994; Witter & Amaral 2004) (Fig. 1.10B).

Owing to its heavy connection with the cortex and the hippocampus, the EC plays an important role in cognition (Coutureau & Di Scala 2009), working memory (Fransen 2005), memory consolidation (Remondes & Schuman 2004) and spatial memory (Fyhn et al. 2004). In fact, the EC acts as a channel for sensory information into the hippocampus, therefore damage to the EC isolates the hippocampus from the cortical sensory input required for spatial memory (Coutureau & Di Scala 2009).
Lesion of the EC (achieved by bilateral injections of ibotenic acid into the EC) impaired both reference and working memory acquisition (measured using MWM and spontaneous object recognition tests) in rats (Galani et al. 1998; Eijkenboom et al. 2000). In addition, impaired attention processing such as sensory gating and selection of relevant information were reported in rats following EC lesion (Oswald et al. 2001). Furthermore, damage to the EC impaired olfactory memory (measured using odour discrimination task) in rats (Staubli et al. 1986), which is also affected in AD patients (Mesholam et al. 1998) and in the 3xTg-AD mouse model of the disease (Cassano et al. 2011). A more recent in vivo study in healthy human volunteers had shown that neurones in the EC are particularly active during the object and spatial encoding tasks (Bellgowan et al. 2009). Taken together, these data indicate that the EC is critical for cognition, independent of its role as a relay station between the cortex and the hippocampus.

Post-mortem studies have reported severe atrophy and neuronal loss in the EC of patients with mild cognitive impairment and early AD (Gomez-Isla et al. 1996; Kordower et al. 2001). Such early degeneration of neurones in the EC may be associated with the presence of Aβ plaques and NFTs neuropathology in this brain region early in the course of AD (Thal et al. 2000). Initially, hyperphosphorylation
of tau protein in AD brains appears in layers II and III of the EC (Braak stage I and II), which provide the major projections to the hippocampus and the subiculum, respectively (Braak & Braak 1991; Thal et al. 2000). Severe loss of neurones in the layer II of the EC disconnects the EC input to the hippocampus (Gomez-Isla et al. 1996; Kordower et al. 2001). These results are supported by an in vivo imaging study (using MRI) that showed reduced EC projection to the hippocampus in patients with mild cognitive impairment (Stoub et al. 2006).

1.1.2.3. Hippocampus

The term “hippocampus” (derived from the Greek word for seahorse) was first coined during the sixteenth century (1587) by an Italian anatomist Giulio Cesare Arantius (1529/1530 – 1589), who considered that the three-dimensional structure of the grossly dissected human hippocampus is analogous to a seahorse (Arantius 1587; Insausti & Amara 2004) (see also Fig. 1.11).

Figure 1.11. An illustrative photograph showing the apparent similarities of a human hippocampus (left) with a seahorse (right).
From (Seress 1980)

The hippocampus appears as a curved cortical structure that is located in the medial temporal lobe of the forebrain (Lorente de Nó 1933; Witter & Amaral 2004). The hippocampus together with the dentate gyrus (DG) as well as the subiculum and the EC are referred to as the hippocampal formation (HF) (Lorente de Nó 1933; Amaral & Witter 1989; Witter & Amaral 2004). The hippocampus is further divided into four subfields namely: the CA3, CA2 and CA1 (CA, cornu ammonis) subfields (Amaral & Witter 1989) as well as the DG.
In the CA subfields, pyramidal neurones are the major component of the pyramidal cell layer (PCL) (Amaral & Witter 1989). In addition to pyramidal neurons, the PCL of CA subfield also encompasses cell bodies of multiple neurones including the basket, axo-axonic, bistratified and radial trilaminar cells (Witter & Amaral 2004). Pyramidal neurones descend their basal dendrites into the deep layer called the stratum oriens (S.Or) (Amaral & Witter 1989; Blasco-Ibanez & Freund 1995). Pyramidal neurones extend their apical dendritic trees to the overlying superficial layer called the stratum radiatum (S.Rad) (Fig. 1.12B). In addition, the S.Or and the S.Rad are also populated by a number of horizontal trilaminar cells as well as basket and bistratified cells (Witter & Amaral 2004). The apical dendritic tree of the pyramidal neurones also reach the stratum lacunosum moleculare (S.Mol) (Amaral et al. 2007). The S.Mol occupies the most superficial portion of the CA subfields, which receives high thalamic projection mainly from the nucleus reuniens (Herkenham 1978; Wouterlood et al. 1990) and 5-HT fibres from the DR and MR nuclei (Vertes 1991; Vertes et al. 1999). In the CA3 subfield of the hippocampus, the region just above the PCL contains the mossy fibres from the DG and is called the stratum lucidum (S.Luc) (Amaral & Witter 1989).

**Figure 1.12 (→).** (A) Toluidine blue stained rat hippocampus showing the distribution of different hippocampal layers. (B) Scheme of the hippocampus illustrating the main types of neurones of the principal layers: Granular cell neurones populate the GCL extending the dendritic tree to the ML and projecting mossy fibres to the pyramidal neurones of the CA3 subfield. Both pyramidal neurones in the CA3 and CA1 subfields display apical and basal dendritic trees. (C) Drawing of the neural circuitry of the young rabbit hippocampus showing the main subfields and the tri-synaptic loop. **Key:** DG: dentate gyrus, GCL: granular cell layer, ML: molecular layer, S. Luc: stratum lucidum, S.Mol: stratum lacunosum moleculare, S.Rad: stratum radiatum, PCL: pyramidal cell layer, S.Or: stratum oriens, EC: entorhinal cortex. A from (Rodríguez et al. unpublished photograph), B from (Rolls & Xiang 2006), C from (Ramón y Cajal 1911).
The DG comprises 2 blades i.e. the upper (the supra pyramidal) and lower (the infra pyramidale) blades that are placed in between both granular layers. The DG is divided into three layers: molecular layer (ML), granule cell layer (GCL) and hilus, which Lorente de Nó originally named as CA4 (Lorente de Nó 1933). The ML of the DG is a relatively cell-free layer that mainly contains the dendrites of the granule cells (Amaral et al. 2007). The other major component of the ML is the axons of the perforant path that originate from the EC. The ML is further divided into three smaller layers namely: outer molecular layer (OML), middle molecular layer (MML) and inner molecular layer (IML) of the DG. The OML is the terminal zone for the perforant path fibres; whilst the MML receives fibres originated from the medial EC (Amaral et al. 2007). The IML is occupied by projections originated from neurones in the hilus (Laurberg & Sorensen 1981; Frotscher et al. 1991).

The principal cell layer of the DG, GCL, is made up largely of densely packed granule cells and their dendrites (Amaral et al. 2007). The granule cells gives rise to very distinctive anatomical axons, the mossy fibres, which project through the hilus and terminate in the CA3 subfield of the hippocampus and more specifically in the S.Luc (Gaarskjaer 1978). The hilus makes up the third layer of the DG and is mainly occupied by cell bodies of the mossy cells (Amaral 1978).

In addition to pyramidal (in the CA subfield) and granule cells (in the DG), there are also a large population of hippocampal interneurones (Insausti & Amara 2004). Whereas the pyramidal and the granule cells are glutamatergic, hippocampal interneurones represent a neurochemically heterogeneous population of neurones, which contain GABA, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), substance P (SP), corticotropin releasing factor (CRF), somatostatin, neuropeptide Y (NPY), enkephalin and dynorphin (Freund & Buzsaki 1996). Hippocampal interneurones play an important role in regulating the activity of the pyramidal and granule neurones in the hippocampus (Freund & Buzsaki 1996). Other types of interneurones found at the boundary of the GCL and the hilus include the dentate pyramidal basket cells, which inhibit the excitability of granule cells (Ribak 1992; Amaral et al. 2007). The terminals of basket cells are GABAergic, which form symmetrical (presumed inhibitory) synapses with the cell bodies and proximal dendrites of the granule cells. Pyramidal baskets cells extend to a large distance that
permit a single basket cell to modulate the activity of up to 10,000 granule cells (Amaral et al. 2007); hence modulating the activity of a small number of hippocampal interneurones has been shown to have a strong effect on the hippocampal activity including learning and memory processes (Gulyas et al. 1999).

The basic connections and anatomy of the HF was initially described in the classical Golgi studies of Santiago Ramón y Cajal (Ramón y Cajal 1911) and Lorente de Nó (Lorente de Nó 1933). Four regions of the HF (EC, DG, CA and subiculum) are linked by a distinctive unidirectional connection called the tri-synaptic circuit (Fig. 1.12C). Fibres arising from the layer II of the EC project to the dendritic trees located in the MML of the DG through the perforant pathway (Witter 2007). Granule cells of the DG project via their mossy fibres to the S.Luc of the CA3 subfield of the hippocampus (Treves et al. 2008). Pyramidal cells in the CA3 subfield provide the major input to the CA1 subfield of the hippocampus through the so-called Schaffer collaterals (Amaral & Witter 1989). Such distinctive unidirectional connection enables the use of extracellular recording techniques to monitor synaptic events in the hippocampus (Neves et al. 2008). In fact, the most commonly accepted (but not proven) mechanism of learning and memory, LTP, was first identified in the DG of the rabbit hippocampus following stimulation of the perforant path (Bliss & Gardner-Medwin 1973; Bliss & Lomo 1973).

The perforant path fibres relay specific sensory cortical input from the EC that is specifically related to the environment. In addition, the hippocampus also receives sub-cortical inputs related to motivation, emotion and the autonomic state of the animal (Gulyas et al. 1999). These sub-cortical inputs of the hippocampus originate from different nuclei containing only few thousands of neurones and include: DA projections form the VTA and the SNc (Gasbarri et al. 1994b), NA projections from the LC (Jones & Moore 1977), ACh fibres from the medial septal nucleus and the nucleus of the diagonal band of Broca (Mesulam et al. 1983b; Wainer et al. 1985) and dense 5-HT projections from the DR and MR nuclei (Azmitia & Segal 1978; Kosofsky & Molliver 1987; Vertes 1991; Vertes et al. 1999). These sub-cortical projections have a strong effect on hippocampal activity via activation of neurotransmitter-specific receptors that are expressed on pyramidal neurones. Sub-
cortical afferents also achieve their robust effect on hippocampal activity by exerting diverse actions on different sub-sets of GABAergic interneurones (Gulyas et al. 1999).

The early report of memory loss in humans following bilateral lesion of the hippocampus was of critical importance in highlighting the significance of the hippocampus in memory function (Scoville and Milner, 1957). Since the early description by Scoville and Milner (1957), multiple animal models with selective lesions of the hippocampus have been developed to uncover its role in memory function. Selective lesions of the hippocampus causes impaired learning and memory performance in rodents (Clark et al. 2000; Prusky et al. 2004), monkeys (Beason-Held et al. 1999; Zola et al. 2000) and humans (Manns et al. 2003). It was proposed that while the cortex maintains various forms of memory representations, the hippocampus mediates the organisation of this information, which extends the persistence of memory (Eichenbaum et al. 1996).

Learning and memory retention are associated with changes in the activity of hippocampal neurones (Suzuki & Eichenbaum 2000; Squire et al. 2004). Electrophysiological recording of the hippocampal pyramidal neurones in rabbit displayed learning-related neural plasticity including an increase in the frequency of firing (Berger et al. 1983). Hippocampal neuronal activity is associated with the establishment of spatial memory, where hippocampal neurones encode the animal’s position in the environment (Hampson et al. 1999a; Hampson et al. 1999b; Wiebe & Staubli 1999; Wood et al. 1999; Kesner 2007). Increased hippocampal activation during associative learning has been also reported in humans using in vivo PET imaging (Henke et al. 1997). In addition, others also found increased hippocampal activity during recognition memory (Stark & Squire 2000) and retrieval of episodic memory (measured using MRI) in humans (Eldridge et al. 2000), suggesting that the hippocampus selectively supports the retrieval of episodic memories.

Selective hippocampal lesions impaired spatial working memory (measured using forced-choice alternation test) in rats (Aggleton et al. 1986). Radio-frequency or ibotenic acid lesions of the hippocampus also impaired visual recognition memory (measured using visual paired comparison task) in rats (Clark et al. 2000; Prusky et
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Similar lesions of the hippocampus in rhesus monkeys impaired visual recognition memory (measured using delayed non-match to sample and delayed recognition span tasks) (Beason-Held et al. 1999; Zola et al. 2000). Patients with bilateral damage limited to the hippocampus also displayed selective impairment of recognition memory (Reed & Squire 1997; Manns & Squire 1999; Manns et al. 2003) and episodic memory (Vargha-Khadem et al. 1997). Other studies in humans have shown that the hippocampus is particularly important for declarative memory, which refers to memories that can be consciously recalled such as facts and knowledge (Cohen & Squire 1980; Ullman 2004). Taken together, these findings highlight the importance of the hippocampus in the acquisition and retention of new information.

In patients with mild cognitive impairment and early AD both the EC and the hippocampus undergo pronounced atrophy (measured using in vivo PET imaging) (Convit et al. 1997; Jack et al. 1997; Jack et al. 1999). Interestingly, hippocampal atrophy was more evident in patients with mild AD compared to that with mild cognitive impairment (Dickerson et al. 2001), suggesting that neurodegenerative processes in the hippocampus is critical for deterioration of cognitive function in AD.

More specifically, the layer II of the EC displayed severe neurodegeneration in patients with mild cognitive impairment and early AD (Gomez-Isla et al. 1996; Kordower et al. 2001). Loss of layer II neurones is particularly important in AD since it would prevent the flow of information to the hippocampus through the perforant path (deToledo-Morrell et al. 2007). Indeed, Hyman and colleagues (1984) had shown that loss of layer II neurones in the EC resulted in disconnection of the hippocampus from the sensory cortical inputs in patients with very mild AD (Hyman et al. 1984). More recent in vivo imaging study using MRI also reported compromised perforant path in patients with mild cognitive impairment (deToledo-Morrell et al. 2007). Altogether, these data suggest that disconnection of the hippocampus from the EC may trigger the memory dysfunction during the early stages of AD. Such hippocampal disconnection results in impaired cognitive function including learning and memory in AD patients.
Post-mortem studies have reported severe loss of synapses particularly in the ML of DG in AD brains (Scheff & Price 1998; Scheff et al. 2006). However, AD-associated neuronal loss is more prominent in the CA1 subfield of the hippocampus (West et al. 1994; West et al. 2000), which may be due to increased presence of NFT neuropathology in this hippocampal region (Van Hoesen & Hyman 1990). In the hippocampus, the AD-related hallmarks become evident relatively early during the course of the disease with NFT pathology appearing during Braak stage I and II; whilst the Aβ plaque deposition becomes evident during stage B (Braak & Braak 1991).

1.1.2.4. Prefrontal cortex

The prefrontal cortex (PFC) is located at the anterior part of the frontal lobe and it is widely connected to different brain regions including the hypothalamus, the brain stem, the basal ganglia and the hippocampus (Barbas 2000). The PFC is subdivided into three major regions: orbital, medial and lateral regions. All three regions of the PFC receive projections from the hippocampus (Barbas & Blatt 1995). In addition, the orbital and medial regions of the PFC also receive projections from the amygdala (Porrino et al. 1981) and the thalamus (Ray & Price 1993). The orbital and medial regions are involved in the regulation of emotional behaviour; whilst lateral PFC is involved in cognition, speech and reasoning (Fuster 2001).

An initial study by Goldman and colleagues (1971) showed that selective lesion of the lateral PFC caused severe impairment in spatial working memory (measured using spatial delayed-alternation task) in monkeys (Goldman et al. 1971). This finding was supported by numerous lesion studies in monkeys that confirmed the importance of the lateral PFC in learning and working memory (Curtis & D'Esposito 2004). Similarly, patients with selective lesions of the lateral PFC displayed impairment in selection and organisation processes that facilitate long-term memory encoding (Blumenfeld & Ranganath 2007).

In vivo imaging studies have reported increased activity of the PFC during tasks that require selective attention and organisation processes (Blumenfeld & Ranganath 2006; Blumenfeld & Ranganath 2007). Another in vivo imaging study
also implicated the PFC in working memory (measured using delayed-response task) in humans (D'Esposito et al. 2000). These data support the involvement of PFC in working memory and long-term memory formation.

Patients with mild cognitive impairment displayed reduced activity of the PFC that was associated with impaired episodic memory (Elgh et al. 2003). Similarly, AD patients also showed reduced PFC activity (measured using in vivo MRI), suggesting that lowered PFC activity may reflect AD-associated neurodegeneration and contribute to the clinical symptoms associated with the disease (Li et al. 2009). In fact, post-mortem studies in AD brains had shown selective loss of large pyramidal neurones in the PFC that correlated with increased NFT neuropathology in these neurones (Hof et al. 1990; Bussiere et al. 2003). Specifically in the PFC, AD-related NFT formation occurs relatively late during the course of the disease following its deposition in the EC and the hippocampus (Vickers et al. 1992).

The experimental work of this thesis had been primarily focused on the hippocampus and in particular the CA1 subfield of the hippocampus, which is the major component of the Schaffer collateral pathway and acts as a neural conduit between the hippocampal subfields, the subiculum and the EC (Amaral & Witter 1989). The CA1 subfield of the hippocampus integrates the incoming information from the CA3 subfield and the EC (via DG) before sending it to the higher cortical areas and the subiculum for further processing and final output. Specifically, the CA1 subfield provides the main hippocampal output to the subiculum. Therefore, neuronal activity in the CA1 subfield of the hippocampus has been strongly linked with memory formation, an aspect of cognitive function that is typically compromised in AD (Walsh & Selkoe 2004). In addition, the CA1 subfield of the hippocampus displayed high susceptibility to Aβ neuropathology in AD brains compared to other hippocampal subfields. Functionally, the CA1 subfield of the hippocampus is involved in associative memory, which is one of the earliest clinical symptoms in AD patients.
1.2. **ANIMAL MODELS OF AD**

No animal suffers from AD, therefore the utilisation of animal models that replicate one or more neuropathological and neurochemical features of AD brains is critically important in order to investigate the disease process in an in vivo paradigm, for review see (Gotz et al. 2004a; Gotz et al. 2004b; Toledano & Alvarez 2004; Cassel et al. 2008; Rodríguez et al. 2009b; Philipson et al. 2010). At first, normally aged animals were used to study AD (Bertoni-Freddari et al. 1986; Biegon et al. 1986). Aged animals displayed a marked atrophy of the basal forebrains, which is associated with degeneration of ACh neurones in this brain region (Decker 1987; Fischer et al. 1992; Sani et al. 2003; Toledano & Alvarez 2004). In this sense and interestingly, in aged monkeys, loss of ACh neurones displayed a close association with the accumulation of Aβ plaques (Toledano & Alvarez 2004).

### 1.2.1. Lesion animal models of AD

Based on the selective loss of the ACh system in AD (Bartus et al. 1982), several models with lesions in the nucleus basalis magnocellularis (NBM), which is the equivalent of the nbM in humans (Pepeu & Marconcini Pepeu 1994; Wellman & Pelleymunter 1999), in the diagonal band of Broca and the septum (Toledano & Alvarez 2004) have been used as animal models of AD (see also Table 1.3).

Early studies relied on electrolytic lesions, which damage both the intended lesion area such as the basal forebrain but also more distal regions, hence causing extended global lesions (Lescaudron & Stein 1999; Toledano & Alvarez 2004). In addition, early global lesion models used non-specific neurotoxins including: ibotenic acid (a potent AMPA receptor agonist), quisqualic acid (another AMPA and group I metabotropic glutamate receptor agonist), quinolic acid (neuroexcitatory tryptophan metabolite), vincristine and colchicine (microtubule poisons) as well as NMDA and Aβ (Beninger et al. 1986; Dunnett et al. 1987; Nilsson et al. 1992; Giovannelli et al. 1998; Eijkenboom & Van Der Staay 1999). Administration of these toxins trigger various forms of neuronal dysfunction such
as inhibition of microtubule polymerisation, impaired axonal transport and excitotoxic neurodegeneration (Olney 1994; Toledano & Alvarez 2004).

More advanced lesion models utilise selective neurotoxins that specifically target ACh neurones in the NBM, the septum and the diagonal band of Broca; whilst preserving non-ACh neurones in the lesioned regions of the brain (Wiley 1992; Toledano & Alvarez 2004). Selective lesions of the ACh neurones was achieved by administration of AF64A cholinotoxin (which binds to the high affinity choline uptake system) and 192 IgG-saporin (a monoclonal antibody against the nerve growth factor receptor coupled to the ribosomal toxin saporin causing selective degeneration of ACh neurones) (Hanin 1996; Toledano & Alvarez 2004). Rats with selective ACh lesions displayed impaired cognitive functions such as learning and memory (Nilsson et al. 1992; Lim et al. 2001).

Lesion studies were especially important in establishing the role of ACh system in cognitive processes (Toledano & Alvarez 2004); however, lesioned animals do not exhibit the neuropathological hallmarks (Aβ plaques and NFTs) associated with AD, which is critical for understating the relation between AD-related neuropathology and its association with cognitive dysfunction (Gotz et al. 2004a; Gotz et al. 2004b).

1.2.2. Single and double transgenic mouse models of AD
Following the development of the first transgenic mouse model of AD in 1995 (Games et al. 1995), over the last 17 years several experimental transgenic animals replicating various neuropathological features associated with the disease have been developed (see Table 1.3).

At first, simple transgenic models were produced that expressed either single mutation in the Aβ-related proteins (APP, PS-1 and PS-2) or tau protein. Games and colleagues (1995) described the first transgenic mouse model of AD carrying the single APP V717F (PDAPP) mutation (Games et al. 1995). PDAPP transgenic mice exhibit extracellular Aβ deposits accompanied by dystrophic neurites, synaptic loss and astrogliosis (Games et al. 1995; Irizarry et al. 1997).
Another study by Hsiao and co-authors (1996) led to the development of Tg2576 transgenic mice carrying the Swedish (KM670/671NL, APP<sub>swe</sub>) mutation (Hsiao et al. 1996). APP<sub>swe</sub> transgenic mice display abundant extracellular Aβ plaques and impaired learning and memory function starting from 9 months of age (Hsiao et al. 1996). However, no correlations were found between Aβ plaque deposition and memory dysfunction in APP<sub>swe</sub> mice (Hsiao et al. 1996; Gotz et al. 2004b). The APP23 transgenic mouse model was generated by inserting human APP751 (Swedish double mutation 670/671 KM→NL) into the murine Thy-1 promoter (Sturchler-Pierrat et al. 1997). In addition to progressively increased Aβ plaques, APP23 mice were the first transgenic mouse model that displayed between 14–25% loss of pyramidal neurones in the CA1 subfield of the hippocampus (Calhoun et al. 1998). Later studies led to the development of other APP transgenic mouse models of AD with severe Aβ neuropathology including the APP<sub>751SL</sub> and APP<sub>V717F</sub> mice, among others (Dodart et al. 2000; Blanchard et al. 2003) (see also Table 1.3).

Subsequently, multiple transgenic mouse models of AD were generated in response to the identification of the PS-1 and PS-2 mutations, which account for the majority of FAD cases (Gotz et al. 2004a; Gotz et al. 2004b). In order to elucidate the role of genetic risk factors in APP processing and memory impairment, double transgenic mouse models of AD have been generated by cross-breeding the PS-1/PS-2 and APP mice (Holcomb et al. 1998; Richards et al. 2003). As a result, multiple double transgenic mouse models, carrying the PS-1/PS-2 and APP double mutations, have been produced including: APP<sub>751SL</sub>/PS<sub>1M146L</sub>, APP<sub>Swe</sub>/PS<sub>1M146L</sub>, APP/PS1KI and PS2/APP<sub>Swe</sub> transgenic mouse models of AD (Janus et al. 2000; Takeuchi et al. 2000; Richards et al. 2003; Casas et al. 2004). All transgenic mouse models of AD carrying the APP/PS-1 or the APP/PS-2 double mutations display early and aggressive Aβ plaque deposition that is associated with learning and memory impairment from younger age compared to single transgenic lines, although these mice do no exhibit evident NFT pathology (Borchelt et al. 1997; Holcomb et al. 1998; Richards et al. 2003). In addition to accelerated Aβ plaque deposition, stereological analysis revealed that the APP<sub>751SL</sub>/PS<sub>1M146L</sub> and APP/PS1KI double transgenic mice also displayed neuronal loss in the hippocampus and the cortex (Casas et al. 2004; Schmitz et al. 2004).
Parallel to the development of APP, PS-1 and PS-2 transgenic mouse models of AD, pathological tau transgenic mice were also generated, with the first transgenic tau model (ALZ7) being created in 1995 (Gotz et al. 1995). The ALZ7 tau transgenic mice demonstrated hyperphosphorylation of tau protein localised in the somatodendritic processes but did not display evident NFT pathology (Gotz et al. 1995). Following the identification of the pathogenic mutations of tau gene in the frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), different transgenic models with clear neuronal NFT pathology were generated (Gotz et al. 2004b; Cassel et al. 2008). The tau<sub>P301L</sub> mutation is most commonly linked with FTDP-17 (Lewis et al. 2000). Transgenic mice over-expressing the tau<sub>P301L</sub> mutation display evident NFT pathology although without Aβ aggregates and/or severe neurodegeneration, except for selective loss of motor neurones in the spinal cord (Lewis et al. 2000; Gotz et al. 2004b).

Taken together, the majority of the animal models mentioned above, fail to express both pathological hallmarks of AD namely: Aβ plaques and NFTs associated with extensive neurodegeneration (Janus & Westaway 2001; Gotz et al. 2004b). In addition, the spatio-temporal distribution of Aβ plaques and NFT pathologies in AD brains have not been reproduced in any of these transgenic mouse models (Kurt et al. 2003). Furthermore, comparisons between two different transgenic lines are extremely difficult, due to differences in the integration sites of the promoter and the genetic background of these animals (Gotz et al. 2004a; Gotz et al. 2004b).
### Table 1.3. Neuropathology in the main AD animal models.

<table>
<thead>
<tr>
<th>Lesion and transgenic mouse, rat and primate models</th>
<th>Neuropathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ageing and lesioned studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ageing and lesioned studies</td>
<td>Cholinergic involution and Aβ deposition</td>
<td>(Michalek et al. 1989; Fischer et al. 1992; Sani et al. 2003)</td>
</tr>
<tr>
<td>Electrolytic lesion</td>
<td>Neuronal death</td>
<td>(Lescaudron &amp; Stein 1999; Vale-Martinez et al. 2002)</td>
</tr>
<tr>
<td>Unspecific toxins (NMDA, Ibotenic acid, Quisalic acid, Quinolic acid, Colchicine, Alkaloids, Alcohol)</td>
<td>Neuronal death</td>
<td>(Boegman et al. 1985; Di Patre et al. 1989; Dunnett et al. 1991; Arendt 1994; Shaughnessy et al. 1994; Winkler et al. 1998)</td>
</tr>
<tr>
<td>Aβ</td>
<td>Cholinergic dysfunction</td>
<td>(Pavia et al. 2000; Giovannini et al. 2002)</td>
</tr>
<tr>
<td><strong>Transgenic mouse models with Aβ pathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDAPP</td>
<td>Plaques</td>
<td>(Games et al. 1995)</td>
</tr>
<tr>
<td>APPswe</td>
<td>Plaques</td>
<td>(Hsiao et al. 1996)</td>
</tr>
<tr>
<td>PS-1/APPswe</td>
<td>Plaques</td>
<td>(Borchelt et al. 1997)</td>
</tr>
<tr>
<td>APP23</td>
<td>Plaques</td>
<td>(Sturchler-Pierrat et al. 1997)</td>
</tr>
<tr>
<td>PSAPP (PS-1/Tg2576)</td>
<td>Plaques</td>
<td>(Holcomb et al. 1998)</td>
</tr>
<tr>
<td>APPswe and PS1M146</td>
<td>Plaques</td>
<td>(Doddart et al. 2000)</td>
</tr>
<tr>
<td>K670N/M671L and V717I</td>
<td>Plaques</td>
<td>(Janus et al. 2000)</td>
</tr>
<tr>
<td>TgAPPswe and PS1M146</td>
<td>Plaques</td>
<td>(Janus et al. 2000)</td>
</tr>
<tr>
<td>Tg-CRNDA</td>
<td>Plaques</td>
<td>(Chishti et al. 2001)</td>
</tr>
<tr>
<td>APPswe/PS1M146</td>
<td>Plaques</td>
<td>(Blanchard et al. 2003)</td>
</tr>
<tr>
<td>PS2APP (PS2/APPswe)</td>
<td>Plaques</td>
<td>(Richards et al. 2003)</td>
</tr>
<tr>
<td>APPswe/PS1s</td>
<td>Plaques</td>
<td>(Savonenko et al. 2005)</td>
</tr>
<tr>
<td>Tg478/Tg111I</td>
<td>Plaques</td>
<td>(Flood et al. 2009)</td>
</tr>
<tr>
<td>K670M/M671L</td>
<td>Plaques</td>
<td>(Klosowska et al. 2010)</td>
</tr>
<tr>
<td><strong>Transgenic mouse models with tangle pathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALZ5</td>
<td>Tangles</td>
<td>(Gotz et al. 1995)</td>
</tr>
<tr>
<td>TauP301L(4R,2-3-)</td>
<td>Tangles</td>
<td>(Lewis et al. 2000)</td>
</tr>
<tr>
<td>P301L</td>
<td>Tangles</td>
<td>(Gotz et al. 2001)</td>
</tr>
<tr>
<td>7Taur1γ</td>
<td>Tangles</td>
<td>(Ishihara et al. 2001)</td>
</tr>
<tr>
<td>P301S</td>
<td>Tangles</td>
<td>(Allen et al. 2002)</td>
</tr>
<tr>
<td>V337M</td>
<td>Tangles</td>
<td>(Tanemura et al. 2002)</td>
</tr>
<tr>
<td>AR2N</td>
<td>Tangles</td>
<td>(Tatebayashi et al. 2002)</td>
</tr>
<tr>
<td>TauP301L</td>
<td>Tangles</td>
<td>(Ramsden et al. 2005)</td>
</tr>
<tr>
<td>P301L.TET-off</td>
<td>Tangles</td>
<td>(Schindowska et al. 2006)</td>
</tr>
<tr>
<td>P301S/2G72V</td>
<td>Tangles</td>
<td>(Eriksen &amp; Janus 2007)</td>
</tr>
<tr>
<td>G272V.P301L.R406W</td>
<td>Tangles</td>
<td></td>
</tr>
<tr>
<td><strong>Transgenic mice with Aβ plaques tangle pathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg2576xJNPL3 (APPswe)</td>
<td>Plaques and Tangles</td>
<td>(Lewis et al. 2001)</td>
</tr>
<tr>
<td>Tg2576 and V150</td>
<td>Plaques and Tangles</td>
<td>(Ribe et al. 2003)</td>
</tr>
<tr>
<td>5x1g-AD</td>
<td>Plaques and Tangles</td>
<td>(Oddo et al. 2003a; Oddo et al. 2003b)</td>
</tr>
<tr>
<td>TauPS2APP</td>
<td>Plaques and Tangles</td>
<td>(Grauerger et al. 2010)</td>
</tr>
<tr>
<td>5x1g-CX3cr1t+ 5x1g</td>
<td>Plaques and Tangles</td>
<td>(Puhrmann et al. 2010)</td>
</tr>
<tr>
<td>5x1g-CX3cr1t+</td>
<td>Plaques and Tangles</td>
<td>(Puhrmann et al. 2010)</td>
</tr>
</tbody>
</table>

Note: Highlighted in yellow are ageing and lesioned studies that involved ACh neuronal death, highlighted in green are transgenic mouse model of AD with Aβ pathology; whilst highlighted in blue are transgenic mice with tangle pathology. In addition, highlighted in red are transgenic mouse models, which displayed both Aβ plaques and tangle pathologies.

Modified from (Rodríguez & Verkhratsky 2011a).
1.2.3. The triple transgenic (3xTg-AD) mouse model of AD

The triple transgenic (3xTg-AD) mouse model of AD was developed in 2003 and harbours three mutant genes for APP\textsubscript{Swe}, PS1\textsubscript{M146V} and tau\textsubscript{P301L} (Oddo \textit{et al.} 2003a; Oddo \textit{et al.} 2003b). The 3xTg-AD mouse model of AD was derived from a single genetic background by introduction of tau\textsubscript{P301L} and APP\textsubscript{Swe} transgenes into a single-cell embryo harvested from the PS1\textsubscript{M146V} mutant mouse (Oddo \textit{et al.} 2003a; Oddo \textit{et al.} 2003b). This method has the advantage of minimising the genetic interactions produced when crossing three independent lines of mice (Gotz \textit{et al.} 2004a; Gotz \textit{et al.} 2004b). Co-integration of APP\textsubscript{Swe} and tau\textsubscript{P301L} mutations at the same loci also reduces the independent assortment of these mutations in subsequent generations (Oddo \textit{et al.} 2003b).

3xTg-AD mice develop both Aβ-containing neuritic plaques and NFTs with a spatio-temporal distribution that closely resembles AD neuropathology in human brains (Oddo \textit{et al.} 2003a; Oddo \textit{et al.} 2003b).

Intraneuronal Aβ deposition becomes evident from 3 months of age in 3xTg-AD mice within the cortex, the amygdala and the pyramidal neurones of the hippocampus (Oddo \textit{et al.} 2003a; Billings \textit{et al.} 2005; Rodríguez \textit{et al.} 2008). These mice display age-associated increase in Aβ accumulation with the first extracellular build up of Aβ plaques appearing between 9 and 12 months of age in the cortex and the subiculum (Oddo \textit{et al.} 2003a). By 18 months of age, numerous extracellular Aβ plaques become evident within the hippocampus (Oddo \textit{et al.} 2003a; Mastrangelo & Bowers 2008; Noristaní \textit{et al.} 2010; Olabarria \textit{et al.} 2010; Rodríguez \textit{et al.} 2010; Olabarria \textit{et al.} 2011) (see also Fig. 1.13A).

Tau neuropathology initially appears within the pyramidal neurones in the CA1 subfield of the hippocampus by 12 months of age in 3xTg-AD mice (Oddo \textit{et al.} 2003a; Mastrangelo & Bowers 2008; Rodríguez \textit{et al.} 2008). Hyperphosphorylation of tau protein continues throughout ageing in 3xTg-AD mice with the first PHF structure appearing at 18 months of age (Oddo \textit{et al.} 2003a; Mastrangelo & Bowers 2008). Similar to the Aβ neuropathology, 3xTg-AD mice also display an age-related increase in NFT neuropathology in the cortex and the hippocampus (Oddo
et al. 2003a; Oddo et al. 2003b; Mastrangelo & Bowers 2008) (see also Fig. 1.13B).

Figure 1.13. Brightfield photomicrographs showing the presence and deposition of extracellular Aβ plaques and intraneuronal accumulation of hyperphosphorylated tau protein within the CA1 subfield of the hippocampus in an 18 months old 3xTg-AD mouse model of AD. From (Rodríguez et al. unpublished photomicrographs).

Recent studies in our laboratory have reported, in addition, reduced adult neurogenesis (Rodríguez et al. 2008; Rodríguez et al. 2009a), alterations in astroglial morphology (Rodríguez et al. 2009b; Olabarria et al. 2010; Yeh et al. 2011), compromised astroglial functionality (Olabarria et al. 2011) and reduced hippocampal perforated synapses (Noristani et al. 2011) in 3xTg-AD mice which, together with depositions of Aβ and NFT neuropathology may also contribute to deficits in learning and memory functions observed in this mouse model of the disease (see chapter 1, section 1.2.4.).

1.2.3.1. The quintuple transgenic (5xTg) mouse model of AD
Latest studies to better understand the interaction between neurones and glia cells led to the generation of a quintuple transgenic (5xTg-CX3cr1+/− or 5xTg-CX3cr1−/−, 5xTg) mice (Fuhrmann et al. 2010). In fact, this transgenic mouse model is identical to the previously described 3xTg-AD mice with additional genetic crossing that led to neurones being fluorescently labelled with yellow fluorescent protein and microglia cells labelled with green fluorescent proteins (Fuhrmann et al. 2010) (Fig. 1.14). Specifically, 5xTg mice were generated by crossing the 3xTg-AD mouse with heterozygous of the YFP-H line and heterozygous CX3CR1+/−GFP
mice. The YFP-H line selectively express yellow fluorescent protein in neurones (Feng et al. 2000), whilst the CX3CR1+eGFP mice selectively express enhanced green fluorescent protein (GFP) in the microglia (Jung et al. 2000; Srivastava et al. 2005) (Fig. 1.14).

**Figure 1.14.** Generation of the 5xTg-Cx3cr1 mouse model of AD. 5xTg-CX3cr1+/- or 5xTg-CX3cr1-/- mice were heterozygous for thy1-YFP+/- and homozygous for PS1M146V APPSwe and tauP301L. The YFP-H line exhibits YFP-expression in subsets of cortical layer III and V neurones. The Cx3cr1 mice carry a GFP knock-in in the cx3cr1-locus. Fluorescence images of the cortex show the neuronal YFP-expression, microglia GFP-staining as well as hyperphosphorylated tau and Aβ deposition. Scale bars = 60 μm, 30 μm (tau, Aβ).

From (Fuhrmann et al. 2010).

Using two-photon in vivo imaging in the 5xTg mouse model of AD, it was shown that there is an increase in microglia numbers that are particularly evident in the close vicinity of degenerative neurones (Fuhrmann et al. 2010).

1.2.4. Cognitive deficits in the 3xTg-AD mouse model of AD
Learning and memory performance in 3xTg-AD mice have been evaluated using multiple behavioural tests including the spatial reference version of the MWM, Barnes maze (BM) and inhibitory avoidance (IA) tests (Billings et al. 2005; Frazer et al. 2008; McKee et al. 2008) (see also Table 1.4). These memory tasks are highly dependent upon normal function of the amygdala and the hippocampus (Billings et al. 2005).
Table 1.4. Summary of age-associated behavioural studies in the 3xTg-AD mouse model of AD and their main findings in relation to cognitive function.

<table>
<thead>
<tr>
<th>Age</th>
<th>Behavioural Test</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Months</td>
<td>MWM and IA</td>
<td>No Difference in spatial and contextual memory</td>
<td>Billings et al. 2005; Billings et al. 2007; Clinton et al. 2007; McKee et al. 2008</td>
</tr>
<tr>
<td>4 Months</td>
<td>MWM</td>
<td>Impaired memory retention</td>
<td>(Billings et al. 2005; Billings et al. 2007; Clinton et al. 2007)</td>
</tr>
<tr>
<td>6 Months</td>
<td>MWM, IA, BM</td>
<td>Impaired spatial and contextual memory</td>
<td>(Billings et al. 2005; Billings et al. 2007; McKee et al. 2008)</td>
</tr>
<tr>
<td>7 Months</td>
<td>Y-maze</td>
<td>Impaired working memory</td>
<td>(Rosario et al. 2006)</td>
</tr>
<tr>
<td>9 Months</td>
<td>MWM, IA, Object Recognition</td>
<td>Impaired spatial memory</td>
<td>(Billings et al. 2007; Clinton et al. 2007)</td>
</tr>
<tr>
<td>12 Months</td>
<td>MWM</td>
<td>Impaired spatial and contextual memory</td>
<td>(Billings et al. 2007)</td>
</tr>
<tr>
<td>13 Months</td>
<td>T-maze</td>
<td>Impaired working memory</td>
<td>(Rosario et al. 2006)</td>
</tr>
<tr>
<td>15 Months</td>
<td>MWM</td>
<td>Impaired spatial memory</td>
<td>(Billings et al. 2007)</td>
</tr>
<tr>
<td>16 Months</td>
<td>T-maze</td>
<td>Impaired working memory</td>
<td>(Caccamo et al. 2007)</td>
</tr>
<tr>
<td>18 Months</td>
<td>MWM</td>
<td>Impaired spatial memory</td>
<td>(Billings et al. 2007)</td>
</tr>
<tr>
<td>23 Months</td>
<td>T-maze</td>
<td>Impaired working memory</td>
<td>(Oddo &amp; LaFerla 2006)</td>
</tr>
</tbody>
</table>

Key: **MWM**: Morris water maze, **IA**: inhibitory avoidance, **BM**: Barnes maze test. Note: Highlighted in green are studies that reported normal cognitive performance in young (2 months old) 3xTg-AD mice; whilst highlighted in yellow are studies that reported age-associated progressive cognitive decline in the 3xTg-AD mouse model of AD. From personal library.

Up to 2 month of age, 3xTg-AD mice display normal synaptic transmission and plasticity including LTP that was associated with normal memory performance at this age (Oddo et al. 2003b; Billings et al. 2005; Billings et al. 2007; McKee et al. 2008). Impaired memory retention is the first sign of cognitive deterioration in 3xTg-AD mice that becomes evident by 4 months of age and correlates with intraneuronal Aβ deposition (Billings et al. 2005). Electrophysiological recordings of 6 months old 3xTg-AD mice have shown impaired LTP that was associated with decline in cognitive performance including spatial memory, fear memory and contextual learning (measured using MWM, BM and IA tests) (Oddo et al. 2003b; Billings et al. 2005; Caccamo et al. 2006; Clinton et al. 2007; McKee et al. 2008). The 3xTg-AD mouse model of AD display age-associated progressive decline in learning and memory function that are linked with increased Aβ plaques and NFTs depositions in the cortex and the hippocampus (Billings et al. 2007; Clinton et al. 2007; McKee et al. 2008).

Taken together, the 3xTg-AD mouse model of AD represents an ideal animal model to study AD-related alterations because they not only express both pathological hallmarks of AD (Aβ plaques and NFTs) in spatio-temporal manner similar to AD brains, but they also display age-related progressive impairment in multiple cognitive tasks, as seen in AD patients.
1.3. **SEROTONERGIC NEUROTRANSMISSION**

1.3.1. The discovery of serotonin

Serotonin (5-hydroxytryptamine or 5-HT) has long been the subject of study in many areas of biomedical and neuroscience research. Two independent laboratories made enormous contributions into the isolation and characterisation of 5-HT almost 75 years ago. 5-HT was initially discovered in 1937 in Italy, where Erspamer and Vialli observed that a substance derived from the enterochromaffin cells of the intestine triggered contraction of the smooth muscles (Vialli & Erspamer 1937-38). Erspamer and Vialli originally named this substance "enteramine" (Vialli & Erspamer 1942). Later on, in 1948 in the United State, Rapport, Green and Page identified and purified a substance from the beef serum, which caused vascular contraction (Rapport et al. 1948b; Rapport et al. 1948a; Rapport 1949). Rapport and colleagues (1948-1949) coined the name serotonin because of its original purification from the serum (sero-) and its effect on the vessel tone (-tonin) (Rapport et al. 1948b; Rapport et al. 1948a; Rapport 1949). Three years later, it was shown that enteramine and serotonin were indeed the same substance (Erspamer & Asero 1952), for review see (Harper 1964). Others reported the presence of 5-HT in the brain and suggested its role as a neurotransmitter (Bogdanski et al. 1956), which initiated the surge of research into the role of 5-HT neurotransmission not only in the central nervous system (CNS) but also in the periphery.

1.3.2. Serotonin neurotransmission in the periphery

1.3.2.1. Serotonin synthesis

The enterochromaffin cells of the intestine is the major site for peripheral 5-HT synthesis, storage and release (Ni & Watts 2006). In fact, over 95% of the total body 5-HT is produced by the enterochromaffin cells of the intestine (Linder et al. 2007). Other sites where 5-HT synthesis takes place include the raphe nuclei of the brain stem, the pineal gland, the heart, the kidney and the neuroendothelial cells lining the lung (Stier & Itskovitz 1985; Ikeda et al. 2005; Ni & Watts 2006).

5-HT is an indoleamine, consisting of an indole ring and a carboxyl-amide side chain (Fig. 1.15). The synthesis of 5-HT is a two-step enzymatic pathway involving two enzymes: tryptophan hydroxylase (TPH) and 5-hydroxytryptophan...
decarboxylase (5-HPDC). The first step involves the addition of a hydroxyl group to the aromatic amino acid l-tryptophan (TrP) by the TPH, which forms 5-hydroxytryptophan (5-HTP) (Fig. 1.15, step 1). The hydroxylation of TrP is the rate-limiting step in 5-HT synthesis (Grahame-Smith 1967). Subsequent decarboxylation of 5-HTP by 5-HPDC results in 5-HT synthesis (Ni & Watts 2006) (Fig. 1.15, step 2).

![Figure 1.15. Serotonin synthesis and metabolism pathway. Key: TPH: tryptophan hydroxylase, 5-HPDC: 5-Hydroxytryptophan decarboxylase, MAO-A: monoamine oxidase type A.](image)

5-HT is synthesised independently in peripheral tissues and in the brain by two different rate-limiting TPH isoforms (Walther & Bader 2003). The TPH1 isoform regulate 5-HT synthesis in the periphery; whilst the TPH2 isoform is responsible for 5-HT synthesis in the brain (Walther & Bader 2003; Walther et al. 2003a) (see also Fig. 1.16). The existence of the two different isoforms of TPH (TPH1 and TPH2) was initially described in 2003 by Walther and colleagues (Walther & Bader 2003; Walther et al. 2003a). Using TPH-knockout mice (TPH−/−), the authors
observed that although TPH\(^{-/-}\) mice displayed deficient 5-HT in the periphery, the level of 5-HT in the brain was close to normal (Walther & Bader 2003). It was shown that the gene for TPH1 is expressed in the gut, the pineal gland, the spleen and the thymus; whilst the gene for TPH2 is predominantly expressed in the brain stem (Walther & Bader 2003).

1.3.2.2. Serotonin storage
In addition to their critical role in 5-HT synthesis, the enterochromaffin cells of the intestine also store 5-HT (Linder et al. 2007). The majority of 5-HT is stored in the platelets, which take up 5-HT from the lungs and the gut through serotonin transporter (SERT) (Ni & Watts 2006). In platelets, 5-HT is stored in granules that requires active uptake of 5-HT from the cytoplasm via vesicular monoamine transporter 2 (VMAT2) (Linder et al. 2007). Platelet 5-HT acts as a buffer and maintains free circulating 5-HT at low concentration (Ni & Watts 2006). Not including platelets, the level of free unbound 5-HT in the plasma is kept between 15 – 120 nM, which are considerably lower compared to the levels of 5-HT in the whole blood (μM range) (Linder et al. 2007). In addition to the enterochromaffin cells and the platelets, minor 5-HT storage is also detected in the aorta, the carotid, the superior mesenteric arteries (Ni et al. 2004) and the saphenous vein (Cohen et al. 1999), although currently it is not clear how 5-HT storage takes place in the aorta and the veins.

1.3.2.3. Serotonin metabolism (catabolism and melatonin synthesis)
The catabolism of 5-HT occurs via monoamine oxidase A (MAO-A) enzyme, which generates the acidic 5-HT metabolite (5-hydroxyindole acetic acid, 5-HIAA) (Fig. 1.15, step 3). The produced 5-HIAA is excreted from the cell by an energy-dependent clearance mechanism before its excretion from the body in the urine (Fig. 1.16). There are two types of MAO enzymes, depending on their relative selectivity for specific substrates (Linder et al. 2007). MAO-A enzyme is involved in the catabolism of NA and 5-HT; whilst MAO-B enzyme preferentially metabolises DA (Linder et al. 2007). The MAO-A enzyme is located inside the mitochondrial membrane (Fig. 1.16), therefore 5-HT must be taken up inside the cells before undergoing catabolism (Ni & Watts 2006). The majority (over 90%) of 5-HT catabolism takes place in the pulmonary circulation of the lungs (Linder et al.
However, any cell that is able to take up 5-HT and possess the necessary enzyme (MAO-A) has the potential to break-down 5-HT into 5-HIAA (Ni & Watts 2006; Linder et al. 2007). Indeed, catabolism of 5-HT have been also reported in the aorta, the carotid, the superior mesenteric arteries (Ni et al. 2004) and in the saphenous vein (Cohen et al. 1999).

In the pineal gland 5-HT undergoes different enzymatic processing that leads to generation of melatonin (Waldhauser et al. 1993). Melatonin is involved in regulation of the sleeping and waking cycles (Waldhauser et al. 1993; Shedpure & Pati 1995). Unlike the CNS, the pineal gland is not isolated from the peripheral system by the blood brain barrier (BBB) and therefore receives its 5-HT input from the periphery (Shedpure & Pati 1995). The first step in melatonin synthesis involves the rate-limiting enzyme 5-HT N-acetyltransferase, which catalyses the conversion of 5-HT into N-acetylserotonin. The latter (N-acetylserotonin) is rapidly converted to melatonin by the enzyme hydroxyindole-omethyltransferase. The activity of 5-HT N-acetyltransferase modulates melatonin synthesis during circadian fluctuations (Klein & Weller 1970; Klein et al. 1997).

1.3.2.4. Uptake and release of peripheral serotonin
Under physiological conditions 5-HT is a protonated molecule (due to a + charge in its amino group), which prevents it from crossing freely the phospholipid bilayer of the cell membrane. The uptake and release of 5-HT is carried out by SERT, which acts as a bi-directional transporter (Ni & Watts 2006). SERT is the major transporter responsible for the uptake and release of 5-HT from cells (Linder et al. 2007). The expression and cloning of SERT gene was initially reported in rats (Blakely et al. 1991; Hoffman et al. 1991), which shortly followed by its identification in humans (Ramamoorthy et al. 1993) and mice (Chang et al. 1996). SERT is an integral protein that is positioned in the plasma membrane with 12 transmembrane domains (Fig. 1.16). SERT protein terminates 5-HT action by removing the liberated 5-HT neurotransmitter from the release site and transporting it back to the 5-HT liberated cells for metabolism and storage (Ni & Watts 2006). In the periphery, high density of SERT is expressed throughout the body including the platelets, the pulmonary arteries (Eddahibi et al. 2000), the smooth muscles, the endothelial cells (Ni et al. 2004), the kidney, the lung, the heart, the liver, the
thyroid gland, the small intestine, the pancreas (Mortensen et al. 1999) and the adrenal glands (Schroeter et al. 1997).

![Figure 1.16. Serotonin release and re-uptake pathways.](image)


*Modified form (Ni & Watts 2006).*

1.3.2.5. The role of serotonin in the periphery

Peripheral 5-HT affects multiple systems including the platelets, the heart, the kidney, the adrenal glands and the blood vessels (Ni & Watts 2006) (see also Fig. 1.14). In platelets, 5-HT promotes continued aggregation that prevents bleeding following tissue damage (Vanhoutte 1991). Increased platelet 5-HT release also acts as a vasoconstrictor and causes narrowing of the airways (Rohrbach et al. 1993).
1984). In the heart, 5-HT induces both an increase (via activation of myocardial 5-HT$_2$, 5-HT$_4$ and 5-HT$_7$ receptors) and a decrease (via activation of 5-HT$_3$ receptors located in the cardiac vagal afferent) on the cardiac rate (Saxena & Villalon 1991; Villalon & Centurion 2007). In the kidney, 5-HT increases renal perfusion (Moran et al. 1997) and promotes phosphate excretion (Berndt et al. 2001). 5-HT also increases the vasoconstriction of renal arteries, which may be mediated by activation of 5-HT$_{2C}$ receptors expressed in the renal artery and the kidney (Mora et al. 2008). In the adrenal glands, 5-HT increases the release of adrenaline and NA, which in turn can affect the cardiovascular system by increasing the total peripheral resistance (Sugimoto et al. 1996).

In the blood vessels, 5-HT has both vasoconstriction and vasodilatation effects depending on the species, the type of the blood vessels and the status of the endothelial cell layer (Ni & Watts 2006). Pharmacological doses of 5-HT caused renal vasoconstriction in rats (Moran et al. 1997) and vasodilatation in dogs (Tian et al. 2002). This effect may be due to activation of different pathways by 5-HT in rats and dogs (Moran et al. 1997; Tian et al. 2002). More specifically, in rats, the vasoconstriction effect of 5-HT is mediated by stimulation of 5-HT$_2$ receptors and activation of angiotensin II (Moran et al. 1997). However, in dogs, the vasodilatation effect of 5-HT is mediated by activation of 5-HT$_2$ receptors and increased nitric oxide release/production (Tian et al. 2002). All in all, 5-HT in the periphery acts as a ubiquitous hormone that is involved in platelet aggregation, regulation of heart rate, increased renal perfusion and vasoconstriction/vasodilatation of the blood vessels (Fig. 1.17).
1.3.2.6. The effect of peripheral 5-HT on central 5-HT signalling

Although 95% of the 5-HT in the body is present in the periphery, it does not affect brain 5-HT neurotransmitter level because the peripheral 5-HT does not cross the BBB (Young 2007). In this sense, TPH1 knock-out mice displayed normal 5-HT levels in the brain even though their peripheral levels of 5-HT were severely depleted (Walther et al. 2003a; Walther et al. 2003b). These findings suggest that the peripheral levels of 5-HT have no effect on central 5-HT levels in the brain (Walther & Bader 2003). The two independent 5-HT synthesis pathways in the

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**Figure 1.17.** Schematic representation of the peripheral effects of serotonin. Note: The TPH1 isoform regulates 5-HT synthesis in the periphery, whilst the TPH2 isoform is involved in regulation of 5-HT synthesis in the brain. This indicates that the peripheral and central functions of 5-HT are differentially regulated and can be targeted independently. Based on (Walther and Bader, 2003).
periphery and in the brain allow therapeutic approaches that target specifically either the peripheral or the central 5-HT neurotransmission.

1.3.3. Tryptophan as a precursor of serotonin

1.3.3.1. Source and effect of tryptophan in the body
Tryptophan (TrP) is one of the 9 essential amino acids that is not synthesised by the body and has to be taken through the diet and supplements (Furst & Stehle 2004). A good nutritional source for TrP includes turkey, chicken, beef, fish, eggs, milk, cheese, nuts, vegetables and fruits, especially banana.

TrP is the only amino acid circulating in the plasma and is loosely bound to the serum albumin (Mc & Oncley 1958). In resting conditions, more than 90% of TrP is bound to the serum albumin and forms a complex that is not able to cross the BBB. On the other hand, the concentration of the free fraction of TrP in the serum regulates the availability of TrP in the brain. This is because only the free TrP is transported across the BBB (Tagliamonte et al. 1971; De Montis et al. 1977). Free TrP is transported into the brain via the competitive and non-specific L-type amino acid transporter (LAT1), which is highly expressed at the BBB (Boado et al. 1999).

Under the normal physiological conditions, the availability of TrP determines the biosynthesis of 5-HT both in the periphery and in the brain (Fernstrom 1983). TrP is taken as part of the normal diet and acts as 5-HT precursor. Therefore, changes in brain TrP content causes an alteration in 5-HT biosynthesis (Knott & Curzon 1972). Acute administration of TrP-free diet reduced serum TrP level and was associated with decreased concentrations of TrP, 5-HT and 5-HIAA in the rat brain (Biggio et al. 1974; Gessa et al. 1974). In addition, TrP-free diet also reduced 5-HT release in the cortex (Fadda et al. 2000) and the hippocampus of freely moving rats (Stancampiano et al. 1997). TrP-depleted diet had no effect on extracellular NA content in the frontal cortex, suggesting that the diet specifically affects 5-HT neurotransmission in the brain and has no effect on other neurotransmitter systems (Fadda 2000; Fadda et al. 2000). These data suggest that alteration of dietary TrP intake (relative to constant level of other amino acids) provides an ideal non-pharmacological mean for effectively modifying the level of 5-HT in the brain.
These diets can be used to study the physiological and behavioral effects of altered 5-HT neurotransmission on the brain function.

The availability of TrP in the brain also depends on the relative plasma concentration of other amino acids that compete with the free plasma TrP for active transport across the BBB into the brain namely: tyrosine, phenylalanine, leucine, isoleucine and valine (Guroff & Udenfriend 1962; Oldendorf 1971; Fernstrom & Wurtman 1972; Fernstrom et al. 1975; Fernstrom 1983). Increased intake of these amino acids that compete with TrP for transport across the BBB produced a modest but significant decline in brain TrP and 5-HIAA levels (Gessa et al. 1974).

Although there is a continued debate as to which of the two mechanisms are more important, it is likely that combination of the two factors (free fraction of TrP and plasma concentration of other amino acids) are critical for regulation of TrP availability in the brain. In the chapter 5 of this thesis, alteration of dietary TrP intake (relative to constant level of other amino acids) has been chosen as an experimental approach to alter 5-HT neurotransmission in the brain of 3xTg-AD mice in order to uncover the role of altered 5-HT neurotransmission on the development of AD-related neuropathology.

1.3.3.2. Other metabolic pathways of tryptophan
In addition to acting as a precursor for 5-HT synthesis, TrP also undergoes another metabolic pathway (called the kynurenine pathway) leading to the production of nicotinamide adenine dinucleotide (NAD) co-enzyme (Ruddick et al. 2006; Chen & Guillemim 2009) (Fig. 1.18). In the first step of the kynurenine pathway, the indole ring of TrP undergoes oxidative opening by two enzymes: the rate-limiting enzyme tryptophan 2,3-dioxygenase (TDO) and the indoleamine 2,3-dioxygenase (IDO).
Figure 1.18. Schematic representation of the kynurenine pathway. Based on (Chen & Guillemin 2009).

Activation of immune response and increased release of stress-related glucorticoid hormones stimulate TrP metabolism through the kynurenine pathway, which lowers TrP concentration and reduces 5-HT biosynthesis in the brain (Schimke et al. 1965; Badawy et al. 1989; Hansen et al. 2000; Wirleitner et al. 2003). On the other hand, administration of commonly prescribed SSRI antidepressants inhibit TrP metabolism through the kynurenine pathway that may contribute to their therapeutic effect via increase in plasma and brain TrP concentrations as well as

In addition to the NAD co-enzyme, the kynurenine pathway of TrP metabolism also produces a number of neuroactive compounds including: 3-hydroxykynurenine and 3-hydroxyanthranilic acid (free radical generators), quinolinic acid (excitotoxic NMDA receptor agonist) and kynurenic acid (neuro-protective NMDA and α7 nAChR antagonist) via specific enzymes (Stone & Perkins 1981; Perkins & Stone 1982; Hilmas et al. 2001; Stone 2001; Stone & Darlington 2002) (Fig. 1.18). Finally, kynurenine pathway generates picolinic acid that has an important role in the leukocyte recruitment and distribution into damaged tissues during inflammatory responses (Bosco et al. 2003).

1.3.3.3. Peripheral effects of tryptophan
In addition to acting as a precursor for 5-HT synthesis and its involvement in the kynurenine pathway, circulating TrP also exert multiple effects on the periphery including protein synthesis, food and water intake, among others, which are briefly described below.

1.3.3.3.1. TrP effect on body weight gain
TrP plays an important role in protein synthesis (Sidransky et al. 1981; Sidransky et al. 1990). Specifically, TrP increased hepatic protein synthesis via promoting DNA-dependent RNA polymerase activity, polyribosomal RNA synthesis and aggregation (Sidransky et al. 1981; Sidransky et al. 1990). The liver uses peripheral TrP during protein synthesis that decreases TrP concentration in the serum and in the brain (Biggio et al. 1974; Gessa et al. 1974). As mentioned earlier, reduced dietary TrP intake decreased TrP levels in the plasma (De Marte & Enesco 1985), which in turn reduced the level of protein synthesis necessary for normal growth (Kantak et al. 1980; De Marte & Enesco 1986; Gonzalez-Burgos et al. 1998). Inhibition of protein synthesis process in rats (by treatment with cycloeximide, a protein synthesis inhibitor) prevent TrP-free diet-induce decrease in serum TrP levels (Gessa et al. 1975), supporting the role of TrP in protein synthesis. Consequently, chronic decrease in dietary TrP intake reduced body weight gain in
normal rats (Gonzalez-Burgos et al. 1998; Orozco-Suarez et al. 2003; Cahir et al. 2007; Jenkins et al. 2010), mice (Kantak et al. 1980; De Marte & Enesco 1986) and chickens (Carew et al. 1983).

Reduced dietary TrP intake also alters thyroid and growth hormone levels that was associated with reduced bone growth and body weight gain in chickens (Carew et al. 1983). On the other hand, peripheral TrP infusion increased 5-HT concentration in the CSF and increased growth hormone release in cattle (holstein steers) (Kasuya et al. 2010), suggesting that TrP-induced increase in growth hormone release is mediated via activation of central 5-HT neurones. Based on reduced plasma thyroid level and lower food conversion efficiencies (weight gain/food intake), Carew and colleagues (1983) postulated that animals fed with low TrP diet displayed energy wastage where the energy intake is being converted to heat rather than protein synthesis (Carew et al. 1983).

1.3.3.3.2. TrP effect on food and water intake
TrP has an inhibitory effect on food consumption. Repeated intraperitoneal injection of TrP suppressed food intake in mice and rats (Ju & Tsai 1995; Amer et al. 2004; Coskun et al. 2006), whereas reduced dietary TrP intake increased food intake in mice (De Marte & Enesco 1986). TrP-induced inhibition of food intake is due to its direct effect on central 5-HT neurotransmission that has been shown to suppress food consumption (Lam et al. 2010). Intraventricular injection of 5-HT neurotoxin, (p-chlorophenylalanine, PCPA) increased food consumption in rats (Breisch et al. 1976). On the other hand, increased 5-HT neurotransmission via administration of SSRI or MAO-A inhibitor (clorgyline) suppressed food intake in mice, rats and hamsters (Feldman 1988; Simansky & Vaidya 1990).

Reduced dietary TrP intake also increased water intake in mice (Kantak et al. 1980). This effect may be mediated by reduced central 5-HT neurotransmission that also exerts a negative effect on drinking behaviour (Castro et al. 2001). Intraventricular injection of 5-HT1C/5-HT2 receptor agonist 6-chloro-2-[1-piperazinyl]-pyrazine (MK-212) induced a decrease in water intake in water deprived rats (Reis et al. 1990). In addition, injection of 5-HT1D receptor agonist (L-694, 247) directly into the third ventricle reduced water intake in dehydrated rats.
(De Castro-e-Silva et al. 1997). Similarly, intraventricular injection of selective 5-HT\textsubscript{3} receptor agonist (m-CPBG) exerted a specific inhibitory effect on water intake induced by pharmacological activation of central ACh pathways in rats (Castro et al. 2002). Furthermore, a recent study has shown that bilateral injection of selective 5-HT\textsubscript{1A/7} receptor agonist -8-hydroxy-2-(di-n-propylamino)-tertralin (8-OH-DPAT) into the lateral septum inhibited water intake in dehydrated rats (de Arruda Camargo et al. 2010).

Altogether, these data suggest that TrP induces an inhibitory effect on food/water intake by modulating central 5-HT neurotransmission.

1.3.4. Serotonin neurotransmission in the brain

1.3.4.1. Serotonin synthesis in the brain

Following its passage through the BBB and into the extracellular fluid or the CSF, TrP is readily available for uptake into the cells of the CNS. TrP from the extracellular fluid is transported into 5-HT neurones by a high-affinity neuronal tryptophan transporter, although the precise molecular identity of this transporter is currently not known (Ruddick et al. 2006). Once inside the 5-HT neuronal cell body, TrP undergoes hydroxylation by the rate-limiting enzyme tryptophan hydroxylase 2 (TPH2), which is located in the cytoplasm and produces 5-hydroxytryptamine (5-HTP) (Grahame-Smith 1967). The activity of TPH2 is increased by the action potential and Ca\textsuperscript{2+}-dependent phosphorylation of the enzyme (Ruddick et al. 2006). The second enzymatic step in 5-HT synthesis is similar to that described for the periphery where 5-HTP undergoes decarboxylation (by 5-HTPDC) and generate 5-HT (Fig. 1.15). Both enzymatic steps involved in central 5-HT synthesis occur within the cell body of 5-HT neurones in the raphe nuclei (Grahame-Smith 1967). The synthesised 5-HT may undergo metabolism by monoamine oxidase A (located on the mitochondrial membrane) that generates the 5-HIAA metabolite (Fig. 1.16). The 5-HIAA is excreted from the neurones through an energy-dependent clearance mechanism. The produced 5-HT is packaged in synaptic vesicles, via the action of VMAT2 (Ruddick et al. 2006). Unlike peripheral cells that only store 5-HT in the cell body, the majority of centrally
produced 5-HT synaptic vesicles are transported through axons to synaptic terminals and are released into the extracellular space by Ca\(^{2+}\)-dependent exocytosis. The released 5-HT can activate post-synaptic 5-HT receptors located on the terminal sites. The 5-HT effect is terminated by the 5-HT neurotransmitter uptake from the release site by the SERT or it may be metabolised to 5-HIAA by post-synaptic cells containing the monoamine oxidase A (Fig. 1.16).

1.3.4.2. Serotonergic neurones in the brain

The first anatomical description of the dorsal raphe (DR) nucleus was made in the early 1900, when Santiago Ramón y Cajal, studying the brain of a newborn kitten, described it as an “intermediate or unpaired nucleus” of the “median sub aqueductal nucleus of the raphe” (Fig. 1.19) (Ramón y Cajal 1904), for review see (Michelsen et al. 2007).

Figure 1.19. Santiago Ramón y Cajal’s drawing illustrating a brain section through the superior colliculus in a newly born kitten. Key: E: dorsal raphe nucleus neurones, A-B: the cells of the trochlear nerve nucleus and their collaterals, C: the medial longitudinal fasciculus, D: the fibres of the superior cerebellar peduncles, F: the ventral cells of the raphe, G: the radicular fibres of the trochlear nerve.

From the annotated and edited translation of Cajal’s 1904 “Texture of the Nervous System of Man and the Vertebrates” by (Pasik & Pasik 2000).
Sixty years later Dahlström and Fuxe (1964), using fluorescence histochemistry, described the anatomical distribution of 5-HT neurones that are mainly located within the midline raphe nuclei of the brain stem (Dahlström & Fuxe 1964). In this study nine clusters of 5-HT-containing neurones were identified, which Dahlström and Fuxe classified alphanumerically as B1 – B9 neurones (Dahlström & Fuxe 1964) (Fig. 1.20).

Monoamine neurotransmitters are characterised by the presence of one amino (-NH₂) group that is covalently bound with an aromatic ring via a two-hydrocarbon chain (-CH₂CH₂-). Monoamine neurotransmitters include: dopamine (DA), adrenaline, noradrenalin (NA), histamine, 5-HT and melatonin. 5-HT system is arguably the most abundant monoamine neurotransmitter in the brain with the highest neuronal population compared to other monoamines including the NA and DA neurones of the brain stem (Sirvio et al. 1994). In human brains, 5-HT neurones are more numerous (>250,000) compared to other species and form a tight, small clusters along the midline raphe nuclei of the brain stem (Tork 1990; Azmitia 2007).
Figure 1.20. Schematic diagram illustrating the major ascending and descending serotonergic pathways in the rat brain. Note: The difference in the innervations pattern between 5-HT projections originating from 5-HT neurons within B7 (dorsal, blue), B8 (median, red) and other (green) brain stem raphe nuclei. 5-HT neurones from the dorsal and the median raphe nuclei innervate the brain stem, the thalamus, the septum, the hippocampus and the cortex, whilst other raphe nuclei innervate the cerebellum and the spinal cord.


Modified from (Ogren et al. 2008).

Afferent inputs to the raphe nuclei include projections from the brain stem (ACh, NA and DA), hypothalamus (peptidergic or histaminergic) and the limbic system (glutamatergic) (Jacobs & Azmitia 1992; Sirvio et al. 1994). More specifically, the
raphe nuclei receive high ACh projections from the pedunculopontine tegmental (PPT) and the laterodorsal tegmental nucleus (LTN) of the brain stem (Woolf & Butcher 1989; Jones 1990). The NA input to the raphe nuclei originate from the LC (Dong & Shen 1986); whilst the ventral tegmental area (VTA) provides the DA input to the raphe nuclei (Peyron et al. 1995). Similarly, the histaminergic input to the raphe nuclei arises from the tuberomammillary nucleus (TMN) of the hypothalamus (Sherin et al. 1998), whereas the glutamatergic input to the raphe nuclei originate from the medial PFC as well as the medullary bodies and the LTN of the brain stem (Lee et al. 2003). Although some of the raphe neurones express other neurotransmitters (e.g. DA, glutamate and GABA) the 5-HT neurones represent the main neuronal population in the raphe nuclei (Sirvio et al. 1994; Michelsen et al. 2007) (Fig. 1.21).

The serotonergic nuclei (B1-B9) of the brain stem are generally divided into the inferior and the superior groups depending on their anatomical localisation and their target projection area (Jacobs & Azmitia 1992; Sirvio et al. 1994). The inferior group consists of the nucleus raphe pallidus (B1), the raphe obscurus (B2), the nucleus raphe magnus (B3), the area postrema and the lateral medulla, all these forming the descending 5-HT projections towards the cerebellum and the spinal cord (Jacobs & Azmitia 1992; Sirvio et al. 1994). The superior group includes: the nucleus pontins central oralis (B4), the median raphe nucleus (B5), the dorsal raphe nucleus (B6-B7) and the caudal linear nucleus (B8) that are all project towards the forebrain and the brain stem (Jacobs & Azmitia 1992). The medial lemniscus (B9) was, for a while, viewed as a minor 5-HT nuclei of the raphe nuclei; however, later studies demonstrated that it also contains large number of 5-HT neurones (Vertes & Crane 1997) that heavily project to the thalamus (Rodríguez et al. 2011). The DR nucleus (B6-B7) is the largest raphe nuclei and contains the densest 5-HT neuronal population in the brain (Wiklund et al. 1981).

Cajal’s description of neuronal morphology within “intermediate or unpaired nucleus”, which was later to be called the raphe nuclei, is still fully applicable (Michelsen et al. 2007). Cajal identified four types of neurones, which he called “voluminous, fusiform, triangular and stellate” (Ramón y Cajal 1904). Subsequent immunohistochemical studies have characterised 5-HT neurones of the raphe
neurones as circular, ovoid, triangular and fusiform, for reviews see (Steinbusch 1981; Steinbusch et al. 1981; Baker et al. 1990; Michelsen et al. 2007; Michelsen et al. 2008).

In the raphe nuclei, 5-HT immunoreactive neurones have large somatas with round, ovoid or fusiform morphology (Fig. 1.21B – 1.21E). For example, 5-HT-immunoreactive neurones from the DR nucleus exhibit somatodendritic profiles characterised by large rounded or ovoid cell bodies with sparse dendritic arborisation (Fig. 1.21D). The median raphe (MR) nucleus contains clusters of small to medium-sized 5-HT neurones, although they constitute only a small fraction of the total neuronal population within this nucleus (Fig. 1.21E and 1.21F). 5-HT positive neurones from the MR nucleus are characterised by the presence of large rounded cell bodies although they have less prominent dendritic arborisation compared to 5-HT neurones from the DR nucleus (Fig. 1.21F).
Figure 1.21. Brightfield photomicrographs showing the distribution of seroton-labelled neurones in the dorsal (C, D) and the median (E, F) raphe nuclei of the mouse brain revealed by toludine blue staining (A, C, E) and specific 5-HT immunohistochemistry. Key: Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part, MR: median raphe nucleus, PMR: paramedian raphe nucleus. From (Rodríguez et al. unpublished photomicrographs).

Anatomically, the DR nucleus is further divided into several sub-nuclei based on their neuronal morphology as well as afferent and efferent connections (Wiklund et al. 1981). In mice the DR nucleus is divided into four sub-nuclei: the dorsal raphe dorsal (DRD), the dorsal raphe ventral (DRV), the dorsal raphe interfascicular part (DRI) and the dorsal raphe ventrolateral part (DRVL) (Fig. 1.22). The DRD
encompasses medium sized or bipolar 5-HT neurones (Van Bockstaele et al. 1993; Commons et al. 2003), whereas the DRV mainly comprises small, round 5-HT neurones (Waterhouse et al. 1986; Kirifides et al. 2001). The DRI encompasses spindle-shaped 5-HT neurones (Azmitia 1981; Kohler et al. 1982), whilst the DRVL comprises of very large multipolar 5-HT neurones (Willoughby & Blessing 1987; Villar et al. 1988) (Fig. 1.22).

**Figure 1.22. Different subdivisions of the dorsal raphe nucleus.** Schematic drawings of a mouse brain section at the level of the dorsal raphe nucleus corresponding to ~4.60 mm posterior to Bregma (A) demonstrating the locations of the different sub divisions of the dorsal raphe nucleus. Brightfield micrographs illustrating 5-HT (B) and toluidine blue (C) labelling in different sub divisions of the dorsal raphe nucleus. **Key:** Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part, DRVL: dorsal raphe ventrolateral part.

A from (Paxinos & Franklin 2004), B and C from personal library.
1.3.4.3. Serotonergic projections in the brain

The raphe nuclei (B1 – B9) give rise to multiple 5-HT projections that are widely distributed throughout the CNS neuroaxis from the olfactory bulb to the spinal cord and from the cortex to the hypothalamus (Azmitia & Whitaker-Azmitia 1991). High density of 5-HT fibres innervate the thalamus, the septum, the putamen, the frontal cortex, the temporal cortex and the hippocampus as well as the cerebellum and the spinal cord (Azmitia & Segal 1978; Vertes 1991; Vertes et al. 1999) (Fig. 1.20). 5-HT neurones from the DR and MR nuclei project to multiple areas of the forebrain in a non-overlapping manner (Vertes 1991; Vertes et al. 1999). These projections rarely innervate separate parts of the same brain structure, which happens in the septum, for example, where 5-HT projections from the DR nucleus innervate the lateral part whereas 5-HT projections from the MR nucleus innervate the medial part (Vertes et al. 1999).

5-HT projections from the DR nucleus invade multiple structures of the forebrain including the frontal cortex, the amygdala, the lateral septum, the striatum and the ventral hippocampus (Vertes 1991). 5-HT neurones located in different sub-nuclei of the DR nucleus give rise to distinct 5-HT projections (Fig. 1.22). Specifically, 5-HT neurones originated from the DRD projects to the hypothalamus, the thalamus, the nucleus accumbens, the amygdala and the PFC (Van Bockstaele et al. 1993; Commons et al. 2003; Rodríguez et al. 2011). The DRV mainly projects to the motor cortex, the sensorimotor cortex and the visual cortex (Waterhouse et al. 1986; Kirifides et al. 2001) (Fig. 1.23). The DRI gives rise to 5-HT projections that innervate the thalamus, the medial septum, the nucleus of the diagonal band of Broca and the hippocampus (Azmitia 1981; Kohler et al. 1982); whilst the DRVL sends 5-HT axonal projections to the superior colliculus, the hypothalamus and the thalamus (Willoughby & Blessing 1987; Villar et al. 1988). Axons arising from the MR nucleus innervate structures located in the midline of the forebrain such as the dorsal part of the diagonal band of Broca nucleus, the septum and the dorsal hippocampus (Vertes et al. 1999) (Fig. 1.23).
The hippocampus receives dense 5-HT projections from both the DR and MR nuclei (Vertes 1991; Vertes et al. 1999). The majority of the hippocampal 5-HT projections arise from the MR nucleus, which reach the hippocampus via two separate routes: (i) through the rostral direction via the fimbria and the dorsal fornix and (ii) through the temporal pole (Azmitia & Segal 1978; Moore et al. 1978). The 5-HT neurones from the DR nucleus provide less dense hippocampal 5-HT projections that reach the hippocampus via the ventral route through the amygdala and the EC (Tohyama et al. 1980; Kohler et al. 1981; Vertes et al. 1999). Immunohistochemical studies revealed that the highest densities of 5-HT projections are evident in the S.Mol of the hippocampus (Vertes 1991; Vertes et al. 1999) (Fig. 1.24). Other hippocampal layers display moderate densities of 5-HT projections including the S.Rad and S.Or; whilst the PCL of the hippocampus has a very low density of 5-HT projections (Vertes et al. 1999). In the DG, moderate density of 5-HT projections are evident in the ML, which is further reduced in the
hilus. Similar to the PCL, the GCL of DG display very low density of 5-HT projections (Fig. 1.24).

![Figure 1.24. Brightfield photomicrograph showing the distribution of SERT fibres in the dorsal hippocampus of a mouse. Key: DG: dentate gyrus, GCL: granular cell layer, ML: molecular layer, S. Luc: stratum lucidum, S.Mol: stratum lacunosum moleculare, S.Rad: stratum radiatum, PCL: pyramidal cell layer, S.Or: stratum oriens. From (Rodríguez et al. unpublished photomicrograph).]

5-HT axons have a distinct morphology depending on their nuclei of origin and projection sites (Kohler et al. 1982; Kosofsky & Molliver 1987; Molliver 1987; Mulligan & Tork 1988; Bjarkam et al. 2005; Keuker et al. 2005; Hensler 2006) (Fig. 1.25). 5-HT neurones from the MR nucleus projects straight, non-varicose axons (stem axons, SA) and thick fibres with large and spherical varicosities that are irregularly spaced (also known as beaded fibres, BF, Fig. 1.25B and 1.25C) (Kosofsky & Molliver 1987; Molliver 1987; Mulligan & Tork 1988; Bjarkam et al. 2005; Keuker et al. 2005; Hensler 2006). 5-HT axons arising from the DR nucleus are represented by fine fibres (FF) with small fusiform or granular varicosities that are regularly spaced (Kosofsky & Molliver 1987) (Fig. 1.25D).
1.3.4.4. Serotonergic neurotransmission in the brain

Through numerous projections into diverse regions of the brain, 5-HT neurotransmission is involved in a great variety of physiological processes. The 5-HT input into the hypothalamus and limbic regions regulates body temperature, food intake and sleep-wakefulness cycle (Weber & Angell 1967; Imeri et al. 1994; Dryden et al. 1996; Yasumatsu et al. 1998). 5-HT innervations of the spinal cord are involved in pain perception (Zhuo & Gebhart 1991; Millan 2002; Dogrul et al. 2009). Cortical 5-HT projections play an important role in motor function and in regulation of emotions (Jacobs & Fornal 1997; Loubinoux et al. 2002; Cools et al. 2008). The 5-HT input into the hippocampus is essential for adult neurogenesis (Brezun & Daszuta 1999; Brezun & Daszuta 2000a). 5-HT neurotransmissions in the septum, the hippocampus and cortical regions play a direct role in cognition via modulation of memory acquisition, consolidation and storage (Buhot et al. 2000; Schmitt et al. 2000).

In all terminal sites, 5-HT neurotransmission takes place mainly via volumetric
transmission. An early study combining electron microscopy and radioautography showed that only 5% of 5-HT terminals displayed typical synaptic junction in the rat neocortex (Descarries et al. 1975). Descarries and colleagues (1975) suggested that 5-HT can be released and taken up from all axonal terminals including those that lack typical synaptic junctions (Descarries et al. 1975). The term “para-synaptic” (i.e. parallel with) was introduced in 1984, which describes that the released neurotransmitters reach their specific target receptors via diffusion from the release site through the extracellular fluid (Schmitt 1984). Anatomical support for “para-synaptic” 5-HT neurotransmission was provided by light microscopic studies, which found a “mismatch” between the 5-HT release sites and the location of corresponding 5-HT receptors (Herkenham 1987). It was not until 1995, when Agnati and co-authors (1995) introduced the term “volume transmission” to describe the mechanism and functional characteristics of “para-synaptic” neurotransmission (Agnati et al. 1995).

Ultrastructural studies using electron microscopy have confirmed that the majority of 5-HT terminals do not display synaptic junctions (Seguela et al. 1989; Oleskevich et al. 1991; Cohen et al. 1995; Miner et al. 2000), suggesting that 5-HT neurotransmission predominantly occur via volumetric transmission. As mentioned above, volumetric transmission involves diffusion of 5-HT neurotransmitter from the release sites through the extracellular space to reach its high affinity receptor targets located on neighbouring neuronal, glial and/or vascular structures.

1.3.4.5. Serotonin transporter in the brain: Localisation and the role of SERT
Serotonergic projections are widely distributed in the brain and the spinal cord where they are in continuous contact with the majority of the cells. A precise regulatory mechanism for the release and the uptake of 5-HT is critical for such widespread neurotransmitter delivery. Serotonin transporter (SERT) is a twelve-transmembrane-domain protein that is responsible for inactivation of 5-HT neurotransmission through re-uptake of 5-HT at release site. Transgenic mice with reduced SERT protein expression (SERT−/−) display decreased 5-HT clearance and elevated extracellular 5-HT levels in the striatum, the cortex and the hippocampus (measured using in vivo microdialysis) (Montanez et al. 2003; Mathews et al. 2004;
Murphy & Lesch 2008). No changes were observed in the DA and NA neurotransmitter levels in SERT−/− knock-out mice, suggesting that altered SERT expression specifically affect 5-HT neurotransmission (Montanez et al. 2003; Mathews et al. 2004).

Immunohistochemical studies using SERT-specific antibodies at light microscopic level have shown that the SERT protein is expressed not only in 5-HT varicosities and terminals but it is evident along 5-HT axons (Qian et al. 1995; Sur et al. 1996; Zhou et al. 1996; Yamamoto et al. 1998; Noristani et al. 2010; Vertes et al. 2010; Noristani et al. 2011; Rodríguez et al. 2011). Electron microscopic studies, using SERT-specific antibodies combined with gold-labelling, have reported that SERT protein is predominantly expressed along plasma membrane of 5-HT axons as well as pre-synaptic membrane of 5-HT axon terminals (Zhou et al. 1998; Pickel & Chan 1999; Huang & Pickel 2002) (Fig. 1.26). In addition, SERT labeling in axonal plasma membrane often occurs near large dense core vesicle (DCVs) that are known to contain 5-HT (Pelletier et al. 1981; Pickel & Chan 1999) (Fig. 1.26A and 1.26B). The majority of SERT-labelled terminals do not display synaptic junctions; however, they rarely form either asymmetric or symmetric synapses (Pickel & Chan 1999; Tao-Cheng & Zhou 1999) (Fig. 1.26C and 1.26D).
In the brain, SERT is mainly expressed pre-synaptically, where it has been localised in the plasma membranes of 5-HT axons and 5-HT terminals either with or with no synaptic junctions (Zhou et al. 1998; Tao-Cheng & Zhou 1999) (Fig. 1.26). An autoradiography binding study, using \[^{3}H\]citalopram as radioligand showed binding throughout SERT fibres, suggesting that axonal SERT are involved in the re-uptake of the released 5-HT neurotransmitter (Zhou et al. 1998). In addition, voltammetry recordings showed that axonal SERT are actively engaged in high affinity 5-HT re-uptake (Zhou et al. 1998).
Altogether, these data suggest that SERT protein is predominantly expressed presynaptically throughout the axonal membrane, far beyond the synaptic junctions, suggesting that axonal SERT mediates the uptake of diffused 5-HT through extra synaptic (volume) transmission (Zhou et al. 1998; Pickel & Chan 1999; Tao-Cheng & Zhou 1999).

1.3.4.6. Serotonin transporter as a marker of serotonergic projections

Following the discovery of 5-HT (Vialli & Erspamer 1937-38; Vialli & Erspamer 1942) and its role as a neurotransmitter in the brain (Bogdanski et al. 1956), different techniques have been developed to describe and localise 5-HT pathways in the brain and the spinal cord. Early studies relied on autoradiography technique, using $[3^H]5$-HT as radioligand (Azmitia & Segal 1978; Parent et al. 1981), to define central 5-HT innervations.

A major advance in the visualisation of 5-HT fibres was followed shortly after the development of 5-HT-specific antibody (Steinbusch 1981), which paved the way for analysing the density and the number of 5-HT-positive fibres in multiple projection areas using immunohistochemistry and light microscopy (Madhav et al. 2000; Mamounas et al. 2000).

Although 5-HT staining is still a widely used technique for the visualisation of 5-HT fibres, a major drawback in using antibody against 5-HT is that the method is sensitive to the level of 5-HT neurotransmitter in the brain (Nielsen et al. 2006). This is particularly important when visualising 5-HT fibres in conditions with compromised 5-HT synthesis/metabolism, where the level of 5-HT content is reduced below the detection levels required for immunohistochemical staining, hence, giving rise to false negative results (Nielsen et al. 2006). In addition, 5-HT undergoes rapid metabolism that in turn underestimates the visualisation and the density of serotonergic fibres using 5-HT specific antibody. Therefore, a decrease in 5-HT-immunoreactivity per se does not necessarily indicate a decrease in serotonergic fibre density since a decrease in 5-HT content might similarly result in reduced fibre staining.
To enhance the visualisation of 5-HT fibres, using 5-HT-specific antibody, some authors increased 5-HT contents by administration of its precursor TrP (Azmitia & Gannon 1983; Birkett & Fite 2005) or administration of MAO-A inhibitor to reduce 5-HT breakdown (Nielsen et al. 2006). However, administration of TrP and MAO-A inhibitors produce another experimental variable in the analysis.

More recent immunostaining procedures have used SERT-specific antibody to visualise and quantify serotonergic fibres in multiple brain regions (Qian et al. 1995; Sur et al. 1996; Zhou et al. 1996; Yamamoto et al. 1998; Noristani et al. 2010; Vertes et al. 2010; Noristani et al. 2011; Rodríguez et al. 2011; Noristani et al. 2012). In situ hybridisation studies have shown that SERT mRNA is exclusively expressed by 5-HT neurones of the raphe nuclei in rat (Rattray et al. 1999) and human brains (Austin et al. 1994), suggesting that SERT is a specific transporter for 5-HT system. Similarly, SERT specific antibodies exclusively recognise serotonergic fibres and show characteristic features similar to that of 5-HT-labelled fibres in the brain (Zhou et al. 1996; Vertes et al. 2010; Rodríguez et al. 2011) (Fig. 1.27). Intracerebral injection of 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) completely removed SERT-labelled fibres in the hippocampus, further supporting its specificity for serotonergic axons (Zhou et al. 1996). In fact, Nielsen and colleagues (2006) showed that, unlike 5-HT antibody, the changes in 5-HT content did not affect serotonergic fibre visualisation and density when using SERT specific antibody (Nielsen et al. 2006).

Specifically, Nielsen and colleagues (2006) had shown that 5-HT staining only reached the quality of SERT labelling for quantifying serotonergic fibres when 5-HT immunohistochemistry was combined with pre-treatment with MAO-A inhibitor to prevent 5-HT degradation (Nielsen et al. 2006). Pre-treatment with MAO-A inhibitor increased the detection of 5-HT-labelled fibres by 200%, which showed almost 100% co-localisation with SERT positive fibres (measured using confocal microscopy) (Nielsen et al. 2006). On the other hand, only 30% of 5-HT fibres showed co-localisation with SERT-positive fibres that was attributed to the loss or the inability to detect 5-HT fibres in non-treated rats (Nielsen et al. 2006). Other immunohistochemical studies, including our own, have shown that immunohistochemical staining using 5-HT and SERT specific antibodies visualised
a comparable set of serotonergic fibres in the rat thalamus, where SERT staining produced a stronger signal compared to that of 5-HT (Vertes et al. 2010; Rodríguez et al. 2011) (see also Fig. 1.27).

**Figure 1.27.** Brightfield photomicrographs illustrating the distribution of serotonin-containing fibres in the RT, the ic and the VAL nucleus of the rat thalamus following immunostaining against 5-HT (A, B) and SERT (C, D). Note: Although both staining using 5-HT and SERT specific antibodies labelled a comparable set of 5-HT fibres in the thalamus, SERT staining produced a stronger signal compared to that of 5-HT. Key: RT: reticular nucleus of the thalamus, VAL: ventral anterior lateral nucleus of the thalamus, ic: internal capsule. From (Rodríguez et al. 2011).

*In vitro* studies have reported SERT mRNA and protein expression in cultured astrocytes derived from neonatal rat brains (Bal et al. 1997; Hirst et al. 1998b; Inazu et al. 2001). Other *in vitro* study also found SERT expression in normal human astrocytes (Kubota et al. 2001). Interestingly, application of stress-related growth factors such as basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) increased SERT mRNA expression in cultured human astrocytes (Kubota et al. 2001). These findings from Kubota and colleagues (2001), led to the proposal that stress conditions in the brain may cause an increase in bFGF expression that in turn stimulate increased SERT expression in glial cells (Kubota
et al. 2001). However, currently there are no published data supporting stress-induced increase in glial SERT expression.

Given the transient expression of SERT mRNA in non-5-HT neurones during brain development (Lebrand et al. 1996; Hansson et al. 1998), one can not rule out that the astrocytic SERT expression may be due to developmental processes (Bal et al. 1997; Hirst et al. 1998b; Inazu et al. 2001). Indeed, in adult brains, SERT mRNA expression is exclusively observed in 5-HT neurones and projections (Lebrand et al. 1996).

There has been no study using immunohistochemical staining of brain section that shown SERT expression in glia cells, using light microscope. The failure to identify SERT protein in glia cells may reflect the lower resolution provided by the light microscope although it can perfectly detect the higher densities of SERT protein expressed by 5-HT neurones and fibres. The expression level of SERT protein in astrocytes may be sufficiently low and escapes detection by the light microscope. In support of this phenomenon, electron microscopic studies, which have much higher resolution and can detect very low level of proteins, have reported low levels of SERT labeling within a few filamentous glial processes in different regions of the rat brain including the medial nucleus of the solitary tract (Huang & Pickel 2002) and the nucleus accumbens (Pickel & Chan 1999). More recent electron microscopy study in our laboratory had shown that no more than 2% SERT labeling was expressed in glia cells within the hippocampus in mice (chapter 4). A better understanding of the structural and functional properties of glial SERT awaits further research.

Taken together, these data suggest that SERT protein is more stable compared to 5-HT and provides a more reliable marker for specific visualisation and quantification of serotonergic fibres because:

- SERT<sup>-/-</sup> knock-out mice display specific increase in 5-HT neurotransmission with no evident changes in the DA and NA neurotransmissions, suggesting that SERT protein expression is specifically associated with 5-HT system (Montanez et al. 2003; Mathews et al. 2004).
• The SERT mRNA is exclusively expressed by 5-HT neurones of the raphe nuclei in the brain stem (Austin et al. 1994; Rattray et al. 1999).

• SERT-specific antibodies exclusively recognise 5-HT projections and show similar morphological features to 5-HT fibres in the brain (Zhou et al. 1996; Vertes et al. 2010; Rodríguez et al. 2011).

• Double immunohistochemical staining showed 100% co-localisation of 5-HT and SERT-positive fibres (measured using confocal microscopy), in rats pre-treated with MAO-A inhibitor (Nielsen et al. 2006).

• Intracerebral injection of 5-HT neurotoxin (5,7-DHT) completely removed SERT-labelled fibres within the hippocampus (Zhou et al. 1996).

• The synthesis and degradation rate of 5-HT does not affect the levels of the SERT protein in the brain (Nielsen et al. 2006).

1.3.4.7. Serotonin receptors in the brain

The remarkable physiological and functional versatility of 5-HT neurotransmission is mediated through an extended family of 5-HT-specific receptors. Shortly after the identification of 5-HT in the brain (Amin et al. 1954), subsequent studies by Gaddum and colleagues (1954 – 1957) identified two types of 5-HT receptors designated as M and D receptors (due to their blockage by morphine and α-adrenergic blocker, dibenzylidine) (Gaddum & Hameed 1954; Gaddum & Picarelli 1957), for historic review see (Green 2006). Further investigations led to the identification of 5-HT₁, 5-HT₂ and 5-HT₃ receptors (Peroutka et al. 1981; Bradley et al. 1986). Molecular cloning identified 7 classes of 5-HT receptors represented by G-protein coupled receptors (5HTR₁,₂,₄,₇) and by ligand-gated cation channels (5-HTR₃), for review see (Barnes & Sharp 1999; Hoyer et al. 2002) (see also Table 1.5).
Table 1.5. Serotonin receptors families and their different sub-types, secondary messenger systems and their expression in different brain regions.

<table>
<thead>
<tr>
<th>5-HT Receptor Family</th>
<th>5-HT Receptor Subtype</th>
<th>Secondary Messenger System</th>
<th>Expression in brain region</th>
<th>Species used</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁</td>
<td>5-HT₁₁A</td>
<td>Receptors are coupled to Gᵢ₁ proteins, which increase K⁺ conductance hence hyperpolarises the membrane at rest</td>
<td>EC, NeC, R, S, T, H</td>
<td>Mouse, rat and human</td>
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<tr>
<td>5-HT₁₁B</td>
<td></td>
<td>5-HT₁₁A receptors activation also inhibit Ca²⁺ current through Gᵢ₁/G₀ proteins</td>
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<td>5-HT₁₁D</td>
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<td>5-HT₁₁F</td>
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<td></td>
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<tr>
<td>5-HT₁₂</td>
<td>5-HT₁₂₂A</td>
<td>Receptors are coupled to Gₛᵢᵢ protein, which hydrolyse inositol phosphates and induce cytosolic [Ca²⁺]ᵢ mobilisation through InsP₃-mediated Ca²⁺ release from the endoplasmic reticulum store</td>
<td>EC, FC, PC, CN, NAc, Ob, A, S, H</td>
<td>Mouse, rats, monkey, and human</td>
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<tr>
<td>5-HT₁₂₂B</td>
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<td>5-HT₁₂₂C</td>
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<tr>
<td>5-HT₁₃</td>
<td>5-HT₁₃ₐ</td>
<td>Ligand-gated cationic channels with pentameric structure permeable to Na⁺, K⁺ and Ca²⁺ Receptor activation result in membrane depolarisation</td>
<td>EC, TC, A, Ob, NAc, H</td>
<td>Mouse, rat, guinea-pig, pig, monkey and human</td>
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<td>5-HT₁₃ₐ</td>
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<td>5-HT₁₃₅B</td>
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<tr>
<td>5-HT₁₄</td>
<td>5-HT₁₄ₐ</td>
<td>Receptors are coupled to Gₛ protein that stimulates adenylate cyclase and cAMP synthesis upon activation</td>
<td>FC, ST, SN, GP, Pu, Ob, H</td>
<td>Mouse, rat, guinea-pig and human</td>
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<td>5-HT₁₄ₐ</td>
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<td>5-HT₁₄₅C</td>
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<tr>
<td>5-HT₁₅</td>
<td>5-HT₁₅ₐ</td>
<td>Receptors are coupled to Gₛ/G₀ protein, where receptor activation inhibits cAMP synthesis and accumulation</td>
<td>EC, PerC, Hy, A, cb, H</td>
<td>Mouse, rat and human</td>
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<td>5-HT₁₆</td>
<td>nr</td>
<td>Receptors are coupled to Gₛ/G₀ proteins that stimulates adenylate cyclase and cAMP synthesis upon activation</td>
<td>EC, FC, TC, NAc, A, cb, Pu, H</td>
<td>Mouse, rat, pig and human</td>
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<td>5-HT₁₆</td>
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<tr>
<td>5-HT₁₇</td>
<td>5-HT₁₇ₐ</td>
<td>Receptors are coupled to Gₛ proteins that stimulates adenylate cyclase and cAMP synthesis upon activation</td>
<td>EC, FC, TC, T, S, ST, SC, H</td>
<td>Mouse, rat, guinea-pig and human</td>
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1.3.4.7.1. 5-HT₁A receptors

The 5-HT₁ family of the receptors comprise five members designated as 5-HT₁ₐ, 5-HT₁₉, 5-HT₁₁D, 5-HT₁₁E and 5-HT₁₁F receptors (Hoyer et al. 2002) (Table 1.5). 5-HT₁ₐ receptors were the first 5-HT receptor gene to be identified and characterised (Kobilka et al. 1987; Fargin et al. 1988). 5-HT₁ₐ receptors are highly expressed.
throughout multiple brain regions including the EC, the raphe nuclei, the septum and the hippocampus, whilst lower expression levels of 5-HT\textsubscript{1A} receptors are evident in the neocortex and the thalamus (Chalmers & Watson 1991; Pompeiano \textit{et al.} 1992; Wright \textit{et al.} 1995) (Table 1.5). 5-HT\textsubscript{1A} receptors are expressed both pre-synaptically in somatas and dendrites of 5-HT neurones within the raphe nuclei as well as post-synaptically in the synaptic terminals at 5-HT projection sites such as the hippocampus (Sotelo \textit{et al.} 1990; Azmitia \textit{et al.} 1996; Kia \textit{et al.} 1996a) (Fig. 1.28A).

\textbf{Figure 1.28.} Brightfield photomicrographs showing hippocampal 5-HT\textsubscript{1A} (A) and 5-HT\textsubscript{2A} receptor (B) expression and localisation in the rat hippocampus. Key: DG: dentate gyrus, GCL: granular cell layer, ML: molecular layer, f: hippocampal fissure, PCL: pyramidal cell layer, S.Or: stratum oriens.

From (Rodríguez \textit{et al.} unpublished photomicrographs).

Simulation of 5-HT\textsubscript{1A} receptors generally inhibit neuronal excitability through the G\textsubscript{i} protein-mediated opening of K\textsuperscript{+} channels and an increase in K\textsuperscript{+} conductance that hyperpolarises the membrane at rest (Aghajanian & Lakoski 1984; Williams \textit{et al.} 1988). Activation of pre-synaptic 5-HT\textsubscript{1A} receptors within the raphe nuclei reduce 5-HT neuronal firing that in turn decreases 5-HT release at terminal sites such as the cortex and the hippocampus (Sprouse & Aghajanian 1987; Romero \textit{et al.} 1994; Bagdy & Harsing 1995). Similarly, activating post-synaptic 5-HT\textsubscript{1A} receptors, by endogenous 5-HT or 5-HT\textsubscript{1A} receptor agonist, induce hyperpolarisation in cells that expresses 5-HT\textsubscript{1A} receptors (Andrade & Chaput 1991; Grunschlag \textit{et al.} 1997; Deng \textit{et al.} 2007b).

Post-synaptic 5-HT\textsubscript{1A} receptors are localised in the hippocampal pyramidal glutamatergic neurones and inhibit glutamate release (Matsuyama \textit{et al.} 1996). This
is particularly important in neurodegenerative disorders such as AD, where excess glutamate release causes excitotoxicity via increased $[Ca^{2+}]_i$ current (Lipton & Rosenberg 1994). Activation of 5-HT$_{1A}$ receptors blocks Ca$^{2+}$ current (Williams et al. 1998; Lin et al. 2001; Hill et al. 2003) and may counteract Ca$^{2+}$ influx-induced excitotoxicity. The 5-HT$_{1A}$ receptor-mediated inhibition of Ca$^{2+}$ current is mediated primarily through activation of G$i$/G$o$ sub-types of G-proteins because it was prevented by N-ethylmaleimide, which specifically block pertussis-sensitive G-proteins (Williams et al. 1998).

In addition to their expression in neurones, 5-HT$_{1A}$ receptors presence has also been reported in astrocytes (Whitaker-Azmitia et al. 1993; Azmitia et al. 1996; Hirst et al. 1998a; Patel & Zhou 2005). Double immunocytochemistry showed colocalisation of 5-HT$_{1A}$ receptors with the glial fibrillary acidic protein (GFAP) and S-100β-expressing astrocytes in the rat hippocampus (Whitaker-Azmitia et al. 1993; Patel & Zhou 2005). Astrocytic 5-HT$_{1A}$ receptors mediate the release of neurotrophic agents such as S-100β from astrocytes that promote 5-HT fibre sprouting (Azmitia et al. 1990; Whitaker-Azmitia et al. 1990).

The 5-HT$_{1B}$ receptors are highly expressed in the 5-HT terminal sites, where activation of the receptors suppresses glutamatergic neurotransmission by reducing glutamate release from pre-synaptic terminals (Guo & Rainnie 2010). 5-HT$_{1B}$-mediated pre-synaptic inhibition of glutamatergic neurotransmission has been demonstrated in multiple brain regions, including the suprachiasmatic nucleus (Pickard et al. 1999), the nucleus accumbens (Muramatsu et al. 1998) and the EC (Schmitz et al. 1998).

1.3.4.7.2. 5-HT$_2$ receptors

The 5-HT$_2$ receptor family is represented by 7-transmembrane domain metabotropic 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (Hoyer et al. 2002) (see also Table 1.5). 5-HT$_{2A}$ receptors are coupled to $G_{q/11}$ protein, which hydrolyse inositol phosphates and induce cytosolic Ca$^{2+}$ $[Ca^{2+}]_i$, mobilisation through the InsP$_3$-mediated Ca$^{2+}$ release from the endoplasmic reticulum store (Conn & Sanders-Bush 1986; Barnes & Sharp 1999; Hoyer et al. 2002). 5-HT$_{2A}$ receptor-mediated increase in $[Ca^{2+}]_i$, is followed by the influx of external Ca$^{2+}$ into the cells through Ca$^{2+}$
channels (Yang et al. 1994).

Autoradiographic, in situ hybridisation and immunohistochemical studies revealed high densities of 5-HT2A receptors in the frontal cortex, the parietal cortex and the EC as well as in the caudate nucleus, the nucleus accumbens, the olfactory bulb, the amygdala, the septum and the hippocampus (Lidow et al. 1989; Mengod et al. 1990; Morilak et al. 1993; Burnet et al. 1995; Lopez-Gimenez et al. 1997; Cornea-Hebert et al. 1999) (Table 1.5). Cortical 5-HT2A receptors are predominantly postsynaptic and are largely localised in the soma and the apical dendrites of the pyramidal neurones (Barnes & Sharp 1999). In the hippocampus post-synaptic 5-HT2A receptors are mainly localised in the dendritic processes of pyramidal neurones and granule cell of the DG (Peddie et al. 2008a) (Fig. 1.28B).

1.3.4.7.3. Other 5-HT receptors

The 5-HT3 receptor family belongs to the super-family of ligand-gated ion channels with a pentameric structure similar to the nAChR, the GABA\textsubscript{A} and glycine ionotropic receptors (Derkach et al. 1989; Maricq et al. 1991). The 5-HT3 receptor family is cationic channels (permeable to Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+}) and their activation results in membrane depolarisation (Sugita et al. 1992; Roerig et al. 1997). At least two distinct subunits of 5-HT3 receptors have been cloned and classified as 5-HT3\textsubscript{A} and 5-HT3\textsubscript{B} receptors (Maricq et al. 1991; Davies et al. 1999) that are expressed in multiple brain regions (Table 1.5). The precise function of 5-HT3 receptor is still under consideration, although it was suggested to be involved in pain processing because of high expression of the receptor in the spinal cord and in the medulla oblongata (Fonseca et al. 2001).

The 5-HT4 receptor family is coupled to G\textsubscript{s} proteins that stimulate adenylyl cyclase upon receptor activation (Claeysen et al. 1998). There are at least 9 different splice variants of 5-HT4 receptor that were cloned from mouse, rat and human tissues (Claeysen et al. 1998; Claeyesen et al. 1999; Bender et al. 2000; Vilaro et al. 2002; Vilaro et al. 2005) and are widely expressed throughout the brain of multiple species (Table 1.5). At cellular level (in rats and guinea-pigs), 5HT4\textsubscript{A}, 5-HT4\textsubscript{B} and 5-HT4\textsubscript{E} receptors are located in 5-HT neuronal soma and throughout 5-HT axons (Vilaro et al. 2005). 5-HT4 receptor is also expressed in the non-neuronal tissues,
where it regulates cardiac inotropy, gastric motility and the release of adrenal hormone, for reviews see (Hegde & Eglen 1996; Taniyama et al. 2000).

The 5-HT$_5$ receptor family belongs to the seven transmembrane domains G-protein coupled receptor super-family (Erlander et al. 1993). Although currently the secondary messenger system for the 5-HT$_5$ receptor family remains ambiguous (Glennon 2003), several studies demonstrated that 5-HT$_{5A}$ receptors activate G$_i$/G$_o$ proteins, where receptor activation inhibits cAMP synthesis and accumulation (Carson et al. 1996; Hurley et al. 1998; Francken et al. 2000; Thomas et al. 2000). The 5-HT$_5$ receptor is exclusively expressed in the CNS with little or no expression in the peripheral tissues (Erlander et al. 1993; Rees et al. 1994). There is some inconclusive evidence suggesting that the 5-HT$_5$ receptor is involved in motor coordination and in the regulation of circadian rhythms (Pasqualetti et al. 1998; Oliver et al. 2000; Kinsey et al. 2001). Genetic deletion of 5-HT$_{5A}$ receptors produced a phenotype with increased exploratory behaviour and modified the effect of lysergic acid diethylamide (LSD), suggesting that the receptor may modulate the activity of neural circuits involved in the exploratory behaviour and may also be responsible for some of the psychotropic effects associated with LSD (Grailhe et al. 1999).

The 5-HT$_6$ receptor family is positively coupled with adenylate cyclase via G$_s$ protein thus stimulating cAMP synthesis upon activation (Conner & Mansour 1990; Sebben et al. 1994; Kohen et al. 1996) and is primarily expressed in the CNS (Table 1.5). Ultrastructural analysis revealed that 5-HT$_6$ receptor is mainly localised post-synaptically in the dendritic processes (Gerard et al. 1997). The development of the CNS is associated with an increase in 5-HT$_6$ receptor expression, suggesting that the 5-HT$_6$ receptor may play a role in the regulation of axonal growth (Grimaldi et al. 1998; Mitchell & Neumaier 2005). In the adult brain, 5-HT$_6$ receptor modulates the release of several neurotransmitters including ACh, glutamate and GABA (Dawson et al. 2001; Hirst et al. 2006; West et al. 2009).

The 5-HT$_7$ family of 5-HTRs was the last to be identified, although initial studies erroneously assigned 5-HT$_7$ receptor to 5-HT$_1$ family referring to them as “5-HT$_1$-like” receptor (Feniuk et al. 1983; Bradley et al. 1986; Trevethick et al. 1986). The
5-HT$_7$ receptor family is coupled to G$_s$ protein that stimulates adenylate cyclase and cAMP synthesis upon receptor activation (Bard et al. 1993; Lovenberg et al. 1993; Adham et al. 1998; Baker et al. 1998). The mRNA encoding the 5-HT$_7$ receptor undergoes alternative splicing that generates at least four isoforms (5-HT$_{7A/B/C/D}$) (Heidmann et al. 1997), which are widely expressed in the brain and the spinal cord (Table 1.5). The 5-HT$_7$ receptor is implicated in regulation of the affective behaviour, circadian rhythms, thermoregulation and modulation of sensory information inflow (Lovenberg et al. 1993; To et al. 1995; Hagan et al. 2000). The 5-HT$_7$ receptor is also expressed in the peripheral tissues including vascular smooth muscles (Bard et al. 1993; Ullmer et al. 1995; Krobert et al. 2001), where it mediates muscle relaxation (Bard et al. 1993; Carter et al. 1995; Ullmer et al. 1995; Krobert et al. 2001).

1.3.5. Serotonergic regulation of behavioural processes

Central 5-HT neurotransmission participates in numerous behavioural processes including food/water intake, aggression, sleep-wake cycle as well as locomotor activity, sexual function, thermoregulation and cognition. On the other hand, impaired 5-HT neurotransmission contributes to the pathophysiology of numerous neurological disorders including schizophrenia, stress and mood disorder as well as depression (Fig. 1.29). The first three mentioned functions, namely: food/water intake, aggression and sleep-wake behaviour are described below, whilst the role of 5-HT in cognition is described in the following section (section 1.3.6.).
Figure 1.29. Schematic representation of the central effects of 5-HT. The TPH2 isoform mediates 5-HT synthesis in the brain. Note: Whilst normal 5-HT neurotransmission regulates numerous behavioural processes including food intake, aggression and sleep, among others, alerted 5-HT neurotransmission has been implicated in pathophysiology of many neurological disorders such as schizophrenia, stress and mood disorder as well as depression.

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1.3.5.1. Serotonin in food intake
Alterations in 5-HT synthesis, bioavailability and metabolism affect food intake (Lam et al. 2010). In general, increased 5-HT neurotransmission inhibits food intake, whilst reducing 5-HT neurotransmission stimulates food consumption.

Increased 5-HT synthesis (via intraperitoneal injection of 5-HT precursor, TrP) inhibited food intake in mice (Coskun et al. 2006) and rats (Ju & Tsai 1995; Amer et al. 2004). On the other hand, reduced 5-HT neurotransmission (via reduced dietary TrP intake) increased food consumption in mice (De Marte & Enesco 1986). In addition, inhibiting 5-HT synthesis (via intraventricular injection of PCPA) increased food intake in rats (Breisch et al. 1976). Interestingly, PCPA-induced increase in food intake was particularly evident in rats with more severe depletion of 5-HT synthesis (Breisch et al. 1976), further supporting the inhibitory role of central 5-HT neurotransmission on food intake.

Intraperitoneal injection of SSRI (sertraline), which increases 5-HT bioavailability, reduced food intake by stimulating the onset of normal satiety in rats (Simansky & Vaidya 1990). Clinical trials in human obesity have reported that D-fenfluramine (which disrupts vesicular 5-HT storage and increases its release through SERT) reduced food intake and prevented body weight gain (Guy-Grand 1995). In addition, administration of MAO-A inhibitor (which increases 5-HT level by inhibiting its metabolism) decreased food intake in mice, rats and Chinese hamsters (Feldman 1988). Taken together, these data support an inhibitory effect of increased 5-HT neurotransmission on food intake.

In addition to 5-HT synthesis, bioavailability and metabolism, pharmacological and genetic targeting of 5-HT receptors also affect food intake (Lam et al. 2010). Activation of 5-HT_{1A} receptor (by administration of 5-HT_{1A} agonist 8-OH-DPAT), which decreases 5-HT release through inhibition of 5-HT neuronal firing, increased food intake in rats (Dourish et al. 1985). On the other hand, administration of 5-HT_{1A} receptor antagonist ((S)-UH-301) decreased palatable food intake in rats (Moreau et al. 1992).

Selective 5-HT_{2C} receptor agonist reduced food intake in mice and rats that was
reversed by the antagonist of the receptor (Schreiber & De Vry 2002). Similarly, genetic deletion of 5-HT2C receptors caused lifelong increase in food intake leading to obesity in 5-HT2C knock-out mice (Tecott et al. 1995; Nonogaki et al. 2003).

Currently little information is available regarding the role of 5-HT3, 5-HT4, 5-HT5 and 5-HT7 receptors on food intake. However, selective antagonist of 5-HT6 receptors have been shown to decrease food intake (Heal et al. 2008), which is inconsistent to the generalised concept of increasing 5-HT neurotransmission on inhibition of food intake (Lam et al. 2010).

1.3.5.2. Serotonin in water intake
In addition to its inhibitory effect on food intake, central 5-HT neurotransmission also exerts a negative effect on drinking behaviour (Castro et al. 2001). Reducing 5-HT neurotransmission (via a decrease in dietary TrP intake) increased water consumption in mice (Kantak et al. 1980). On the other hand, bilateral injection of selective 5-HT1A/7 receptor agonist (8-OH-DPAT) into the lateral septum decreased water intake in dehydrated rats (de Arruda Camargo et al. 2010). In addition, selective activation of 5-HT1C/5-HT2 receptor (via intraventricular injection of receptor agonist, MK-212) reduced water consumption in water-deprived rats (Reis et al. 1990). Furthermore, intraventricular injection of selective 5-HT1D and 5-HT3 receptor agonists inhibited water intake in rats (De Castro-e-Silva et al. 1997; Castro et al. 2002).

1.3.5.3. Serotonin in aggression
High densities of 5-HT projections are evident in brain regions involved in regulation of aggressive behaviour including the frontal cortex, the hypothalamus and the amygdala (Vertes et al. 1999). In general, increased 5-HT neurotransmission has an inhibitory effect on aggression (Miczek et al. 1989; Popova 2008), whereas lower brain 5-HT level has been linked with increased aggressive behaviours (Linnoila & Virkkunen 1992).

Reduced 5-HT neurotransmission (achieved by acute TrP depletion) increased aggressive responses in healthy males (Moeller et al. 1996) and females (Bond et al. 2001). On the other hand, increased 5-HT neurotransmission (by increased TrP
intake) reduced aggressive response in healthy female volunteers (Marsh et al. 2002). In addition, treatment with SSRI (fluoxetine) also reduced aggression in clinically aggressive individuals (Coccaro & Kavoussi 1997).

Highly aggressive rats displayed reduced 5-HT level in the midbrain, which may be due to reduced activity of the TPH2 (the enzyme responsible for 5-HT synthesis in the brain) (Popova et al. 1991a; Popova et al. 1991b). Similarly, low brain 5-HT level (due to reduced TPH2 activity) was also reported in highly aggressive foxes compared to their domesticated counterparts (Popova et al. 1991b). In vivo microdialysis recording showed reduced 5-HT neurotransmitter level in the prefrontal cortex of male rats before, during and after a 10-min aggressive confrontation (van Erp & Miczek 2000), suggesting that aggressive behaviours are associated with reduced 5-HT neurotransmission.

Interestingly, genetic deletion of MAO-A (the enzyme responsible for 5-HT metabolism) led to increased aggression in MAO-A knock-out (MAO-A−/−) mice (Cases et al. 1995; Popova et al. 2000). The MAO-A−/− mice displayed increased 5-HT level but reduced 5-HT metabolite (5-HIAA) in the brain, suggesting a reduction in the functional activity of the 5-HT system (Cases et al. 1995; Popova et al. 2001), which may account for the increased aggressive behaviour in these mice. These latter studies are supported by clinical findings that also reported reduced 5-HIAA in the CSF samples from aggressive individuals (Brown et al. 1979; Linnoila et al. 1983; Kruesi et al. 1990), supporting hypofunction of 5-HT system in aggression.

Increasing evidence highlight the involvement of 5-HT1A receptors in modulating aggression due to their special function in auto-regulation of 5-HT neurones in the raphe nuclei (Sprouse & Aghajanian 1987; Romero et al. 1994; Bagdy & Harsing 1995). Selective 5-HT1A receptor agonists decreased aggression in rats and mice (Olivier et al. 1995; Miczek et al. 1998). The anti-aggressive effect of 5-HT1A receptors was blocked by selective 5-HT1A receptor antagonists (Mendoza et al. 1999; de Boer et al. 2000), which further support the selective involvement of these receptors in the regulation of aggressive behaviours. Aggressive rats displayed reduced 5-HT1A receptor mRNA in the midbrain as well as reduced 5-HT1A
receptor binding sites in the frontal cortex, the hypothalamus and the amygdala (Popova et al. 2005). Taken together, these results suggest that reduced 5-HT$_{1A}$ receptor expression and function may result in compromised activity of the 5-HT neurones and contribute to the increased aggressive behaviours.

In addition to 5-HT$_{1A}$ receptors, selective activation of 5-HT$_{1B}$ receptors led to highly specific anti-aggressive effect in aggressive residential mice and in mice that were made more aggressive by administration of low-doses of alcohol (Fish et al. 1999; Miczek & de Almeida 2001). On the other hand, genetic deletion of 5-HT$_{1B}$ receptors increased aggressive behaviour in 5-HT$_{1B}$ receptor knock-out mice (Saudou et al. 1994; Brunner & Hen 1997; Bouwknecht et al. 2001), which further support the importance of 5-HT$_{1B}$ receptor in the regulation of aggression. Further studies are required to clarify the role of other 5-HT receptors in the regulation of aggression (Olivier 2004).

1.3.5.4. Serotonin in sleep
Extensive evidence acquired using electrophysiology, neurochemistry, neuropharmacology and genetic studies suggest that increased 5-HT neurotransmission increases wakefulness and prevents rapid eye movement (REM) sleep, for recent review see (Monti 2011).

Increasing 5-HT neurotransmission (via administration of SSRIs) inhibited sleep in rats (Pastel & Fernstrom 1987; Neckelmann et al. 1996), Syrian hamsters (Gao et al. 1992) and depressed patients (Van Bemmel et al. 1993). Electrophysiological recording from the DR nucleus in unanesthetised cats showed increased neuronal firing during wakefulness, which progressively decreased during the early, middle and the late phases of sleep (McGinty & Harper 1976; Trulson & Jacobs 1979).

Inhibition of 5-HT neuronal activity (by microdialysis perfusion of 8-OH-DPAT into the DR nucleus) decreased 5-HT release and promoted sleep in freely moving cats (Portas et al. 1996). Similarly, injection of 5-HT$_{1A}$ receptor agonists (flesinoxan and 8-OH-DPAT) into the DR nucleus increased sleep in freely moving rats that was reversed by selective 5-HT$_{1A}$ receptor antagonist WAY 100635 (Monti et al. 2002). On the other hand, intra-dorsal raphe nucleus injection of 5-HT$_{1A}$
receptor antagonists (WAY 100635 or p-MPPI) prevented sleep, suggesting that activation of pre-synaptic 5-HT_{1A} receptors within the DR nucleus (which inhibits 5-HT neuronal firing and reduces 5-HT release at terminal sites) increases sleep, whereas selective blockade of these receptors induces the opposite effect (Sorensen et al. 2001; Monti et al. 2002).

Systemic injection of 5-HT_{1A} receptor agonist (flesinoxan) reduced sleep in rats (Monti & Jantos 2003), which may be due to activation of post-synaptic 5-HT_{1A} receptors that mediate the inhibitory effect of 5-HT on sleep. Similarly, systemic administration of low doses of 8-OH-DPAT (which mainly activate pre-synaptic 5-HT_{1A} receptors) increased sleep, whilst high doses of 8-OH-DPAT (which activate post-synaptic 5-HT receptors) inhibited sleep in rats (Monti & Jantos 1992; Monti et al. 1994). Furthermore, genetic deletion of 5-HT_{1A} receptors increased sleep in 5-HT_{1A} receptor knock-out mice (Boutrel et al. 2002) that may be due to absence of the inhibitory effect of post-synaptic 5-HT_{1A} receptors on sleep. Although the precise inhibitory mechanism of post-synaptic 5-HT_{1A} receptors on sleep is not clear, it is likely to involve inhibition of sleep active GABAergic neurones in the ventrolateral preoptic area of the hypothalamus (Monti 2011).

Altogether, these data suggest that activation of somatodendritic 5-HT_{1A} receptors in the DR nucleus increases sleep via suppression of 5-HT neuronal activity and reduced 5-HT release, whilst activation of post-synaptic 5-HT_{1A} receptors inhibit sleep possibly via inhibition of GABAergic neurones in the hypothalamus.

Other studies also support the involvement of 5-HT_{1B}, 5-HT_{2}, 5-HT_{6} and 5-HT_{7} receptors in sleep regulation, where intraperitoneal administration of 5-HT_{1B}, 5-HT_{2} and 5-HT_{7} receptor agonists as well as 5-HT_{6} receptor antagonist reduced sleep in rats (Dugovic et al. 1989; Bjorvatn & Ursin 1994; Monti 2011; Monti & Jantos 2011), although their precise mechanisms of action awaits further research.
1.3.6. Serotonergic transmission in cognition

1.3.6.1. Serotonin in short-term and long-term memory

Serotonin plays a critical role in cognitive function including learning, short-term and long-term memory as well as cognitive flexibility (Meneses 1999; Schmitt et al. 2000; Evers et al. 2007). The cognitive effect of 5-HT is mediated by its interaction with other neurotransmitter systems including the glutamatergic, GABAergic, DA-ergic and in particular ACh-ergic systems, for reviews see (Meneses 1999; Buhot et al. 2000; Olvera-Cortes et al. 2008) (Fig. 1.30).

Anatomically, the 5-HT/ACh interactions occur at their nuclei of origin namely: the raphe nuclei (which encompasses all 5-HT neurones) and the forebrain nuclei with high density of ACh neurones including the medial septum (MS), the ventricular limb of the diagonal band of Broca (vDBB), the nucleus basalis magnocellularis (NBM), the pedunculopontine tegmental (PPT) nucleus and the laterodorsal tegmental nucleus (LTN) (Steckler & Sahgal 1995). 5-HT projections from the raphe nuclei terminate in multiple forebrain regions with high densities of ACh neurones such as the MS, the vDBB, the NBM, the PPT and the LTN (Kohler et al. 1982; Semba et al. 1988; Vertes 1988; Jones & Cuello 1989; Vertes 1991; Milner & Veznedaroglu 1993; Vertes et al. 1999). These forebrain structures, in turn, send dense ACh projections to the hippocampus, the cerebral cortex, the amygdala, the thalamus and the PFC (Mesulam et al. 1983a; Amaral & Kurz 1985; Senut et al. 1989). ACh projections to the raphe nuclei originate from ACh neurones located within the PPT and the LTN (Woolf & Butcher 1989; Jones 1990). The 5-HT/ACh interaction also occurs at their projection sites (i.e. the neocortex and the hippocampus that are intimately involved in learning and memory), which receive converging 5-HT and ACh inputs from the raphe nuclei and the forebrain nuclei, respectively (Senut et al. 1989; Steckler & Sahgal 1995; Vertes et al. 1999).
Figure 1.30. Schematic diagram illustrating the possible role of 5-HT transmission in cognition, which may be mediated through modulation of ACh system (left) and direct (right) role of 5-HT system affecting either short-term or long-term memory processes. Increased 5-HT neurotransmission in the septum strongly activates 5-HT1A receptors, which in turn reduces the activation of ACh neurones as well as memory acquisition and consolidation (far left). During reduced 5-HT neurotransmission in the septum, 5-HT1A receptors undergo weak activation, which in turn increases the activation of ACh neurones as well as memory consolidation (middle left). Based on (Jeltsch-David et al. 2008).

Increased 5-HT neurotransmission in the hippocampus (via oral administration of 5-HT precursor l-tryptophan, TrP) improved memory acquisition, memory consolidation and memory storage (Haider et al. 2006; Khaliq et al. 2006; Haider et al. 2007) (middle right). Selective lesion of 5-HT neurones in the raphe nuclei reduced 5-HT neurotransmission in the hippocampus that was also associated with reduced memory acquisition and memory consolidation (Lieben et al. 2006) as well as compromised adult neurogenesis (Brezun & Daszuta 1999; Brezun & Daszuta 2000a; Brezun & Daszuta 2000b) (far right).

Combined pharmacological blockade of 5-HT (by intraperitoneal injection of PCPA, which selectively and irreversibly inhibits TPH2 [the rate limiting enzyme for 5-HT synthesis]) and ACh (by M1 receptor antagonist scopolamine or M2 antagonist atropine) neurotransmission, induced a severe deficit in spatial and working memory (measure using MWM task) (Richter-Levin & Segal 1989; Riekkinen et al. 1991).
Lesion studies further support the importance of the 5-HT/ACh interaction in cognitive function (Richter-Levin et al. 1993; Lehmann et al. 2002). Although the individual lesion of 5-HT (via intracerebroventricular injection of neurotoxic agent 5,7-dihydroxytryptamine, 5,7-DHT) and ACh (via intracerebroventricular injection of ACh-neurones specific immunotoxin 192 IgG-saporin) systems resulted in minor behavioural alterations, concomitant lesions of 5-HT and ACh systems induced severe deficit in learning (Nilsson et al. 1988; Richter-Levin & Segal 1991a; Richter-Levin et al. 1993; Lehmann et al. 2002) and working memory (Lehmann et al. 2000; Jeltsch-David et al. 2008). Lesion-induced memory impairments were reversed by (i) embryonic raphe grafts (rich in 5-HT neurones) into the hippocampus but not into the EC or the hypothalamus (Richter-Levin et al. 1993; Richter-Levin et al. 1994) and (ii) simultaneous transplantation of septal grafts (rich in ACh neurones) and raphe grafts into the hippocampus (Nilsson et al. 1990) but not into the EC (Richter-Levin et al. 1993). In fact, transplanting a co-grafts containing both septal and raphe grafts had an additive effect and produced greater improvement in spatial memory compared to transplanting them separately in pre-lesioned rats (Nilsson et al. 1990).

Neurochemical studies demonstrated that the activation of 5-HT projections have an inhibitory effect on ACh release in the forebrain (Rada et al. 1993; Steckler & Sahgal 1995). Increased 5-HT input inhibits ACh release in the forebrain thus compromising memory acquisition and memory consolidation (Jeltsch-David et al. 2008) (Fig. 1.30).

Increasing evidence suggest that reduced 5-HT neurotransmission is associated with impaired memory and cognition. Selective lesions of 5-HT neurones (via intraraphe injection of 5,7-DHT) impaired object memory in rats (Lieben et al. 2006). Incidentally, selective memory impairment associated with the recreational use of ecstasy (3,4-methylenedioxyamphetamine, MDMA) may be due to the specific neurotoxic effect of the drug on 5-HT projections (O’Hearn et al. 1988; Wilson et al. 1989; Morgan 1999; de Sola Llopis et al. 2008). Selective lesion of the raphe nuclei was also associated with reduced adult neurogenesis (which is likely to be involved in memory function) in the sub granular layer of the DG and the sub ventricular zone (Brezun & Daszuta 1999; Brezun & Daszuta 2000a; Brezun &
Daszuta 2000b). The intracisternal administration of 5,7-DHT also prevented environmental enrichment-induced adult neurogenesis in the rat hippocampus (Ueda et al. 2005). Inhibiting 5-HT synthesis (by administration of PCPA) hampered the proliferation and survival of adult stem cells in vitro (Benninghoff et al. 2010). Depressed neurogenesis was reversed by transplantation of foetal raphe grafts in the hippocampus followed by graft-derived 5-HT re-innervations of the hippocampus (Brezun & Daszuta 2000b).

A decrease in 5-HT neurotransmission (via reduced dietary TrP intake) impaired object memory in rodents (Lieben et al. 2004; Jenkins et al. 2010). TrP-deficient diet also impaired short- and long-term memory by reducing memory encoding, consolidation and storage in healthy volunteers (Riedel et al. 1999; Schmitt et al. 2000; Rubinsztein et al. 2001; Scholtissen et al. 2006; Sambeth et al. 2007; Merens et al. 2008; Sambeth et al. 2009) and in AD patients (Porter et al. 2000; Newhouse et al. 2002; Porter et al. 2003) (Fig. 1.30).

Conversely, daily TrP injection improved spatial memory in aged rats (Levkovitz et al. 1994; Richter-Levin & Segal 1996). Likewise, chronic increase in 5-HT neurotransmission (by oral administration of TrP) has been associated with improved memory acquisition, consolidation and storage in rats (Haider et al. 2006; Khaliq et al. 2006; Haider et al. 2007). Acute intravenous injection of SSRI (citalopram) improved memory consolidation and long-term memory in healthy volunteers (Harmer et al. 2002). Chronic treatment with other SSRIs including fluoxetine also improved memory function and reduced cognitive decline in AD patients (Mowla et al. 2007; Mossello et al. 2008; Rozzini et al. 2010). Incidentally, the increase in 5-HT content in the brain (following chronic treatment with fluoxetine) promoted adult neurogenesis and synaptogenesis in the hippocampus that may be involved in memory improvement (Malberg et al. 2000; Hajszen et al. 2005; Marcussen et al. 2008), see also (Aboukhatwa et al. 2010) for review.

1.3.6.2. Serotonin receptors in learning and memory

5-HT-mediated effect on cognitive function depends on activation of 5-HT-specific receptors that are widely expressed in multiple brain regions involved in cognitive
function, including the neocortex and the hippocampus (Barnes & Sharp 1999; Hoyer et al. 2002). Although the majority of 5-HT receptors have been implicated in regulation of learning and memory processes, most studies have focused on 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptors due to their earlier characterisation. The role of these two receptors in learning and memory function is described in greater details below, followed by a brief description of the role of other 5-HT receptors in cognition (Table 1.6).

1.3.6.2.1. 5-HT\textsubscript{1A} receptors in learning and memory

In general, activation of 5-HT\textsubscript{1A} receptors impair cognitive function by interfering with memory acquisition and consolidation, whereas blockade of 5-HT\textsubscript{1A} receptors improve cognition by increasing ACh and glutamate neurotransmitter release (Harder et al. 1996; Harder & Ridley 2000; Misane & Ogren 2003; Luttgen et al. 2005a; Koenig et al. 2008) (see also Table 1.6).

The introduction of selective 5-HT\textsubscript{1A/7} receptor agonist -8-hydroxy-2-(di-n-propylamino)-tertralin (8-OH-DPAT) has been of major importance in addressing the role of 5-HT\textsubscript{1A} receptors in cognitive function (Arvidsson et al. 1981). Systemic treatment with 8-OH-DPAT reduced LTP induction in rat primary visual cortex (Edagawa et al. 1998). Administration of 8-OH-DPAT also impaired spatial memory, recognition memory, object recognition and fear memory (measured using MWM, object recognition and passive avoidance tests) in rats (Carli et al. 1992a; Carli & Samanin 1992; Carli et al. 1992b; Meneses 1999; Pitsikas et al. 2005; Elvander-Tottie et al. 2009).

The detrimental effect of 8-OH-DAPT on memory function is mediated by activation of post-synaptic 5-HT\textsubscript{1A} receptors in the septum and the hippocampus (Egashira et al. 2006; Elvander-Tottie et al. 2009). In particular (i) local injection of 8-OH-DPAT into the dorsal hippocampus impaired memory function in a dose-dependent manner (Egashira et al. 2006) and (ii) 8-OH-DPAT-induced memory impairment was not affected by 5-HT depletion of the brain (using 5,7-DHT), suggesting that the absence of pre-synaptic 5-HT\textsubscript{1A} receptors are not obligatory for detrimental effect of 8-OH-DPAT on memory function (Carli & Samanin 1992).
Table 1.6. Mechanisms of cognitive-enhancing effects of different serotonin receptors.

<table>
<thead>
<tr>
<th>5-HT Receptor</th>
<th>Agonist/Antagonist</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>Antagonist</td>
<td>Increase in ACh and glutamate release</td>
<td>Borg et al. 2008; Koening et al. 2008; Koenig et al. 2008</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>Agonist</td>
<td>Increase in ACh and glutamate release</td>
<td>Hirano et al. 1995; Meneses et al. 2002; Harvey et al. 2003</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Antagonist</td>
<td>Increase in ACh release and suppression of GABA</td>
<td>Staubli &amp; Xu 1995; Arnsten et al. 1997; Paus et al. 2004</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Agonist/Partial agonist</td>
<td>Increase in ACh release and reduced Aβ accumulation</td>
<td>Orsetti et al. 2003; Mohler et al. 2007</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;5&lt;/sub&gt;</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Antagonist</td>
<td>Increase in ACh and glutamate release</td>
<td>Dawson et al. 2001; West et al. 2009</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Agonist</td>
<td>nr</td>
<td>nr (Martin-Cora &amp; Pazos 2004; Ballaz et al. 2007)</td>
</tr>
</tbody>
</table>

Note: The increase in ACh and glutamate release is mediated by antagonists of 5-HT<sub>1A</sub>, 5-HT<sub>3</sub> and 5-HT<sub>6</sub> receptors (highlighter in red), whilst agonist of 5-HT<sub>2A</sub> and agonist/partial agonist of 5-HT<sub>4</sub> receptors (highlighted in green) induce increase in ACh and glutamate release. Key: nr: not reported.

From (Rodríguez et al. 2012).

Immunohistochemical studies demonstrated the presence of 5-HT<sub>1A</sub> receptors in ACh neurones within the septum and the diagonal band of Broca (Kia et al. 1996b; Luttgen et al. 2005b), suggesting regulation of ACh neurotransmissions by 5-HT<sub>1A</sub> receptors. In fact, 5-HT<sub>1A</sub> receptor antagonists and partial agonists increased ACh release in the cortex and in the hippocampus that were associated with improved memory performance (Millan et al. 2004; Schechter et al. 2005; Hirst et al. 2008; Kehr et al. 2010). In addition, 5-HT<sub>1A</sub> receptor antagonists reversed memory impairments induced by ACh and glutamate deficits (achieved by the intrahippocampal injection of M<sub>1</sub> receptor antagonist scopolamine and NMDA receptor antagonist 7-chloro-kynurenic acid) (Carli et al. 1997a). The 5-HT<sub>1A</sub> receptor antagonist WAY100635 also reversed learning deficits induced by intraperitoneal injection of AMPA receptor blocker NBQX (Schiapparelli et al. 2006). Schiapparelli and colleagues (2005) showed that inhibition of hippocampal 5-HT<sub>1A</sub> receptors (by intraperitoneal injection of WAY-100635) promoted the molecular cascades implicated in memory formation including (i) increased phosphorylation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII), (ii) enhanced Ca<sup>2+</sup>-independent CaMKII and protein kinase A (PKA) enzyme activity
as well as (iii) increased membrane expression of GluR1 subunit of the AMPA receptors in the hippocampus (Schiapparelli et al. 2005).

1.3.6.2.5-HT2A receptors in learning and memory

Conceptually, 5-HT2A receptor agonists improve learning and memory consolidation whereas 5-HT2A receptor antagonists induce a negative effect on learning and memory functions (Harvey 1996; Harvey 2003) (Table 1.6). At the behavioural level, 5-HT2A receptor activation improves working memory (as indicated by delayed-response task in monkeys) (Williams et al. 2002).

Double in situ hybridisation, immunohistochemistry and ultrastructural studies found that 5HT2A receptors co-localise with NMDA receptors and with GABA receptors in the hippocampus and the cortex, further favouring the modulatory role of 5-HT2A receptors in glutamatergic and GABAergic neurotransmissions (Willins et al. 1997; Jakab & Goldman-Rakic 1998; Santana et al. 2004; de Almeida & Mengod 2007; Peddie et al. 2008a). Activation of cortical 5-HT2A receptors increased glutamate and GABA release from pre-synaptic terminals (Abi-Saab et al. 1999; Aghajanian & Marek 1999; Marek & Aghajanian 1999; Scruggs et al. 2003). This effect is mediated by 5-HT2A-induced inhibition of voltage-gated K+ channel that in turn increases neuronal firing (Lambe & Aghajanian 2001). On the contrary, another study reported 5-HT2A receptor-mediated depression of glutamate release (Wang et al. 2006). However, the latter study used isolated nerve terminals (synaptosomes) mainly consisting of pre-synaptic receptors whereas the majority of hippocampal 5-HT2A receptors are post-synaptic (Peddie et al. 2008a) (Fig. 1.28). This may explain the apparent discrepancy in 5-HT2A receptor-mediated glutamate release in the isolated pre-synaptic nerve terminals (Wang et al. 2006) as opposed to previous studies performed on in vitro slice preparations (Aghajanian & Marek 1999) or employing in vivo microdialysis (Scruggs et al. 2003). Interestingly, 5-HT2A receptors also exhibit high affinity for hallucinogenic and antipsychotic drugs (Scruggs et al. 2003). In fact, the cognitive-enhancing effect of atypical antipsychotic is partially attributed to their effect on 5-HT2A receptors (Tyson et al. 2004).
1.3.6.2.3. Other 5-HT receptors in learning and memory

Pharmacological inhibition of 5-HT$_3$ receptor (by intraperitoneal injection of selective 5-HT$_3$ receptor antagonist ondansetron) promoted LTP induction, memory retention and spatial memory (Staubli & Xu 1995). In addition, 5-HT$_3$ receptor antagonists reversed cognitive deficits associated with the M$_1$ receptor antagonist scopolamine (Carey et al. 1992; Carli et al. 1997b; Pitsikas & Borsini 1997) and age-associated deficit in memory function in rats, marmosets and monkeys (Pitsikas & Borsini 1996; Arnsten et al. 1997). The majority of 5-HT$_3$-immunoreactive neurones also express GABA, possibly indicating an interaction between 5-HT and GABA neurotransmissions (Morales et al. 1996; Morales & Bloom 1997; Puig et al. 2004). The cognitive-enhancing effects of 5-HT$_3$ receptor antagonists are mediated indirectly via suppression of GABA neurotransmission (Choi et al. 2007) and a concomitant increase in ACh neurotransmitter release (Ramirez et al. 1996) (Table 1.6).

Activation of 5-HT$_4$ receptor by selective agonists and parital agonists improved LTP induction as well as attention, learning and memory performances in rodents (Moser et al. 2002; Marchetti et al. 2004; Mohler et al. 2007; Hille et al. 2008). 5-HT$_4$ receptor agonists and partial agonists were also effective in reversing the cognitive impairment associated with the M$_1$ receptor blockade (Galeotti et al. 1998; Lelong et al. 2003), ageing (Moser et al. 2002; Marchetti et al. 2011) and intracerebroventricular injection of Aβ (Micale et al. 2006). Interestingly, 5-HT$_4$ receptor agonists promote dendritic spine growth in the hippocampus (Restivo et al. 2008) and rescued memory impairments induced by hippocampal damage (following intragyral infusion of tubulin inhibitor colchicine) (Marchetti et al. 2008). Although the precise mechanisms involved in cognitive-enhancing properties of 5-HT$_4$ receptor remains unknown, it may be mediated through stimulation of ACh release (Consolo et al. 1994; Mohler et al. 2007) (Table 1.6). Agonist and parital agonists of 5-HT$_4$ receptor stimulate the non-amyloidogenic processing of APP both in vitro (Robert et al. 2001; Lezoualch & Robert 2003; Cho & Hu 2007; Mohler et al. 2007) and in vivo (Cachard-Chastel et al. 2007; Russo et al. 2009). This suggests that manipulation of 5-HT$_4$ receptor may be useful not only for improving cognitive impairments but it may also be effective in
reducing the Aβ load associated with neurodegenerative disease such as AD (Russo et al. 2009).

Pharmacological activation of 5-HT₆ receptor attenuated LTP induction (West et al. 2009) and impaired both short- and long-term memory (Meneses et al. 2008). On the other hand, selective 5-HT₆ receptor antagonists improved memory acquisition, retention and consolidation in rats (Rogers & Hagan 2001; Woolley et al. 2001; Perez-Garcia & Meneses 2005; Marcos et al. 2008). Administration of 5-HT₆ receptor antagonist reversed scopolamine-induced deficits in object recognition, fear memory and learning as well as the age-dependent deficits in special memory (Foley et al. 2004; Perez-Garcia & Meneses 2005; Hirst et al. 2006). Although the underlying mechanism of 5-HT₆ receptor antagonist-mediated improvement in memory function is not clear, it is likely to be due to stimulation of ACh and glutamate release (Dawson et al. 2001; Hirst et al. 2006; West et al. 2009). Recently, several 5-HT₆ receptor antagonists have advanced into clinical trials for the treatment of cognitive deficits associated with neurodegenerative diseases (Kwon et al. 2004; Geldenhuys & Van der Schyf 2008; Upton et al. 2008).

Finally, pharmacological stimulation of 5-HT₇ receptor (using a selective agonist, AS 19) enhanced memory consolidation and reversed scopolamine-induced amnesia in rats (Perez-Garcia et al. 2006). Conversely, pharmacological inhibition of 5-HT₇ receptor reduced object-recognition memory in rats (Ballaz et al. 2007) and spatial memory in mice (Sarkisyan & Hedlund 2009). Similarly, 5-HT₇ receptor knock-out mice displayed reduced hippocampal LTP induction that was associated with impaired contextual learning and spatial memory performance in these mice (Roberts et al. 2004; Sarkisyan & Hedlund 2009). Further research is required to uncover the cognitive-enhancing mechanism mediated by 5-HT₇ receptor activation.
1.4. SEROTONIN DURING BRAIN DEVELOPMENT AND AGEING PROCESS

1.4.1. Development of serotonergic system

In humans, 5-HT neurones emerge between 5 – 6 weeks of gestation (Sundstrom et al. 1993), whereas in mice and rats 5-HT neurones are detected between embryonic days 11 – 12 (E11 – E12) (Lauder 1990; Briscoe et al. 1999; Zhou et al. 2000; Whitaker-Azmitia 2001; Deng et al. 2007a). In rats, 5-HT projections appear at E12 as soon as 5-HT cell bodies become visible and they are increasingly evident during the growth of 5-HT axons from the raphe nuclei (Lidov & Molliver 1982a; Lidov & Molliver 1982b; Bruning et al. 1997). In rats, descending 5-HT projections reach the spinal cord between E12 – E14, whereas ascending 5-HT projections attain their destinations in the thalamus, the amygdala and the frontal cortex much later between E16 through postnatal day 21 (P21) (Lidov & Molliver 1982a; Lauder 1990; Zhou et al. 2000).

The ontogeny of 5-HT projections in rats can be divided into (i) initial axon elongation [E13 – E16], (ii) selective pathway development [E15 – E19] and (iii) maturation of 5-HT terminal field [E19 – P21] (Lidov & Molliver 1982a). High levels of 5-HT innervations (measured using 5-HT transporter binding and autoradiography) during brain development continues for the first 4 weeks after birth that is followed by a pronounced decrease at P28 and remains at this decreased level throughout adulthood in rats (Galineau et al. 2004). The density of 5-HT projections during brain development is regulated by neurotrophic factors and 5-HT itself that control growth cone motility and synaptogenesis (Ahmad & Zamenhof 1978; Haydon et al. 1984; Shemer et al. 1991). The developmental effect of 5-HT is primarily mediated by activation of 5-HT1A receptors (which regulate 5-HT neuronal firing) and 5-HT1B receptors (which regulate 5-HT release at the terminal regions) (Whitaker-Azmitia & Azmitia 1986; Whitaker-Azmitia et al. 1987). In particular, the outgrowth of 5-HT projections requires S-100β protein, a neurotrophic factor that is synthesised, stored and released by astrocytes (Azmitia et al. 1990; Whitaker-Azmitia et al. 1990; Liu & Lauder 1992).
1.4.2. Serotonergic transmission in ageing

Age-related alterations in 5-HT system occur at multiple levels including changes in (i) the density of 5-HT-positive neurones in the raphe nuclei, (ii) 5-HT neurotransmitter level in the CNS, (iii) the density of 5-HT projections, (iv) 5-HT transporter expression and (v) 5-HT receptors, for review see (Palmer & DeKosky 1993; Meltzer et al. 1998a) (Table 1.7).

1.4.2.1. Serotonin neurotransmission in ageing

Most commonly, rats at different ages have been used to study age-associated changes in 5-HT system (Table 1.7). Aged rats displayed stable 5-HT neurones in the raphe nuclei up to 19 – 24 month of age (van Luijtelaar et al. 1992). Likewise, 5-HT neurones remain stable in aged humans (62 – 84 years) (Kloppel et al. 2001). In addition to stable 5-HT neurones, the rate of 5-HT synthesis (measured by accumulation of 5-hydroxytryptophan after decarboxylase blockade) is also unchanged in aged (24 months) rats (Herrera et al. 1991).

Despite the steady number of 5-HT neurones and stable 5-HT synthesis, the majority of studies in aged rats have reported reduced 5-HT neurotransmitter levels in multiple brain regions (Table 1.7). Specifically, aged (25 – 27 months) rats displayed reduced 5-HT release in the hippocampus and the frontoparietal cortex (measured using electrically evoked tritium in slices preloaded with \([^3\text{H}]5\text{-HT}\)) (Birthelmer et al. 2003a; Birthelmer et al. 2003b). In addition, increased 5-HT metabolism (measured by an increase in 5-HIAA/5-HT ratio) in the hypothalamus, the median eminence, the frontal cortex, the amygdala and the striatum have been reported in aged (24 months) rats (Rodríguez-Gomez et al. 1995; Miguez et al. 1999). Therefore, age-associated decrease in 5-HT neurotransmitter level may be due to reduced 5-HT release and accelerated 5-HT metabolism.
Table 1.7. Age-associated changes of serotonergic system in rats.

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Brain Region</th>
<th>Ageing effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 - 24</td>
<td>DR</td>
<td>No change</td>
<td>van Luijtelaar et al. 1992</td>
</tr>
</tbody>
</table>

5-HT Neurotransmission

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Brain Region</th>
<th>Ageing effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Me, R</td>
<td>Reduced</td>
<td>Bhaskaran &amp; Radha 1983</td>
</tr>
<tr>
<td>25 - 26</td>
<td>Hy</td>
<td>Reduced</td>
<td>Simpkins 1984</td>
</tr>
<tr>
<td>24 - 30</td>
<td>ST</td>
<td>Reduced</td>
<td>Machado et al. 1986</td>
</tr>
<tr>
<td>22</td>
<td>FC, ST, Hy</td>
<td>Reduced</td>
<td>Petkov et al. 1987</td>
</tr>
<tr>
<td>24</td>
<td>SuCo</td>
<td>Reduced</td>
<td>Herrera et al. 1991</td>
</tr>
<tr>
<td>25 - 26</td>
<td>SC</td>
<td>Reduced</td>
<td>Ko et al. 1997</td>
</tr>
<tr>
<td>24</td>
<td>FC</td>
<td>Reduced</td>
<td>Miguez et al. 1999</td>
</tr>
<tr>
<td>26</td>
<td>OC, ST</td>
<td>Reduced</td>
<td>Stemmelin et al. 2000</td>
</tr>
<tr>
<td>24</td>
<td>PFC</td>
<td>Reduced</td>
<td>Mizoguchi et al. 2010</td>
</tr>
<tr>
<td>29</td>
<td>H, cb, ST</td>
<td>Non-change</td>
<td>Ponzie et al. 1982</td>
</tr>
<tr>
<td>24 - 35</td>
<td>R, Hy, H</td>
<td>Increased</td>
<td>van Luijtelaar et al. 1992</td>
</tr>
</tbody>
</table>

5-HT Projections

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Brain Region</th>
<th>Ageing effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 - 32</td>
<td>FC, PC, Pu</td>
<td>Reduced 5-HT fibre density</td>
<td>van Luijtelaar et al. 1988</td>
</tr>
<tr>
<td>28</td>
<td>FC, PC, ST</td>
<td>Reduced 5-HT fibre density</td>
<td>Steinbusch et al. 1990</td>
</tr>
<tr>
<td>28</td>
<td>NeoC, H, ST, NAc, T</td>
<td>Reduced 5-HT fibre density</td>
<td>Davidoff &amp; Lolova 1991</td>
</tr>
<tr>
<td>18</td>
<td>Me</td>
<td>Reduced 5-HT fibre density</td>
<td>Behan &amp; Brownfield 1999</td>
</tr>
</tbody>
</table>

5-HT Transporter

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Brain Region</th>
<th>Ageing effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>FC</td>
<td>Reduced</td>
<td>Brunello et al. 1985</td>
</tr>
</tbody>
</table>

5-HT Receptors

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Brain Region</th>
<th>Ageing effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>DR, Hy, Pu</td>
<td>Reduced 5-HT_{1A} receptors</td>
<td>Halpern et al. 1989</td>
</tr>
<tr>
<td>18</td>
<td>SuCo, PR</td>
<td>Reduced 5-HT_{2A} receptors</td>
<td>Parsons et al. 2001</td>
</tr>
</tbody>
</table>

Note: Highlighted in yellow are studies that reported age-related decrease in 5-HT system, highlighted in green are studies that reported no changes, whilst highlighted in red is a single study that found an increase in 5-HT neurotransmitter level. 


From Rodríguez et al. 2012.

Age-related decrease in 5-HT neurotransmission is accompanied with morphological alterations of 5-HT projections in aged rats (van Luijtelaar et al. 1992; Rodríguez-Gomez et al. 1995). 5-HT projections displayed degenerative profiles in aged rats as indicated by swollen and tortuous varicosities or abnormally thickened, ballooned or spherical axon terminals (van Luijtelaar et al. 1988;
Steinbusch et al. 1990; van Luijtelaa et al. 1992). These degenerative features are similar to that produced following intracerebroventricular injection of 5-HT neurotoxin (5,7-DHT) (van Luijtelaa et al. 1989). These findings suggest that age-associated aberrant fibre morphologies represent degeneration of ascending 5-HT projections (van Luijtelaa et al. 1988; van Luijtelaa et al. 1989; van Luijtelaa et al. 1992). In senescent rat brains, the incidence of degenerative fibres increased with advanced age (36 months) in the cortex and the hippocampus (Nishimura et al. 1998).

Aged rats also exhibited a general decrease in 5-HT fibre densities throughout the brain including the neocortex, the caudate nucleus, the putamen, the medulla and the hippocampus (van Luijtelaa et al. 1988; Davidoff & Lolova 1991; Behan & Brownfield 1999) (Table 1.7). Reduced 5-HT fibre density was initially observed in rats at 18 months of age and progressively decreased with advanced age (Behan & Brownfield 1999). The reduction in 5-HT fibre density occurred without the appearance of aberrant 5-HT fibre morphology in the hippocampus (van Luijtelaa et al. 1988; Steinbusch et al. 1990).

The precise mechanism of age-associated decrease in 5-HT projection is unclear, although Nishimura and colleagues (1995) proposed an association with S-100β-expressing astrocytes (Nishimura et al. 1995). The authors reported reduced 5-HT fibres in aged rats that showed a positive correlation with reduced number of S-100β-expressing astrocytes in the hippocampus (Nishimura et al. 1995). Transgenic mice with reduced S-100β expression displayed decreased 5-HT fibres in the cortex and the hippocampus (Ueda et al. 1994), whereas S-100β-over expressing mice displayed an increase in serotonergic fibres in the hippocampus (Shapiro et al. 2010). These data suggest that the age-related reduction in 5-HT fibres may be due to the decrease in the S-100β neurotrophic factor released from the neighbouring astrocytes.

Alternatively, other reports suggest that age-associated decrease in 5-HT fibres could be linked to a decline in the brain derived neurotrophic factor (BDNF) signalling system (Luellen et al. 2007; Aznar et al. 2010). Transgenic mice with reduced BDNF expression (BDNF+/−) show accelerated age-associated loss of 5-HT
projections in the hippocampus that starts from 12 months of age (Luellen et al. 2007). Similar accelerated age-associated decrease in 5-HT projections was observed in the congenital learned helplessness rat (cLH), an animal model of depression that was also linked to a reduced BDNF signalling (Aznar et al. 2010).

Aged (24 months) rats displayed reduced SERT binding sites (measured using [3H] imipramine binding) when compared to young adult controls (Brunello et al. 1985) (Table 1.7). Binding studies using [3H] imipramine also showed ~50% age-associated reduction in SERT binding sites in the human cingulate cortex (Marcusson et al. 1987). On average, there was ~10% decrease per decade in SERT binding sites in human brain stem and the thalamus (Yamamoto et al. 2002). In vivo imaging studies in human using PET and SPECT also found age-related decline in SERT binding potential (BP) in the thalamus, the midbrain and the brain stem (Pirker et al. 2000; van Dyck et al. 2000; Yamamoto et al. 2002). However, it is important to note that BP represents transporter density (B\text{max}), affinity (K\text{D}) or mixture of both (Meltzer et al. 1999). This is particularly important when interpreting studies using BP, because abnormalities in either (B\text{max} or K\text{D}) or both measures may contribute to the reported difference. Reduction in transporter affinity decreases BP, which might be interpreted as reduced transporter density. Therefore, it is critical to take into account K\text{D}, when measuring transporter density using BP (Meltzer et al. 1999).

1.4.2.2. Serotonin receptors in ageing
1.4.2.2.1. 5-HT\textsubscript{1A} receptors
A decrease in 5-HT\textsubscript{1A} receptor binding sites (measured using \textsuperscript{3}H[5-HT] binding potential) was found in the DR nucleus and the hypothalamus but not in the frontal cortex in aged (18 months) rats (Halpern et al. 1989), suggesting region-specific alteration in 5-HT\textsubscript{1A} receptor expression during ageing (Table 1.7). Similarly, autoradiographic studies (using \textsuperscript{3}H[5-HT] and 5-HT\textsubscript{1A/7} receptor agonist \textsuperscript{3}H8-OH-DPAT as radioligand) demonstrated age-related decrease in 5-HT\textsubscript{1A} receptor density in multiple regions of the human brains including the raphe nuclei, the temporal cortex, the frontal cortex and the hippocampus (Marcusson et al. 1984a; Dillon et al. 1991). Reduced number of 5-HT\textsubscript{1A} receptors (assessed by
autoradiography with $^3$H[5-HT] radioligand) was also reported in the somatosensory cortex of aged rhesus monkeys (Bigham & Lidow 1995).

*In vivo* PET studies (using selective 5-HT$_{1A}$ receptor antagonist as radioligand [$^{11}$C–carbonyl] WAY-100635) further support the age-related decline in 5-HT$_{1A}$ receptor BPs in multiple brain regions including the neocortex and the hippocampus of monkeys (Tsukada et al. 2001) and humans (Cidis Meltzer et al. 2001; Tauscher et al. 2001). In the human cortex, Tauscher and colleagues (2001) found a decline of approximately 10% in 5-HT$_{1A}$ receptor BP per decade (Tauscher et al. 2001). An immunocytochemical analysis also showed age-associated decrease in 5-HT$_{1A}$ receptor density in the cerebella of both humans and mice (Yew et al. 2009).

### 1.4.2.2.2. 5-HT$_{2A}$ receptors

Aged rats displayed reduced 5-HT$_{2A}$ receptor density in various brain regions including the superior colliculus and the pretectum (measured using quantitative immunohistochemistry) (Parsons et al. 2001). Similar age-associated decline in 5-HT$_{2A}$ receptor density have been reported in rhesus monkeys (Bigham & Lidow 1995) and in humans (Marcusson et al. 1984b; Gross-Isseroff et al. 1990; Arranz et al. 1993). The decline in 5-HT$_{2A}$ receptor density commences from ~60 years of age in humans and shows a negative correlation with advanced age, suggesting that comparatively greater receptor loss is observed in younger ages (Marcusson et al. 1984b).

*In vivo* PET studies found an age-related loss of 5-HT$_{2A}$ receptors in the caudate putamen, the neocortex, the occipital cortex, the frontal cortex, the parietal cortex and the occipital cortex of humans (Wong et al. 1984; Blin et al. 1993; Iyo & Yamasaki 1993; Rosier et al. 1996; Meltzer et al. 1998b). Immunocytochemical analysis identified age-associated decrease in 5-HT$_{2A}$ receptor density in the cerebella of both humans and mice (Yew et al. 2009).

### 1.4.2.2.3. Other 5-HT receptors in ageing

Currently the available data on age-related alterations of other 5-HT receptor types is rather limited. An *in situ* hybridisation study reported stable 5-HT$_{2C}$ receptor mRNA in aged (22 – 24 months) rats (Yau et al. 1999). More recently a PET study,
using selective 5-HT$_4$ receptor antagonist [\textsuperscript{11}C] SB207145 as radioligand, had found age-associated decrease in human 5-HT$_4$ receptor binding sites throughout the brain (Marner et al. 2010b). At present there are no reports on age-associated alterations on 5-HT$_5$ and 5-HT$_6$ receptors, whilst the evidence for 5-HT$_7$ receptor also remains inconclusive (Yau et al. 1999; Kohen et al. 2000).

In summary, ageing has a complex effect on 5-HT neurotransmission throughout multiple brain regions. Although there are no clear alterations in the total number of 5-HT neurones, accumulated evidence suggest compromised 5-HT neurotransmission and alterations in the expression of 5-HT transporter and receptors during aging. These age-related alterations in 5-HT neurotransmission may contribute (at least partially) to the ageing of the brain.
1.5. **SEROTONIN IN AD**

1.5.1. **Serotonergic neurones in AD**

The neurodegenerative processes associated with AD is closely linked with decreased number of 5-HT neurones in the DR and MR nuclei of the brain stem (Yamamoto & Hirano 1985; Aletrino *et al.* 1992; Halliday *et al.* 1992; Chen *et al.* 2000a; Kovacs *et al.* 2003) (see also Table 1.8). Post-mortem studies on AD brains have consistently reported reduced densities of 5-HT neurones in the raphe nuclei (Lyness *et al.* 2003). AD brains also exhibit increased Aβ plaques and NFTs in the DR and MR nuclei (Curcio & Kemper 1984; Ebinger *et al.* 1987; German *et al.* 1987; Halliday *et al.* 1992; Hendricksen *et al.* 2004), which may explain AD-related loss of 5-HT neurones. In AD, loss of 5-HT neurones was linked with rapid progression of clinical symptoms associated with the disease (Halliday *et al.* 1992).

Post-mortem AD studies have primarily focused on the DR and MR nuclei, which encompass the majority of 5-HT neurones that project to the neocortex and the hippocampus, among others (Vertes 1991; Vertes *et al.* 1999). The main loss of 5-HT neurones were reported in the caudal part of the DR nucleus (Zweig *et al.* 1988), which is the origin of 5-HT projection that innervate the septum and the hippocampus; brain regions that are severely affected in AD (Braak & Braak 1991). Earlier post-mortem studies on AD-related neuronal loss in the raphe nuclei relied upon traditional stains including the haematoxylin, the cresyl violet and the Nissl staining (Curcio & Kemper 1984; Yamamoto & Hirano 1985; Wilcock *et al.* 1988; Zweig *et al.* 1988; Burke *et al.* 1990; Aletrino *et al.* 1992; Halliday *et al.* 1992). AD-related loss of 5-HT neurones was confirmed in subsequently post-mortem studies that used immunohistochemistry and specific antibody against TPH2 (Kovacs *et al.* 2003; Hendricksen *et al.* 2004). A 3-dimensional study of the DR nucleus reported up to 40% loss of 5-HT neurones in post-mortem AD brains (Aletrino *et al.* 1992).
### Table 1.8. AD-associated changes in the number of serotonergic neurones: post-mortem and animal model studies.

<table>
<thead>
<tr>
<th>Method used</th>
<th>Age (AD)</th>
<th>N (AD)</th>
<th>Brain Region/changes in number of neurones in serotonergic nuclei</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post-mortem studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nissl staining</td>
<td>75</td>
<td>5</td>
<td>DR - reduced to 23% of the control</td>
<td>(Yamamoto &amp; Hirano 1985)</td>
</tr>
<tr>
<td>Nissl staining</td>
<td>78</td>
<td>12</td>
<td>DR - decrease in total number of neurones by ~40%</td>
<td>(Wilcock et al. 1988)</td>
</tr>
<tr>
<td>Cresyl violet staining</td>
<td>73</td>
<td>25</td>
<td>LC - reduction to 30% 19% and 33% of the control for rostral, mid and caudal levels, DR - reduction to 64% of the control only in caudal level, CSN - no change</td>
<td>(Zweig et al. 1988)</td>
</tr>
<tr>
<td>Nissl staining</td>
<td>74</td>
<td>8</td>
<td>DR - overall number of neurones was reduced by 39%</td>
<td>(Aletrino et al. 1992)</td>
</tr>
<tr>
<td>Nissl staining</td>
<td>75</td>
<td>11</td>
<td>DR, MR - total number of neurones reduced by 25%</td>
<td>(Halliday et al. 1992)</td>
</tr>
<tr>
<td>Immunohistochemistry - TPH2</td>
<td>82</td>
<td>12</td>
<td>DR - density of neurones was reduced by 41%, MR - density of neurones was reduced by 29%</td>
<td>(Chen et al. 2000a)</td>
</tr>
<tr>
<td>Immunohistochemistry - TPH2</td>
<td>73</td>
<td>10</td>
<td>MR - significant decrease in the number of 5-HT synthetising neurones</td>
<td>(Kovacs et al. 2003)</td>
</tr>
<tr>
<td>Immunohistochemistry - TPH2</td>
<td>83</td>
<td>15</td>
<td>DR - 5-HT neurones density was reduced by 45%</td>
<td>(Hendricksen et al. 2004)</td>
</tr>
<tr>
<td>Haematoxylin and eosin</td>
<td>88</td>
<td>7</td>
<td>DR - no change</td>
<td>(Curcio &amp; Kemper 1984)</td>
</tr>
</tbody>
</table>
| PET with 
{\[^{18}\text{F}\]}altanserin and 
{(11)C}\text{N,N-Dimethyl-2-(2-aminoo-4-cyanophenylthio)benzylamine (}\text{C\text{DASB}\text{)}}  | 74       | 12     | DR - in PET scanning no change in SERT binding probe was found (suggesting no change in number of 5-HT neurones/projections)  | (Marner et al. 2010a)                         |
| **Animal model studies**     |          |        |                                                                                                                                 |                                                |
| Immunohistochemistry - 5-HT  |          |        | Transgenic mouse model of AD with Aβ pathology - ~50% loss of monoaminergic neurones in the forebrain                           | (Liu et al. 2008)                             |
| Immunohistochemistry - TPH2  |          |        | Canine dog model of AD with Aβ pathology DR and MR ~33% reduction on serotonergic neurones in dogs with Aβ deposits            | (Beredo et al. 2009)                          |
| Immunohistochemistry - 5-HT  |          |        | Transgenic mouse model of AD with Aβ and tangle pathology DR and MR - no changes                                                | (Noristani et al. 2010)                       |

**Key:** Age: mean age, N: number of AD samples included in the study, LC: locus coeruleus, DR: dorsal raphe nucleus, CSN: Central superior raphe nucleus, MR: median raphe nucleus, TPH2: tryptophan hydroxylase isoform 2. Note: Highlighted in yellow are studies reported decrease in either total number of neurones or specific serotonergic neurones and highlighted in green are studies reported no changes these neurones.

Modified from (Rodríguez et al. 2012).
AD-related increase in NFT pathology in the raphe nuclei has been reported early during the course of the disease (Ishii 1966; Curcio & Kemper 1984; Yamamoto & Hirano 1985; German et al. 1987; Zweig et al. 1988; Chen et al. 2000a; Kovacs et al. 2003). In AD brains, hyperphosphorylation of tau cytoskeleton protein (measured by immunohistochemical staining against AT8) was evident during Braak stage I – II in the DR nucleus (Rub et al. 2000; Hendricksen et al. 2004). In addition to 5-HT neuronal loss, the remaining 5-HT neurones displayed severe shrinkage in AD brains (Aletrino et al. 1992).

Although early studies suggested greater 5-HT neuronal loss in AD patients with concomitant depression (Yamamoto & Hirano 1985; Zweig et al. 1988; Halliday et al. 1992), a more recent investigation had demonstrated the same degree of 5-HT neuronal death regardless of previous depressive symptoms (Hendricksen et al. 2004). These discrepancies may result from differences in methodological analysis of 5-HT neurones in the raphe nuclei (Kovacs et al. 2003). Indeed, whereas Zweig and colleagues (Zweig et al. 1988) relied upon cresyl violet staining to quantify total raphe neurones, Hendricksen and co-authors (Hendricksen et al. 2004) employed immunohistochemistry technique using specific antibody against TPH2 (Table 1.8). It is likely that the earlier profound neuronal loss reported by (Zweig et al. 1988) may be due to degeneration of non-5-HT rather than 5-HT-expressing neurones (Michelsen et al. 2008). Interestingly, a recent PET study in AD patients at early stage of disease reported no overall changes in 5-HT neuronal density in the raphe nuclei (Marner et al. 2010a).

It is important to note that AD-associated findings from post-mortem studies are affected by multiple factors such as the severity of underlying dementia, brain volume reduction and the quantitative methods applied to study 5-HT neurones. AD brains exhibit a significant decrease in the volume of gray matters throughout the brain (Thompson et al. 2001; Dickerson et al. 2009). The inclusion of partial volume correction (as a measure of volume atrophy) is critical when quantifying neuronal density using stereology, for review see (Coggeshall & Lekan 1996). Furthermore, post-mortem studies are prone to misinterpretation due to multiple experimental variables such as: (i) exclusive use of brain tissues derived from patients at the end stage of the disease, (ii) differences in pharmacological treatment
(e.g. AChE inhibitors, anti-depressants and anti-psychotics) and (iii) post-mortem delay (Chen et al. 1996; Meltzer et al. 1998a; Versijpt et al. 2003). Therefore, the use of animal models that reproduce AD pathology is of major importance for understanding specific alterations in neurotransmitter systems (including 5-HT) throughout the disease progression and for the development of potential therapies (Gotz et al. 2004a; Rodríguez et al. 2008; Rodríguez et al. 2009a; Rodríguez et al. 2009b) (see also chapter 1, section 1.2.).

Conflicting results have been reported on AD-related changes in 5-HT neurones using animal models of the disease. AβPP<sub>swe</sub>/PS1<sub>ΔE9</sub> double transgenic mouse with severe Aβ neuropathology displayed up to 50% loss of 5-HT neurones in the DR nucleus (Liu et al. 2008). Similarly, a study using the canine dog model of AD with Aβ neuropathology also reported reduced 5-HT neurones in the DR and MR nuclei (Bernedo et al. 2009). Conversely, other transgenic mouse models expressing either APP<sub>swe</sub> or PS1<sub>ΔE9</sub> single mutations displayed stable 5-HT neurones in the DR nucleus (Liu et al. 2008). Furthermore, a recent study in our laboratory reported no changes in the total number of 5-HT neurones in the DR and MR nuclei in 3xTg-AD mice (Noristani et al. 2010). These discrepancies may be due to differences in transgenic mouse lines (AβPP<sub>swe</sub>/PS1<sub>ΔE9</sub> vs. 3xTg-AD) (Liu et al. 2008; Noristani et al. 2010) or differences in species (dog vs. mouse) (Bernedo et al. 2009; Noristani et al. 2010).

Early post-mortem studies have proposed that AD-associated neurodegeneration is initiated at the axon terminals due to the accumulation of neurotoxins such as Aβ at terminal sites (Marcyniuk et al. 1986; Burke et al. 1988). At the affected terminals, these neurotoxins are taken up by afferent axons and undergo retrograde transport to the neuronal cell bodies, for review see (Hardy et al. 1986). The accumulation of neurotoxins within neuronal cell bodies triggers neuronal loss (Hardy et al. 1986). Similarly, in the AβPP<sub>swe</sub>/PS1<sub>ΔE9</sub> mice, the deposition of Aβ pathology is followed by degeneration of 5-HT axons at projection site, which is then progressed to 5-HT cell bodies in a retrograde manner (Liu et al. 2008).

Reduced densities of 5-HT neurones in the raphe nuclei may contribute to the cognitive deficits associated with AD (Hendricksen et al. 2004). Pronounced
deficits in learning and memory in rodents are only observed with combined double lesions of both 5-HT (by intracerebroventricular injection of 5,7-DHT) and ACh-ergic (by radiofrequency lesion of the septum or intracerebroventricular injection of ACh immunotoxin 192 IgG-saporin) neurones (Richter-Levin & Segal 1991a; Richter-Levin & Segal 1991b; Richter-Levin & Segal 1992; Richter-Levin et al. 1993; Lehmann et al. 2002; Jeltsch-David et al. 2008). Restoration of 5-HT and ACh neurones by simultaneous transplantation of the septal and the raphe grafts into the hippocampus is necessary for improved cognitive performance including learning and memory (Nilsson et al. 1990).

1.5.2. Serotonergic neurotransmission in AD
In addition to reduced 5-HT neurones, AD-related neuropathology is also associated with reduced 5-HT neurotransmitter levels in multiple brain regions (Table 1.9). Decreased 5-HT neurotransmitter levels were detected in the temporal cortex, the frontal cortex and the parietal cortex as well as in the amygdala, the caudate nucleus, the putamen and the raphe nuclei (Burke et al. 1990; Nazarali & Reynolds 1992; Lai et al. 2002; Garcia-Alloza et al. 2005; Bowen et al. 2008). Biopsies taken from the neocortex of AD patients also displayed severe deficits in 5-HT neurotransmitter levels in the temporal cortex and the frontal cortex (Palmer et al. 1987a). In AD, reduced 5-HT neurotransmitter levels was associated with progressive decline in cognitive performance (measured using MMSE scores) (Lai et al. 2002) as well as with increased behavioural symptoms including depression, aggressive behaviour and psychosis (Garcia-Alloza et al. 2005). In vivo PET studies (using selective 5-HT1A antagonist as radioligand [18F] MPPF) reported increased 5-HT1A receptor BPs in patients with mild cognitive impairment before the onset of AD symptomatic (Truchot et al. 2007; Truchot et al. 2008), suggesting an up-regulation of 5-HT metabolism in these patients. Similar finding have been reported in pre-clinical study, using the same radioligand, where reduced extracellular 5-HT content (achieved via intraperitoneal injection of tryptophan hydroxylase inhibitor, p-EPA) triggered an increase in 5-HT1A receptor BPs in the rat hippocampus (Zimmer et al. 2003). Interestingly, the deficit in 5-HT neurotransmission is specific for AD as in the frontotemporal dementia both 5-HT level and 5-HT projections were relatively preserved despite massive death of pyramidal neurones (Bowen et al. 2008).


**Table 1.9.** AD-associated changes in the levels of 5-HT/5-HIAA: post-mortem and animal model studies.

<table>
<thead>
<tr>
<th>Marker/ (HPLC)</th>
<th>Age (AD)</th>
<th>N (AD)</th>
<th>Brain Region/levels of 5-HT/5-HIAA% of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post-mortem studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>82</td>
<td>46</td>
<td>FC - 65 for 5-HT, TC - 49 for 5-HT</td>
<td>(Palmer et al. 1987a)</td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>76</td>
<td>17</td>
<td>A - 59 for 5-HT, 50 for 5-HIAA, DR - 215 for 5-HT, 118 for 5-HIAA</td>
<td>(Burke et al. 1990)</td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>81</td>
<td>13</td>
<td>TC - 37 for 5-HT, 63 for 5-HIAA, CN - 45 for 5-HT, 51 for 5-HIAA, A - 51 for 5-HT, 57 for 5-HIAA, Pu - 64 for 5-HT, 81 for 5-HIAA</td>
<td>(Nazarali &amp; Reynolds 1992)</td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>81</td>
<td>20</td>
<td>FC - 94 for 5-HT, 144 for 5-HIAA, TC - 70 for 5-HT, 117 for 5-HIAA</td>
<td>(Chen et al. 1996)</td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>81</td>
<td>20</td>
<td>FC - 45 for 5-HT, 47 for 5-HIAA, TC - 48 for 5-HT, 40 for 5-HIAA</td>
<td>(Garcia-Alloza et al. 2005)</td>
</tr>
<tr>
<td>5-HT 5-HIAA</td>
<td>74</td>
<td>9</td>
<td>FC - 75 for 5-HT, 99 for 5-HIAA, TC - 118 for 5-HT, 140 for 5-HIAA, PC - 55 for 5-HT, 87 for 5-HIAA</td>
<td>(Bowen et al. 2008)</td>
</tr>
<tr>
<td><strong>Animal model studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HIAA</td>
<td>Transgenic mouse model of AD with Aβ Pathology</td>
<td>C - 74 *</td>
<td></td>
<td>(Liu et al. 2008)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>Intrahippocampal Aβ injection</td>
<td>H - 151</td>
<td></td>
<td>(Verdurand et al. 2011)</td>
</tr>
</tbody>
</table>

**Key:** **Age:** mean age, **HPLC:** high performance liquid chromatography, **5-HT:** 5-hydroxytryptamine, **5-HIAA:** 5-hydroxyindoiatic acid, **N:** number of AD sample included in the study, **FC:** frontal cortex, **TC:** temporal cortex, **PC:** parietal cortex, **A:** amygdala, **CN:** caudate nucleus, **Pu:** putamen, **DR:** dorsal raphe nucleus, **C:** cortex, **H:** hippocampus, nr: not reported. *Numbers were estimated from the graphs. Note: Highlighted in yellow are studies reported decrease in the levels of 5-HT/5-HIAA and highlighted in red are studies reported increase in the levels of 5-HT/5-HIAA. Modified from (Rodríguez et al. 2012).
1.5.2.1. Serotonin projections in AD

Early reports had shown sprouting of hippocampal ACh axons in post-mortem AD brains (Geddes et al. 1985; Hyman et al. 1987a). However, to our knowledge, no dedicated post-mortem study has used 5-HT specific markers to analyse 5-HT projections in AD.

A previous study in the AβPP<sub>swe</sub>/PS1<sub>ΔE9</sub> double transgenic mouse model of AD found degeneration of 5-HT fibres in multiple brain regions including the cortex, the amygdala and the hippocampus between 12 – 18 months of age (Liu et al. 2008) (Table 1.10). However, more recent study in the same transgenic line up to 11 months of age revealed no alterations in 5-HT transporter binding sites (measured using [<sup>3</sup>H]-citalopram radioligand) despite Aβ deposition in the cortex and the hippocampus (Holm et al. 2010). Similarly, APP<sub>Sw,Ind</sub> mice displayed stable 5-HT fibre densities in the parietal cortex and the hippocampus (Aucoin et al. 2005), whilst the APP-23 transgenic mouse model showed aberrant non-5-HT axonal sprouting in the hippocampus (Phinney et al. 1999).

Experimentally-induced neurotoxin lesions (achieved by injection of ibotenic acid and NMDA into the NBM) and Aβ accumulation in the striatum and in the hippocampus triggered 5-HT fibre sprouting in rats (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001). Pronounced 5-HT fibre sprouting was also reported in monkeys with permanent hemi parkinsonism (achieved via experimental lesions of DA neurones) (Gaspar et al. 1993), supporting the existence of 5-HT fibre sprouting in a primate model of neurodegenerative disease. In addition to the experimentally-induced lesions, 5-HT fibre sprouting was also reported in multiple neurodegenerative disease mouse models including the brindled mottled mutant mouse model of Menkes' disease, a fatal neurodegenerative disorder (Martin et al. 1994) and the Wobbler mouse model of human motoneuron diseases including amyotrophic lateral sclerosis (ALS) (Bose & Vacca-Galloway 1999). Furthermore, an increased density of SERT-immunolabelled fibres and terminals were detected in the hippocampus of the 3xTg-AD mouse model of AD (Noristani et al. 2010; Noristani et al. 2011; Noristani et al. 2012) (chapters 3 - 5).
Table 1.10. Serotonergic system in animal models of AD.

<table>
<thead>
<tr>
<th>AD Model</th>
<th>Neuropathology</th>
<th>Brain Region</th>
<th>5-HT alteration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPswe/PS1ΔE9</td>
<td>Plaques</td>
<td>C, A, H</td>
<td>5-HT fibre degeneration (12 and 18 months)</td>
<td>(Liu et al. 2008)</td>
</tr>
<tr>
<td>APPswe/PS1ΔE9</td>
<td>Plaques</td>
<td>FC, PFC</td>
<td>No change</td>
<td>(Holm et al. 2010)</td>
</tr>
<tr>
<td>hAPPsw</td>
<td>Plaques</td>
<td>H</td>
<td>No change</td>
<td>(Aucoin et al. 2005)</td>
</tr>
<tr>
<td>Intrastratal ibotenic acid injected rat model</td>
<td>Excitotoxic lesion</td>
<td>ST</td>
<td>Vigorous sprouting of 5-HT fibres</td>
<td>(Zhou et al. 1995)</td>
</tr>
<tr>
<td>MBN injected NMDA rat model</td>
<td>Increased APP expression</td>
<td>NBM</td>
<td>Abundant sprouting of 5-HT fibres within damaged area</td>
<td>(Harkany et al. 2000b)</td>
</tr>
<tr>
<td>NBM injected Aβ1-42 rat model</td>
<td>Cholinergic lesion</td>
<td>NBM</td>
<td>5-HT fibre sprouting</td>
<td>(Harkany et al. 2001)</td>
</tr>
<tr>
<td>Aβ1-40 injected rat model</td>
<td>Plaques and Tangles</td>
<td>H</td>
<td>65% increased SERT-IR Fibre density (3 and 18 months)</td>
<td>(Noristani et al. 2010)</td>
</tr>
<tr>
<td>Intrahippocampal Aβ1-40 injected rat model</td>
<td>Aggregated amyloid</td>
<td>H</td>
<td>Increased 5-HT activity within the vicinity of injection site</td>
<td>(Verdurand et al. 2011)</td>
</tr>
</tbody>
</table>

Key: C: cortex, A: amygdala, H: hippocampus, ST: striatum, NBM: nucleus basalis magnocellularis, NMDA: N-methyl-D-aspartate, APP: amyloid precursor protein, FC: frontal cortex, PFC: prefrontal cortex. Note: Highlighted in yellow is a single study that reported degeneration of serotonergic fibres, highlighted in red are studies reported serotonergic fibre sprouting and a single study that reported increased 5-HT activity within the vicinity of the Aβ1-40 injection site.

Modified from (Noristani et al. 2011).

Several processes may account for 5-HT fibres sprouting. Injection of neurotoxins such as ibotenic acid, NMDA and Aβ1-42 can stimulate 5-HT fibre sprouting due to damage of neighbouring 5-HT fibres (homotypic sprouting). 5-HT fibre sprouting can also occur in response to damage of non-5-HT fibres (heterotypic sprouting) in the forebrain, the striatum and the hippocampus (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001). Chronic build-up of Aβ plaques may likewise induce neurotoxicity resulting in neuronal damage that in turn may also stimulate sprouting of 5-HT fibres (Harkany et al. 2000b; Harkany et al. 2001).

Glial derived neurotrophic factors play a critical role in promoting 5-HT fibre
sprouting. Increased Aβ plaque deposition triggers local inflammation in the brain that is associated with increased astrocyte and microglia activation surrounding Aβ plaques (Rodríguez et al. 2009b; Olabarria et al. 2010; Verdurand et al. 2011). These activated glia cells surrounding the Aβ plaques release numerous inflammatory cytokines as well as trophic factors such as S-100β that promote 5-HT fibre sprouting in AD (Fig. 1.31). S-100β is a neurotrophic factor released by astrocytes that is required for the outgrowth of 5-HT fibres (Azmitia et al. 1990; Whitaker-Azmitia et al. 1990; Liu & Lauder 1992; Azmitia 2001). Transgenic mice with increased S-100β expression display a concomitant increase in S-100β-positive astrocytes that was also associated with increased serotonergic fibre density in the hippocampus (Shapiro et al. 2010). In the rat striatum, 5-HT fibres sprouting was more evident in areas where the reactive astrocytes showed an increased staining for S-100β protein (Zhou et al. 1995). On the other hand, transgenic mice with reduced S-100β expression displayed reduced hippocampal 5-HT fibres (Ueda et al. 1994). Furthermore, a concomitant decrease in the number of S-100β positive astrocytes and the density 5-HT projections has been reported in the hippocampus of aged rats (Nishimura et al. 1995).

The gene for S-100β is located on chromosome 21, which is triplicated in patients with Down's syndrome (DS) (Gulesserian et al. 2000; Azmitia 2001). The neuropathological manifestations of DS are comparable to AD (Gulesserian et al. 2000; Azmitia 2001). Interestingly, a post-mortem study in DS and in AD brains revealed increased S-100β expression in the temporal lobe (Griffin et al. 1989). Western blot analysis of the frontal cortex also showed increased SERT protein expression in DS brains (Gulesserian et al. 2000).

The role of microglia-derived inflammatory factors that promote 5-HT fibre sprouting is not clear at the current stage. However, increased release of brain derived neurotrophic factor (BDNF) following experimental lesions have been shown to promote 5-HT fibre sprouting (Mamounas et al. 2000; Azmitia 2001) (Fig. 1.31, see also chapter 6).
Figure 1.31. Sprouting of serotonergic fibres and the potential neuro-protective mechanism of serotonin in AD. The sprouting is directly related with the presence of Aβ plaques that triggers a local inflammatory response associated with glia activation and increase the release of neurotrophic and inflammatory factors by astrocytes, microglia and degenerative neurones. Increased 5-HT input at the vicinity of Aβ plaques may hyperpolarise neurones through activation of 5-HTアン/アン receptors and subsequent opening of K⁺ channels. Hyperpolarisation in turn limits Ca²⁺ entry (and hence excitotoxicity) by closing voltage-gated Ca²⁺ channels (VGCCs) and favouring Mg²⁺ block of NMDA receptors (NMDARs).
From (Rodríguez et al. 2012).
The Aβ-induced neurotoxicity involves the increased activation of glutamatergic system with subsequent glutamate excitotoxicity (Brorson et al. 1995; Miguel-Hidalgo et al. 2002) and aberrant Ca\(^{2+}\) homeostasis (Stutzmann 2007; Supnet & Bezprozvanny 2010). 5-HT inhibits glutamatergic neurotransmission in various regions of the brain including the hippocampus (Schmitz et al. 1998) and the spinal cord (Takahashi et al. 2001). Increased 5-HT input in AD brains, in particularly at the vicinity of Aβ plaques, may counteract the NMDA-induced neurotoxicity via inhibition of Ca\(^{2+}\) currents and membrane hyperpolarisation (Harkany et al. 2000b) (Fig. 1.31). Electrophysiological recordings have shown inhibition of Ca\(^{2+}\) current by 5-HT in different brain regions such as the cortex, the caudal raphe nucleus, hypoglossal motoneurones and the ventromedial hypothalamus (Koike et al. 1994; Bayliss et al. 1995; Foehring 1996; Bayliss et al. 1997). These effects are mediated by the activation of 5-HT\(_{1A}\) and 5-HT\(_{1B}\) receptors (Patel & Zhou 2005; Peddie et al. 2008b). Therefore, an increased sprouting of 5-HT fibres may be an intrinsic protective mechanism in response to Aβ-induced excitotoxic damage in AD (see also chapter 6).

1.5.2.2. Serotonin receptors in AD

Increasing evidence support AD-related alterations in 5-HT specific receptors including 5-HT\(_{1A}\), 5-HT\(_{1B}\), 5-HT\(_{1D}\), 5-HT\(_{2A}\) and 5-HT\(_{6}\) receptors (Tables 1.11 and 1.12), for reviews see (Cross 1990; Meltzer et al. 1998a; Salmon 2007; Xu et al. 2012). Given the pivotal role of 5-HT receptors in learning and memory (section 1.3.6.2.), different agonists and antagonists of 5-HT receptors may be effective for treatment of cognitive impairment associated with AD (Terry et al. 2008; Upton et al. 2008).

1.5.2.2.1. 5-HT\(_{1A}\) receptors in AD

An early binding study (using \(^{3}\)H]-5-HT as radioligand) reported reduced 5-HT\(_{1A}\) receptor densities in the frontal and the temporal cortex of AD brains (Bowen et al. 1983). The finding of Bowen and colleagues (1983) was supported by subsequent binding studies (using the same radioligand) that demonstrated between 20 – 80% AD-associated decrease in 5-HT\(_{1A}\) receptor densities in the frontal cortex, the temporal cortex, the parietal cortex, the raphe nuclei, the amygdala and the hippocampus (Cross et al. 1984; Perry et al. 1984) (Table 1.11).
Table 1.11. AD-associated changes in 5-HT_1A receptors: post-mortem, PET and immunohistochemical studies.

<table>
<thead>
<tr>
<th>Radioligand/Marker</th>
<th>Age (AD)</th>
<th>N (AD)</th>
<th>Brain Region/5-HT_1A Receptors</th>
<th>% of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding studies</td>
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<td></td>
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<td></td>
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<tr>
<td>[3H]5-HT</td>
<td>72</td>
<td>nr</td>
<td>FC - 53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]5-HT</td>
<td>79</td>
<td>nr</td>
<td>PC - 74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]5-HT</td>
<td>79</td>
<td>12</td>
<td>FC - 85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td>TC - 53</td>
<td></td>
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<td></td>
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<td>H - 60</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A - 48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>82</td>
<td>24</td>
<td>H - 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td></td>
<td></td>
<td>PC - No changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]5-HT</td>
<td>78</td>
<td>13</td>
<td>PC - No changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]5-HT</td>
<td>72</td>
<td>8</td>
<td>PC - No changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>81</td>
<td>8</td>
<td>PC - No changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>83</td>
<td>85</td>
<td>PC - No changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>82</td>
<td>11</td>
<td>PC - Increased density</td>
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<td></td>
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<tr>
<td>[18F]MPPF</td>
<td>75</td>
<td>14</td>
<td>H - 73</td>
<td>R - 59</td>
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</tr>
<tr>
<td>[18F]MPPF</td>
<td>77</td>
<td>14</td>
<td>H - 87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18F]MPPF</td>
<td>72</td>
<td>11</td>
<td>H - 65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18F]MPPF</td>
<td>70</td>
<td>10</td>
<td>H, InfOG - Decreased binding potential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18F]MPPF</td>
<td>8</td>
<td>11</td>
<td>H - increased binding potential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunohistochemical studies</td>
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</tr>
<tr>
<td>5-HT_1A</td>
<td>83</td>
<td>8</td>
<td>PN - Reduced density</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VN - Reduced density</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R - Reduced density</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT_1A</td>
<td>77</td>
<td>11</td>
<td>H - Reduced immunoactivity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: Age: mean age, N: number of AD samples included in the study, BS: binding studies, Au: autoradiography, PET: positron emission tomography, aMCI: amnesic mild cognitive impairment, FC: frontal cortex, TC: temporal cortex, PC: parietal cortex, A: amygdala, H: hippocampus, Neo: neocortex, R: raphe nuclei, InfOG: inferior occipital gyrus, PN: pontile nuclei, VN: vagal nucleus, nr: not reported. Note: Highlighted in yellow are studies reported decrease in 5-HT_1A receptor binding potential/densities, highlighted in green are studies reported no changes in 5-HT_1A receptor binding potential/densities and highlighted in red are studies reported increase in 5-HT_1A receptor binding potential/densities. Modified from (Rodríguez et al. 2012).
Contrary to the earlier reports, other binding studies (using $[^3\text{H}]-5$-HT and 5-HT$_{1A/7}$ receptor agonist $[^3\text{H}]8$-OH-DPAT as radioligand) reported no AD-related alterations in 5-HT$_{1A}$ receptor binding sites in the temporal cortex, the neocortex and the hippocampus (Cross et al. 1986; Jansen et al. 1990; Lai et al. 2003b; Tsang et al. 2010). In addition, increased 5- HT$_{1A}$ receptor binding sites were reported in the frontal cortex of post-mortem AD brains (Lai et al. 2002), suggesting region-specific alterations in 5- HT$_{1A}$ receptor binding sites.

In vivo imaging studies (using PET combined with selective 5-HT$_{1A}$ antagonist $[^{18}\text{F}]$ MPPF as radioligand) support AD-related decrease in 5-HT$_{1A}$ receptor binding sites in the raphe nuclei and the hippocampus of patients with mild AD (Kepe et al. 2006; Truchot et al. 2008). Subsequent in vitro autoradiography measurement (using $[^{18}\text{F}]$ MPPF) confirmed earlier PET observation in the same group of patients and found up to 60% decrease in 5-HT$_{1A}$ receptor binding sites in the hippocampus (Kepe et al. 2006). AD patients with pronounced decrease in hippocampal 5-HT$_{1A}$ receptor binding sites displayed a more severe disease progression as shown by pronounced decline in their MMSE scores (Kepe et al. 2006). Similarly, progressive decrease in 5-HT$_{1A}$ receptor density in the temporal cortex correlated with increased aggressive behaviour in patients with AD (Lai et al. 2003b). However, the latest binding study (using 5-HT$_{1A/7}$ receptor agonist $[^3\text{H}]8$-OH-DPAT as radioligand) also had shown that the reduced hippocampal 5-HT$_{1A}$ receptor binding sites specifically correlates with depressive symptoms associated with AD (Lai et al. 2011).

Recent immunohistochemical study confirmed previous binding assays and in vivo PET imaging studies showing AD-related decrease in 5-HT$_{1A}$ receptor immunoreactivity (measured using optical density) in the hippocampus (Mizukami et al. 2011). In addition, the later immunohistochemical study supports earlier suggestion that 5-HT$_{1A}$ receptors are mainly affected at the late-stage of the AD pathology (Cross et al. 1984), with severe decrease in 5-HT$_{1A}$ receptor immunoreactivity observed only in patients at Braak stage V – VI (Mizukami et al. 2011).

Interestingly, three in vivo PET imaging studies (with $[^{18}\text{F}]$MPPF as a radioligand)
reported divergent alterations in 5-HT$_{1A}$ receptor binding sites in patients with mild cognitive impairments (Kepe et al. 2006; Truchot et al. 2007; Truchot et al. 2008). The first report demonstrated a decrease (Kepe et al. 2006), whereas subsequent studies have shown increased in the hippocampal 5-HT$_{1A}$ receptor binding sites in patients with mild cognitive impairment (Truchot et al. 2007; Truchot et al. 2008). It is likely that the difference between the reported studies may be due to inclusion of partial volume effect correction in the latter study to compensate for the atrophy of the hippocampus in AD brains (Truchot et al. 2007). In addition, the members of the control group used in the first PET study by Kepe and colleagues (Kepe et al. 2006) were of a younger age compared to that of patients with mild cognitive impairment (60 years in control vs. 77 years in mild cognitively impaired group) that may account for the difference between reported studies. This is particularly relevant when considering the age-associated decrease in hippocampal 5-HT$_{1A}$ receptor binding sites in humans (Marcusson et al. 1984a; Dillon et al. 1991).

AD-associated decrease in 5-HT$_{1A}$ receptors may reflect a compensatory mechanism to balance the compromised ACh and glutamatergic neurotransmissions (section 1.1.1.6.). Given that ACh hypofunction is the major pathological hallmark of AD and that pharmacological inhibition of 5-HT$_{1A}$ receptors increased ACh release in multiple brain regions concomitant with improved cognitive function; AD-related decrease in 5-HT$_{1A}$ receptor densities may result in reducing the inhibitory tone of 5-HT$_{1A}$ receptors on the remaining ACh neurones, hence, maintaining the functional activity of ACh neurotransmission (Rada et al. 1993; Millan et al. 2004; Schechter et al. 2005; Hirst et al. 2008; Kehr et al. 2010). It is however important to note that reduced 5-HT$_{1A}$ receptor densities have not been shown consistently in all post-mortem studies of AD brains (Table 1.11). Administration of 5-HT$_{1A}$ receptor antagonists have demonstrated promising results in pre-clinical studies in rodents and non-human primates as well as in clinical trials on AD patients (Misane & Ogren 2003; Millan et al. 2004; Luttgen et al. 2005a; Madjid et al. 2006; Hirst et al. 2008).

1.5.2.2.2. 5-HT$_{2A}$ receptors in AD
Unlike studies in 5-HT$_{1A}$ receptors, binding studies (using 5-HT$_{2A}$ receptor antagonist $[^{3}$H]-ketanserin as radioligand) have consistently demonstrated reduced
5-HT\textsubscript{2A} receptor binding sites in the temporal cortex, the frontal cortex, the parietal cortex, the amygdala, the EC and the hippocampus of post-mortem AD brains (Crow et al. 1984; Cross et al. 1986; Procter et al. 1988; Dewar et al. 1990; Cheng et al. 1991; Blin et al. 1993) (Table 1.12). Reduced 5-HT\textsubscript{2A} receptor densities have also been reported by numerous in vivo imaging studies using PET (Blin et al. 1993; Meltzer et al. 1999; Santhosh et al. 2009; Marner et al. 2010a), SPECT (Versijpt et al. 2003) and MRI (Hasselbalch et al. 2008). In addition, AD-related deficit in 5-HT\textsubscript{2A} receptors were found at a cellular level using immunohistochemistry with 5-HT\textsubscript{2A} receptor specific antibody (Lorke et al. 2006). AD-associated decrease in 5-HT\textsubscript{2A} receptors in the temporal cortex has been linked with cognitive decline (assessed using MMSE score) in AD patients (Lai et al. 2005) (Table 1.12).

Intrahippocampal injections of insoluble Aβ peptide induced memory impairment associated with decreased 5-HT\textsubscript{2A} receptors in the rat hippocampus (Christensen et al. 2008), suggesting that loss of 5-HT\textsubscript{2A} receptors may be related to accumulation of AD-related neuropathology. Recent in vitro autoradiography study with selective 5-HT\textsubscript{2A} receptor antagonist [\textsuperscript{3}H]-MDL100907 as radioligand also reported reduced 5-HT\textsubscript{2A} receptor binding sites in the median PFC of the AβPP\textsubscript{swe}/PS1\textsubscript{dE9} double transgenic mouse model of AD (Holm et al. 2010). Reduced 5-HT\textsubscript{2A} receptor binding sites showed direct correlation with increased Aβ plaque burdens (measured using [\textsuperscript{11}C]-PIB binding to Aβ plaques) in the AβPP\textsubscript{swe}/PS1\textsubscript{dE9} mice (Holm et al. 2010). Taken together, the two preclinical studies suggest that AD-related decreases in 5-HT\textsubscript{2A} receptors may be due to neuropathological deposition of Aβ aggregates (Christensen et al. 2008; Holm et al. 2010).
Table 1.12. AD-associated changes in 5-HT$_{2A}$ receptors: post-mortem, imaging and immunohistochemical studies.

<table>
<thead>
<tr>
<th>Radioligand/Marker</th>
<th>Age (years)</th>
<th>N (AD)</th>
<th>Brain Region/5-HT$_{2A}$ Receptors % of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binding studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>79</td>
<td>12</td>
<td>TC - 35, FC - 54, H - 67, A - 60</td>
<td>(Cross et al. 1984)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>79</td>
<td>nr</td>
<td>EC - 42, PC - 65</td>
<td>(Perry et al. 1984)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>78</td>
<td>13</td>
<td>TC - 58</td>
<td>(Cross et al. 1986)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>72</td>
<td>5</td>
<td>TC - 57</td>
<td>(Cross et al. 1988)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>nr</td>
<td>nr</td>
<td>TC - 57 - 65</td>
<td>(Procter et al. 1988)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>77</td>
<td>8</td>
<td>DG - 48, CA1 - 56, CA3 - 48, EC - 55</td>
<td>(Jansen et al. 1990)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>82</td>
<td>7</td>
<td>TC - 52</td>
<td>(Cheng et al. 1991)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>82</td>
<td>29</td>
<td>FC - 62*, PC - 48*</td>
<td>(Lai et al. 2005)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>82</td>
<td>35</td>
<td>TC - 54 - 72</td>
<td>(Tsang et al. 2010)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>74</td>
<td>1</td>
<td>FC, H - No change</td>
<td>(Dewar et al. 1999)</td>
</tr>
<tr>
<td>[H]$\text{H}$ketanserin</td>
<td>82</td>
<td>24</td>
<td>H - No change</td>
<td>(Lai et al. 2011)</td>
</tr>
<tr>
<td>PET - [18F]altanserin</td>
<td>70</td>
<td>9</td>
<td>AnC - 90, PFC - 92, SCo - 96</td>
<td>(Meltzer et al. 1999)</td>
</tr>
<tr>
<td>PET - [18F]altanserin</td>
<td>74</td>
<td>12</td>
<td>OC - 62, PFC - 71</td>
<td>(Marner et al. 2010a)</td>
</tr>
<tr>
<td>PET - [18F]deuteroaltanserin</td>
<td>76</td>
<td>9</td>
<td>AnC - 58</td>
<td>(Santhosh et al. 2009)</td>
</tr>
<tr>
<td>SPECT - $^{123}$I-5-I-R91150</td>
<td>81</td>
<td>9</td>
<td>Orf - 84, PFC - 85, OC - 92</td>
<td>(Versijpt et al. 2003)</td>
</tr>
<tr>
<td>MRI - [18F]altanserin</td>
<td>70</td>
<td>16</td>
<td>Neo - 20 - 30</td>
<td>(Hasselbalch et al. 2008)</td>
</tr>
<tr>
<td><strong>Immunohistochemistry studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT$_{2A}$</td>
<td>88</td>
<td>6</td>
<td>PFC - 67</td>
<td>(Lorke et al. 2006)</td>
</tr>
<tr>
<td>5-HT$_{2A}$</td>
<td>83</td>
<td>8</td>
<td>STN - Reduced density</td>
<td>(Yeung et al. 2010)</td>
</tr>
</tbody>
</table>

**Key:** Age: mean age, N: number of AD samples included in the study, BS: binding study, Au: autoradiography, PET: Positron emission tomography, SPECT: single photon emission computed tomography, MRI: magnetic resonance imaging, IHC: immunohistochemistry, aMCI: amnesic mild cognitive impairment, FC: frontal cortex, TC: temporal cortex, PC: parietal cortex, PFC: prefrontal cortex, EC: entorhinal cortex, OC: occipital cortex, Neo: neocortex, Orf: orbitofrontal, SCo: somatosensory cortex, SM: sensory motor, A: amygdala, H: hippocampus, DG: dentral gyrus, CA1, CA3: cornus ammonus 1,3 areas of hippocampus, AnC: anterior singulate, STN: sensory trigeminal nuclei, nr: not reported. *Numbers were estimated from the graphs. Note: Highlighted in yellow are studies reported decrease in 5-HT$_{2A}$ receptor binding potentials/densities and highlighted in green are studies reported no changes in 5-HT$_{2A}$ receptor binding potentials/densities. Modified from (Rodríguez et al. 2012).
It is not entirely clear whether AD-related reduction in 5-HT_{2A} receptor densities are due to loss of neurones that express 5-HT_{2A} receptors or down regulation of 5-HT_{2A} receptor protein expression in these neurones (Hasselbalch et al. 2008). Given that activation of 5-HT_{2A} receptors increase ACh and glutamate neurotransmitter release and promote learning and memory functions, reduced 5-HT_{2A} receptor densities may contribute to the cognitive deficits associated with AD (Harvey 1996; Meneses & Hong 1997b; Aghajanian & Marek 1999; Marek & Aghajanian 1999; Harvey 2003). Agonists of 5-HT_{2A} receptors have demonstrated pro-cognitive effects in pre-clinical studies using different behavioural tasks (Alhaider et al. 1993; Meneses & Hong 1997a; Welsh et al. 1998; Harvey et al. 2004; Romano et al. 2010).

1.5.2.2.3. Other 5-HT receptors in AD

In addition to alterations in 5-HT_{1A} and 5-HT_{2A} receptors, the progression of AD is also associated with reduced expression of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{6} receptors in the frontal cortex, the temporal cortex and the PFC (Garcia-Alloza et al. 2004; Lorke et al. 2006). Deficits in 5-HT_{1B} and 5-HT_{1D} receptors correlate well with cognitive impairment in AD assessed by MMSE scores (Garcia-Alloza et al. 2004).

Autoradiographic binding study (using the 5-HT_{3} receptor as radioligand [^{3}H]-(S)-zacopride) reported no AD-related alteration in 5-HT_{3} receptor binding sites in the hippocampus and the amygdala (Barnes et al. 1990). Early in vitro binding studies (using radiolabelled 5-HT_{4} receptor antagonist, [^{3}H]-GR 113808) reported decreased 5-HT_{4} receptor binding sites in the temporal cortex, the frontal cortex and the PFC as well as in the hippocampus of AD brains (Reynolds et al. 1995; Wong et al. 1996). On the other hand, another binding study (using the same radioligand) reported no alteration in 5-HT_{4} receptor binding sites in the temporal cortex and the frontal cortex of AD brains (Lai et al. 2003a). One reason for this discrepancy may be related to the smaller sample size in the first two studies (n = 10, Reynolds et al. 1995; Wong et al. 1996) compared to 34 cases included in the latter study (Lai et al. 2003a). More recent binding study (using [^{3}H]-GR 113808) found decreased 5-HT_{4} receptor binding sites in the temporal cortex of relatively small sub-group of AD brains (n = 9) from patients who had been previously diagnosed with
hyperphagia, whereas no changes in 5-HT$_4$ receptor binding sites were identified in the brain tissues from AD patients without hyperphagia (n = 26) (Tsang et al. 2010). Taken together, these studies suggest that differences in sample size and selection of AD patients with specific behavioural abnormalities may account for contradictory findings on AD-related alteration in 5-HT$_4$ receptors.

### 1.5.3. Serotonin transporter in AD

In post-mortem AD brains, significant decrease in SERT binding sites have been reported in the temporal cortex, the frontal cortex and the hippocampus (Table 1.13). Diminished SERT binding sites was shown to be more prominent in AD patients with early onset of the disease (50 – 60 years of age) (Bowen et al. 1983). In vivo imaging studies further support reduced SERT binding sites at the early stages of AD (Ouchi et al. 2009; Marner et al. 2010a). The reduced SERT binding sites did not correlate with the presence of concomitant behavioural symptoms including anxiety and depression in patients with AD (Tsang et al. 2010).
Table 1.13. AD-associated changes in SERT density: post-mortem, PET and animal studies.

<table>
<thead>
<tr>
<th>Radioligand/Marker</th>
<th>Age (AD)</th>
<th>N (AD)</th>
<th>Brain Region/SERT Density</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binding studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[^3H]imipramine</td>
<td>69</td>
<td>13</td>
<td>TC - 72 - 81</td>
<td>(Bowen et al. 1983)</td>
</tr>
<tr>
<td>[^3H]5-HT</td>
<td></td>
<td></td>
<td>TC - 62</td>
<td></td>
</tr>
<tr>
<td>[^3H]paroxetine</td>
<td>81</td>
<td>20</td>
<td>TC - 61</td>
<td>(Chen et al. 1996)</td>
</tr>
<tr>
<td>[^3H]citalopram</td>
<td>81</td>
<td>22</td>
<td>FC - 88*</td>
<td>(Tsang et al. 2003)</td>
</tr>
<tr>
<td>[^3H]CNIMI</td>
<td>82</td>
<td>14</td>
<td>PFC - 53</td>
<td>(Thomas et al. 2006)</td>
</tr>
<tr>
<td>[^3H]paroxetine</td>
<td>74</td>
<td>9</td>
<td>FC - 63</td>
<td>(Bowen et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PC - 42</td>
<td></td>
</tr>
<tr>
<td>[^3H]citalopram</td>
<td>82</td>
<td>24</td>
<td>H - 70</td>
<td>(Lai et al. 2011)</td>
</tr>
<tr>
<td>[^3H]citalopram</td>
<td>82</td>
<td>18</td>
<td>No change</td>
<td>(Tsang et al. 2010)</td>
</tr>
<tr>
<td><strong>Positron emission tomography studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[^18]C]DASB</td>
<td>61</td>
<td>7</td>
<td>Mi - 67, NAc - 63, Pu - 62, T - 68</td>
<td>(Ouchi et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>12</td>
<td>PFC - 88, OC - 77, mTC - 67</td>
<td>(Marner et al. 2010a)</td>
</tr>
<tr>
<td><strong>Western blot study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERT</td>
<td>61</td>
<td>7</td>
<td>cb - 86</td>
<td>(Gulesserian et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC - 101</td>
<td></td>
</tr>
<tr>
<td><strong>Animal model study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC- SERT</td>
<td></td>
<td></td>
<td>H - 161 at 3 months; H - 174 at 18 months</td>
<td>(Noristani et al. 2010)</td>
</tr>
</tbody>
</table>

**Key:** Age: mean age, N: number of AD samples included in the study, AD-D: Alzheimer’s disease patients with depression, BS: binding studies, PET: Positron emission tomography, WB: western blots, IHC: immunohistochemistry, TC: temporal cortex, FC: frontal cortex, PFC: prefrontal cortex, PC: parietal cortex, OC: occipital cortex, mTC: mesial temporal cortex, cb: cerebellum, Mi: midbrain, NAc: nucleus accumbens, Pu: putamen, T: thalamus, H: hippocampus. * Numbers were estimated from the graphs. Note: Highlighted in yellow are studies reported reduced SERT binding potentials/densities, highlighted in green are studies reported no changes in SERT binding potentials/densities and highlighted in red are studies reported increase in SERT protein/fibre densities.

Modified from (Rodríguez et al. 2012).
Transmembrane 5-HT transport depends on a functional polymorphism within the promoter region of the SERT gene (Borroni et al. 2006; Seripa et al. 2008). This polymorphism consists of both “short” and “long” variants of the SERT promoter where the “short” variant displays a significantly reduced SERT transcriptional activity (Assal et al. 2004). The relatively high frequency of the “short” variant of the SERT promoter polymorphism was suggested to be associated with an increased risk of AD in Caucasian population (Hu et al. 2000). Low expression of SERT protein results in abnormal 5-HT homeostasis/transmission in the hippocampus that correlates with the cognitive impairment associated with AD (Hu et al. 2000). The polymorphism of the SERT promoter was also linked with AD-related behavioural symptoms including aggression and psychosis in some (Sukonick et al. 2001; Borroni et al. 2006) but not in all (Assal et al. 2004) studies. Subsequent study in Caucasian population however, failed to find any association between SERT promoter polymorphism and increased risk of AD (Seripa et al. 2008). Similar analysis of Japanese and Latin American (Kunugi et al. 2000; Forero et al. 2006) populations found no correlation between SERT polymorphism and AD either.

1.5.4. Depressive disorders as a risk factor for AD

Increasing evidence suggest that depressive symptoms precede the development of dementia in the elderly population (Geerlings et al. 2000). Longitudinal studies not only proposed depression as a risk factor for the onset of AD but they also demonstrated an increased prevalence of depressive behaviours in patients already diagnosed with AD (Chen et al. 1999; Geerlings et al. 2000; Heun et al. 2003; Zubenko et al. 2003; Dal Forno et al. 2005; van Reekum et al. 2005; Bowirrat et al. 2006). These studies suggest that a late onset of depression in the elderly (> 60 years old) patients may represent an early event before the onset of cognitive impairment associated with AD (Berger et al. 1999; Wetherell et al. 1999; Heun et al. 2003; Heun et al. 2006; Sierksma et al. 2010). In patients with mild cognitive impairment, depressive symptoms are most common in those patients who are most likely to develop AD (Barnes et al. 2006; Gabryelewicz et al. 2007), although the latter has not been a universal finding (Panza et al. 2008).
There are mainly two major hypotheses linking depression and AD progression namely: (i) depression is a psychological reaction to the progressive cognitive impairment and development of AD symptoms and (ii) the neurodegenerative processes linked with AD contribute to the development of depression, where depression results from severe loss of neurones that in turn makes the brain more susceptible to the development of AD (Chen et al. 1999; Geerlings et al. 2000; Lyketsos & Olin 2002). In fact, AD patients with concomitant depressive symptoms displayed greater degeneration of monoamine system compared to AD patients without depression (Lyketsos & Olin 2002). In addition, histopathological studies have shown that AD patients with a major depression history had increased Aβ plaques and NFTs deposition in the hippocampus (Rapp et al. 2006).
1.6. **Increased Serotonergic Neurotransmission as a Potential Therapeutic Approach in AD Treatment**

Several clinical studies have analysed the effect of 5-HT neurotransmission-enhancing drugs on cognitive and behavioural abnormalities associated with AD (Table 1.14). Whereas some clinical studies demonstrated significant improvement in cognitive function following treatment with SSRIs (Schneider et al. 1991; Roth et al. 1996; Taragano et al. 1997; Mowla et al. 2007; Mossello et al. 2008; Rozzini et al. 2010), others have failed to detect major pro-cognitive effects (Nyth & Gottfries 1990; Lyketsos et al. 2003; Munro et al. 2004; Rao et al. 2006). These discrepancies between reported studies are mainly due to great heterogeneity in the clinical designs namely: (i) duration of drug treatment, (ii) number of patients included in the clinical study, (iii) selection of AD patients at different stages of disease progression, (iv) drug dosage and (v) concomitant effect of other drugs (de Vasconcelos Cunha et al. 2007). An international double-blind, placebo-control clinical trial showed protective effect of moclobemide (a reversible monoamine oxidase inhibitor, MOAI) against cognitive impairment in AD subjects with depressive symptoms (Roth et al. 1996). Antidepressant treatment also improved cognitive function in non-demented elderly-depressed subjects (Butters et al. 2000; Rocca et al. 2005) and in depressed elderly patients with concomitant dementia (Nyth et al. 1992).

Independent of their effect on cognitive function, clinical studies using SSRIs have consistently reported improved behavioural abnormalities associated with AD including depression, agitation, irritability, anxiety, affective symptoms and aggressive behaviour (Table 1.14). In addition, Mizukami and colleagues (2009) reported improved behavioural symptoms associated with AD following treatment with milnacipram (a selective 5-HT and NA reuptake blocker) (Mizukami et al. 2009).
Table 1.14. Serotonominetic drugs in AD treatment.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Drug tested (Dose)</th>
<th>Study Duration</th>
<th>N (AD)</th>
<th>Age (years)</th>
<th>Effect on Cognitive Function</th>
<th>Effect on other behaviour</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-B</td>
<td>Citalopram, SSRI, (10- 30mg/day)</td>
<td>4 Weeks</td>
<td>65</td>
<td>72</td>
<td>Improved ADL</td>
<td>Improved behaviour symptoms</td>
<td>Nyth &amp; Gofftires 1990</td>
</tr>
<tr>
<td>AD-D</td>
<td>AChEIs + MAOI (10mg/day)</td>
<td>4 Weeks</td>
<td>14</td>
<td>60</td>
<td>Improved episodic memory</td>
<td>Improved agitation and depressive behaviour</td>
<td>Schneider et al. 1991</td>
</tr>
<tr>
<td>AD-D</td>
<td>Moclobemide, reversible MAOI (400mg/day)</td>
<td>7 Weeks</td>
<td>694</td>
<td>74</td>
<td>Reduced cognitive disturbance</td>
<td>Improved depressive behaviour</td>
<td>Roth et al. 1996</td>
</tr>
<tr>
<td>AD-D</td>
<td>Fluoxetine, SSRI (10mg/day)</td>
<td>6 Weeks</td>
<td>18</td>
<td>72</td>
<td>Improved MMSE</td>
<td>Improved depressive behaviour</td>
<td>Taragano et al. 1997</td>
</tr>
<tr>
<td>AD-D</td>
<td>Setraline, SSRI, (25-150 mg/day)</td>
<td>12 Weeks</td>
<td>22</td>
<td>77</td>
<td>Less decline in ADL</td>
<td>Improved depressive behaviour</td>
<td>Lyketsos et al. 2000</td>
</tr>
<tr>
<td>AD-D</td>
<td>Setraline hydrochloride, SSRI, (25-150 mg/day)</td>
<td>12 Weeks</td>
<td>44</td>
<td>76</td>
<td>Improved ADL</td>
<td>Improved depressive behaviour</td>
<td>Lyketsos et al. 2003</td>
</tr>
<tr>
<td>AD-D</td>
<td>Setraline, SSRI, (nr)</td>
<td>14 Weeks</td>
<td>44</td>
<td>76</td>
<td>Improved verbal learning in women but not men AD patients</td>
<td>Improved depressive behaviour</td>
<td>Munro et al. 2004</td>
</tr>
<tr>
<td>AD</td>
<td>Rivastigmine, AChEI (6-12mg/day) + Fluoxetine, SSRI (20mg/day)</td>
<td>12 Weeks</td>
<td>41</td>
<td>55-85</td>
<td>Improved MMSE, memory, ADL and global functioning</td>
<td>Significant improvement in depressive behaviour</td>
<td>Mowla et al. 2007</td>
</tr>
<tr>
<td>AD-D</td>
<td>AChEIs + SSRI (nr)</td>
<td>9 Months</td>
<td>72</td>
<td>78</td>
<td>Improved cognitive performance and less cognitive decline</td>
<td>Improved depressive behaviour</td>
<td>Mossello et al. 2008</td>
</tr>
<tr>
<td>AD-D</td>
<td>AChEIs + SSRI, (5-60mg/day)</td>
<td>9 Months</td>
<td>66</td>
<td>78</td>
<td>Preserved cognitive function</td>
<td>Improved depressive behaviour</td>
<td>Rozzini et al. 2010</td>
</tr>
</tbody>
</table>
### Table 1.14. cont.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Drug tested (Dose)</th>
<th>Study Duration</th>
<th>N (AD)</th>
<th>Age (years)</th>
<th>Effect on Cognitive Function</th>
<th>Effect on other behaviour</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-D</td>
<td>Setraline, SSRI, (25-100 mg/day)</td>
<td>8 Weeks</td>
<td>17</td>
<td>88</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(Magai et al. 2000)</td>
</tr>
<tr>
<td>AD-D</td>
<td>Fluoxetine, SSRI (10-40 mg/day)</td>
<td>6 Weeks</td>
<td>41</td>
<td>70</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(Petracca et al. 2001)</td>
</tr>
<tr>
<td>AD-B</td>
<td>Citalopram, SSRI, (10-20mg/day)</td>
<td>17 Days</td>
<td>31</td>
<td>81</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(Pollock et al. 2002)</td>
</tr>
<tr>
<td>AD-B</td>
<td>AChEI + Setraline, SSRI, (50-200 mg/day)</td>
<td>36 Weeks</td>
<td>124</td>
<td>&gt; 50</td>
<td>nr</td>
<td>Improved irritability, anxiety, agitation, affective symptoms and aggressive behaviour</td>
<td>(Finkel et al. 2004)</td>
</tr>
<tr>
<td>AD-D</td>
<td>Citalopram, SSRI, (10-20mg/day)</td>
<td>8 Weeks</td>
<td>15</td>
<td>50-90</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(Rao et al. 2006)</td>
</tr>
<tr>
<td>AD-D</td>
<td>Venlafaxine, SNRI, (38-131 mg/day)</td>
<td>6 Weeks</td>
<td>14</td>
<td>78</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(de Vasconcelos Cunha et al. 2007)</td>
</tr>
<tr>
<td>AD-D</td>
<td>Milnacipram, SNRI, (15-30 mg/day)</td>
<td>12 Weeks</td>
<td>14</td>
<td>74</td>
<td>nr</td>
<td>Improved depressive behaviour</td>
<td>(Mizukami et al. 2009)</td>
</tr>
<tr>
<td>AD-B</td>
<td>Citalopram, SSRI, (5-30mg/day)</td>
<td>36 Weeks</td>
<td>34</td>
<td>UD</td>
<td>nr</td>
<td>Improved irritability without sedation</td>
<td>(Siddique et al. 2009)</td>
</tr>
</tbody>
</table>

**Key:** *SSRI:* selective serotonin reuptake inhibitor, *AChEIs:* acetylcholinesterase inhibitors, *SNRI:* selective serotonin and noradrenalin reuptake inhibitor, *AD-D:* Alzheimer’s disease patients with depression, *AD-B:* Alzheimer’s disease patients with behavioural symptoms (irritability, apathy and delusion but without depression), *MMSE:* mini-mental state examination, *MAOI:* monoamine oxidase inhibitor, *ADL:* activity of daily living, *nr:* not reported. Note: Highlighted in green are studies reported improved cognition, improved ADL and less cognitive decline as well as improved depressive behaviour in AD patients following treatment with SSRI/AChEI + SSRI. Highlighted in yellow are studies reported improved depressive behaviour with no reported effect on cognition following treatment with SSRI/AChEI + SSRI. Modified from (Rodríguez et al. 2012).
An alternative approach to improve cognitive deficits associated with AD involves simultaneous stimulation of both ACh and 5-HT neurotransmitter systems (Dringenberg 2000; Abe et al. 2003; Toda et al. 2003; Smith et al. 2009). Combined treatment with inhibitors of AChE and SERT increased cerebral metabolism and improved memory and cognition in AD patients (Finkel et al. 2004; Mossello et al. 2008; Smith et al. 2009; Rozzini et al. 2010). Clinical studies also reported better performance of daily activity in AD patients following concomitant treatment with AChE and SERT inhibitors (Nyth & Gottfries 1990; Lyketsos et al. 2003; Mowla et al. 2007). Studies by Abe and co-workers (2003) led to the development of a compound with dual increase in ACh and 5-HT neurotransmitter levels (Abe et al. 2003). This drug works by simultaneously inhibiting both AChE and SERT in the brain, for recent review see (Toda et al. 2010). Oral administration of this compound simultaneous increased both 5-HT and ACh neurotransmitter levels in the hippocampus that was associated with improved memory performance in mice and rats (Abe et al. 2003; Toda et al. 2010).

Currently it is not known whether the combined treatment with AChEIs and SSRIs act independently and/or synergistically in AD (Lyketsos et al. 2003; Finkel et al. 2004). There are two main hypotheses explaining the possible mechanisms of serotonominimetic drugs as cognitive-enhancers in AD (Rozzini et al. 2010) namely: (i) this effect may be mediated by direct action of 5-HT-specific drugs on neurotransmitter imbalance in AD brains, where increased 5-HT will interact with ACh system and subsequently improve cognition. Alternatively, (ii) it may be due to a decline in depressive symptoms that may account for improved cognitive function in AD patients (Nyth et al. 1992; Roth et al. 1996). Further randomised clinical trials of adequate design, duration and sample size are required to uncover the precise mechanisms responsible for improved cognition following treatment with serotonominimetic drugs in AD.

Interestingly, in vitro studies have shown that increased 5-HT neurotransmission stimulates the non-amyloidogenic processing of the APP and reduced pathogenic Aβ peptide secretion (Nitsch et al. 1996; Robert et al. 2001; Payton et al. 2003; Morse et al. 2004; Pakaski et al. 2005). Similarly, an in vivo study reported that
chronic administration of 5-HT\textsubscript{2A/2C} agonist dexnorfenfluramine (DEXNOR) increased SAPP\textbeta in the CSF and reduced A\textbeta secretion in primary basal forebrain neuronal culture of guinea pigs (Arjona et al. 2002). Given that the released APP is no longer available for the amyloidogenic deposition, mediated by \beta- and \gamma-secretases cleavage, increased 5-HT neurotransmission may decrease AD-related neuropathology (Arjona et al. 2002). Indeed, acute administration of SSRIs including fluoxetine, desvenlafaxine and citalopram reduced A\textbeta level in brain interstitial fluid (measured using \textit{in vivo} microdialysis) in PS1APP mice (Cirrito et al. 2011). Direct 5-HT infusion into the hippocampus (by adding 5-HT into the microdialysis probe perfusion buffer, reverse microdialysis) reduced interstitial fluid A\textbeta level in PS1APP mice (Cirrito et al. 2011). Chronic treatment with SSRI (citalopram) reduced A\textbeta plaque burdens in the cortex and the hippocampus in the PS1APP transgenic mouse model (Cirrito et al. 2011). In addition, chronic treatment with SSRI (paroxetine) reduced AD-related histopathology (A\textbeta plaques and NFT) in the cortex and the hippocampus associated with improved memory performance in 3xTg-AD mice (Nelson et al. 2007b). A recent preliminary clinical study had found that exposure to SSRI antidepressants (for a minimum of 5 years) reduced A\textbeta plaque load in cognitively normal individuals (measured by PET imaging with Pittsburgh Compound B, which is a radioligand that binds to A\textbeta plaques and enables quantification of cortical A\textbeta plaque load in the living human brains) (Cirrito et al. 2011).

In summary (Fig. 1.32), the beneficial effect of serotoninmimetic drugs include an improvement in behavioural/depressive symptoms that subsequently may (i) promote learning and memory functions, (ii) reduce cognitive decline and (iii) improve the quality of life, which ultimately leads to improved cognitive performance in AD patients. Early indications from \textit{in vitro} and \textit{in vivo} studies suggest that serotoninmimetic drugs may also influence accumulation of A\textbeta and NFT neuropathology in AD brains. The use of transgenic animal models with AD-related neuropathological characteristics and cognitive impairment is of critical importance in uncovering the exact link between altered 5-HT neurotransmission and its effect on cognition and AD-related neuropathology.
Patients with Alzheimer’s Disease (AD)

AD Patients With Behavioural Symptoms
- Improved Behavioural Symptoms
- Improved Depressive Symptoms
- Improved Activity of Daily Living

AD Patients Without Behavioural Symptoms
- Improved Learning and Memory
- Less Cognitive Decline
- Improved Activity of Daily Living

Treatment with SSRI and/or SSRI + AChEI

Figure 1.32. Schematic illustration of the clinical effects of serotonomimetic drugs in AD patients with and without behavioural symptoms, respectively. In AD patients with behavioural symptoms, combined SSRI and AChEI treatment not only reduces behavioural/depressive symptoms but it also improves the activity of daily living (left). In AD patients without behavioural symptoms, combined SSRI and AChEI treatment reduces cognitive decline whilst promoting learning, memory and improved activity of daily living (right). Ultimately, combined SSRI and AChEI treatment improves cognition in AD patients.
From (Rodríguez et al. 2012).
1.7. **SEROTONERGIC NEUROTRANSMISSION IN NON-AD DEMENTIA**

Vascular dementia (VaD) is the second most common form of dementia (Geldmacher & Whitehouse 1996). Epidemiological studies on the occurrence of dementia reported various incidence rates of VaD depending on the geographical location, ranging between 15 – 20% in Europe and Canada to 27 – 38% in Asia. Similar to AD, there is increased prevalence of VaD in people with lower level of education (Ott *et al.* 1995; Liu *et al.* 1998; Di Carlo *et al.* 2002). However, unlike AD, the incidence of VaD does not increase with advanced age (Brayne *et al.* 1995; Ott *et al.* 1995; Liu *et al.* 1998). VaD is triggered by a cerebrovascular injury including stroke and ischemia (Kalaria *et al.* 2004; Elliott *et al.* 2009). Although few studies have reported impaired 5-HT neurotransmission in VaD (Gottfries 1990; Tohgi *et al.* 1995; Elliott *et al.* 2009), others have reported no changes (Hansson *et al.* 1996).

More specifically, patients with VaD displayed reduced 5-HT neurotransmitter level in the CSF (Tohgi *et al.* 1995). A post-mortem study reported reduced levels of 5-HT metabolite (5-HIAA) in the caudate nucleus and the hippocampus of VaD brains (Wallin *et al.* 1989). However, early binding study (using radioligands [3H]8-OH-DPAT, [3H]-ketanserin and [3H]-paroxetine) reported no VaD-related changes in 5-HT1A and 5-HT2A receptors as well as SERT binding sites in the frontal cortex, the temporal cortex and the caudate nucleus (Hansson *et al.* 1996). Interestingly, a more recent *in vitro* binding study (using [3H]-WAY 100635 and [3H]-ketanserin as radioligands) demonstrated increased 5-HT1A and 5-HT2A receptor binding sites in the temporal cortex of VaD brains (Elliott *et al.* 2009). In VaD, increased 5-HT1A receptor bindings correlate with preserved global cognition and memory function (Elliott *et al.* 2009), suggesting that up-regulation of 5-HT1A receptors may be associated with preserved cognitive function.

Frontotemporal lobar degeneration is a progressive form of dementia that is characterised by a relatively selective atrophy of the frontotemporal cortex (Garibotto *et al.* 2011). In frontotemporal lobar degeneration, the changes in 5-HT neurotransmission are primarily associated with reduced post-synaptic 5-HT1A and 5-HT2A receptors that are mainly due to degeneration of cortical pyramidal
neurones (Franceschi et al. 2005; Lanctot et al. 2007; Bowen et al. 2008). The levels of 5-HT, 5-HTIAA and SERT remain stable in the frontotemporal lobar degeneration, which may possibly explain the minor therapeutic effect of SSRI (paroxetine) in treatment of this disorder (Deakin et al. 2004; Bowen et al. 2008).

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterised by selective degeneration of motor neurones in the corticospinal, the brain stem and the spinal cord (Rowland & Shneider 2001). ALS-associated changes in 5-HT neurotransmission include alterations in 5-HT and 5-HIAA concentrations and a decrease in 5-HT1A and 5-HT2A receptor binding sites in the spinal cord, for review see (Sandyk 2006).

Contradictory results have been reported on ALS-associated alterations in 5-HT system. Post-mortem studies reported stable levels of 5-HT and 5-HIAA in the spinal cord tissues of ALS patients (Ohsugi et al. 1987; Forrest et al. 1996). Similarly, Bertel and colleagues (1991) reported no changes in 5-HT concentration but reduced 5-HIAA levels in the spinal cord tissue of ALS patients (Bertel et al. 1991). Another study also revealed ALS-associated decrease in 5-HT and 5-HIAA concentrations in the spinal cord (Sofic et al. 1991). ALS patients showed reduced levels of 5-HT precursor (TrP) in the plasma and CSF that may account for reduced 5-HT synthesis in the brain (Monaco et al. 1979). Quantitative autoradiographic study (using [3H]8-OH-DPAT and [3H]ketanserin as radioligands) reported reduced 5-HT1A/7 and 5-HT2A receptor binding sites in post-mortem ALS spinal cord (Forrest et al. 1996). Recent in vivo PET studies (using a more selective 5-HT1A receptor radioligand, [11C]-WAY100635) also reported reduced 5-HT1A receptor binding sites in the frontotemporal cortex, the cingulate cortex and the raphe nuclei in ALS patients (Turner et al. 2005; Turner et al. 2007).
Scope and outline of the thesis

Alzheimer's disease (AD) is an age-related, progressive neurodegenerative disease that forms the most common type of dementia. Pathologically, AD is characterised by accumulation of extracellular amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) accompanied by synaptic and neuronal loss (Selkoe 2001b; Selkoe 2002). AD affects specific brain regions involved in learning and memory processes such as the cortex and the hippocampus. Clinically AD is characterised by deterioration of cognitive function including learning and memory impairment as well as the presence of behavioural abnormalities such as anxiety, agitation, irritability, delusion and depression. Although classically the loss of cholinergic (ACh) system has been considered as the main neurochemical alteration associated with AD, more recent studies highlight the involvement of other neurotransmitter systems. Serotonin (5-HT) is widely distributed in the periphery and throughout the brain and plays an important role in regulating numerous behavioural processes including food intake, aggression, sleep, learning and memory. Impaired 5-HT neurotransmission has been linked to the development of multiple neurological disorders such as schizophrenia, depression, stress and mood disorders as well as neurodegenerative disease including AD.

The main research question of this thesis involved investigating alterations in 5-HT neurotransmission in AD using the triple transgenic (3xTg-AD) mouse model of the disease, which resembles closely the development of AD neuropathology in humans. Specifically, the research questions included: (i) How is the 5-HT neurotransmission altered in AD, (ii) What is the exact alteration of 5-HT neurotransmission during the progression of the disease in the 3xTg-AD mouse model of AD and (iii) What is the effect of altered 5-HT neurotransmission on the development of AD-related neuropathology in 3xTg-AD mice?

The first part of the introductory chapter (chapter 1) described in detail the neuropathological hallmarks associated with AD, the interaction between Aβ and NFTs as well as AD-associated alterations in major neurochemical systems followed by a detailed description of the anatomy of brain regions that are deeply affected in AD. The introductory chapter also includes a comprehensive description
of different animal models of AD used to study the disease in an in vivo setting including the 3xTg-AD mouse model. In addition, the distribution of both the peripheral and the central 5-HT neurotransmission were discussed together with their different roles in the periphery and the central nervous system (CNS). Furthermore, the introductory chapter (Chapter 1) provides a comprehensively review of the current literature in relation to altered 5-HT neurotransmission in AD including: (i) 5-HT neurones within the raphe nuclei, (ii) 5-HT neurotransmission, (iii) 5-HT projections, (iv) 5-HT reuptake transporter, (v) 5-HT receptors and (vi) the effect of drugs acting on 5-HT neurotransmission as a therapeutic approach in AD.

All the literature described in chapter 1 has been covered by Harun Najib Noristani (H. N. N.) and corrected by José Julio Rodríguez Arellano (J. J. R. A.).

Chapter 2
Materials and methods
Chapter 2 comprehensively describes the methodological approaches and techniques used to obtain the results described in this PhD thesis including: detailed description of the method used for generation of 3xTg-AD mice, fixation and tissue processing, antibodies used, immunohistochemistry-peroxidase, optical density (OD) measurement, cell count of 5-HT neurones in the raphe nuclei, immunohistochemistry-fluorescence, electron microscopy, labelling profiles and nomenclature, numerical density, surface area measurement and statistical analysis. Furthermore, chapter 2 supplementary materials provides the step-by-step protocols for preparation of the solutions used for mice perfusion, toluidine blue staining, solutions for immunohistochemistry, immunohistochemistry-peroxidase, measuring optical density using ImageJ, immunohistochemistry-fluorescence, electron microscopy processing, calculation of surface area and volume measurements using electron micrographs as well as detailed description of the Konigsmark equation and the Cavalieri principle.
Chapter 3
Controversial results have been reported in different animal models of AD in relation to alteration in 5-HT neurotransmission. Whereas few AD animal models have showed deficits in 5-HT neurotransmissions (Liu et al. 2008), others have reported no changes (Aucoin et al. 2005; Holm et al. 2010) or even increased 5-HT neurotransmissions (Harkany et al. 2000b; Harkany et al. 2001; Verdurand et al. 2011).

The primary study in chapter 3 investigates the AD-specific alteration in 5-HT neurotransmission by analysing (i) the expression, density and distribution of serotonin transporter immunoreactive (SERT-IR) fibres, (ii) the specific morphological characteristics of SERT-IR fibres and their relation to Aβ plaques and (iii) the total number of 5-HT neurones within the dorsal and median raphe nuclei in the 3xTg-AD mouse model of AD compared to sex and age-matched non-transgenic (non-Tg) control mice.


All the experimental procedures for the above manuscript was carried out by H. N. N. including: immunohistochemistry, optical density measurement of SERT-IR fibre density using imageJ, morphological analysis of hippocampal SERT-IR fibres, cell count of 5-HT neurones in the raphe nuclei and the majority of the confocal imaging of SERT-IR fibres and Aβ plaques. In addition H.N.N. also carried out all data analysis, prepared all the figures and wrote the drafts for the above manuscript.

Markel Olabarria (M. O.) contributed to the confocal imaging of SERT-IR fibres and Aβ plaques as well as preparation of the graphs for the manuscript.

Alexj Verkhratsky (A. V.) contributed to the writing of the manuscript.
J. J. R. A. perfused all the mice used for the study, coordinated the research, supervised the project and wrote the final draft of the manuscript.

Chapter 4
Loss of synapses occurs prior to the development of AD pathology and coincides with cognitive deficits including learning and memory in AD. Post-mortem studies have shown reduced synaptic density within the neocortex (DeKosky & Scheff 1990; Terry et al. 1991) and the hippocampus of AD brains (Sze et al. 1997; Scheff & Price 1998). However, studies using different transgenic models of AD have reported variable results (Boncristiano et al. 2005; Zhong et al. 2008). Whereas some AD models with increased Aβ pathology displayed increased synaptic densities (King & Arendash 2002; Hu et al. 2003), others have reported no changes (Irizarry et al. 1997a; Irizarry et al. 1997b; Takeuchi et al. 2000; Boncristiano et al. 2005) or reduced synaptic densities (Games et al. 1995; Hsia et al. 1999; Buttini et al. 2002; Zhong et al. 2008). Transgenic mouse models with severe tangle pathology also displayed reduced synaptic densities in the cortex and the hippocampus (Schindowski et al. 2006; Yoshiyama et al. 2007; Mocanu et al. 2008). Earlier reports in 3xTg-AD mice found no changes in total synaptic densities within the cortex and the hippocampus (Yao et al. 2005; Bertoni-Freddari et al. 2008; Julien et al. 2010), although the numerical density (Nv, #/µm³) of perforated synapses were reported to decrease in this mouse model (Bertoni-Freddari et al. 2008).

Based on the findings from chapter 3, the second experimental chapter (chapter 4), investigates the ultrastructural localisation, distribution and Nv of the hippocampal SERT axons (SERT-Ax) and terminals (SERT-Te). In addition, chapter 4 also examines alterations in hippocampal synaptic Nv in 3xTg-AD mice compared to age- and sex-matched non-Tg controls.

The majority of the experimental procedures for the above manuscript was carried out by H.N.N. including: immunohistochemistry, electron microscopic processing of the brain sections, all data analysis, surface area measurement of the SERT-Te and confocal imaging of SERT-IR fibres and Aβ plaques. In addition, H.N.N. also prepared all the figures, tables and wrote drafts of the manuscript.

Roger S. Meadows (R. S. M.) carried out the ultra-thin cutting of the brain sections and electron microscopic imaging of the brain samples.

M. O. contributed to the preparation of graphs and the tables for the manuscript.

A. V. contributed to the writing of the manuscript.

J. J. R. A. perfused all the mice used for the study, flat embedded the immunostained brain sections, verified all the counting of SERT labelled profiles, coordinated the research, supervised the project and wrote the final draft of the manuscript.

Chapter 5
AD patients have shown improvement of clinical symptoms following treatment of drugs known to act on 5-HT system (Marksteiner et al. 2003; Mowla et al. 2007; Mossello et al. 2008). Increased 5-HT neurotransmission also induced a stimulating effect on adult neurogenesis, which in turn is suggested to play an important role in learning and memory processes (Marcussen et al. 2008). Despite the clear positive effects of 5-HT drugs on clinical symptoms associated with AD, it is not clear how these drugs affect the underlying neuropathology.

Previous *in vitro* studies have shown that increased 5-HT neurotransmission promoted the non-amyloidogenic processing of APP metabolite (APP$_S$) and lowered pathogenic Aβ peptide secretion, which may also potentially prevent Aβ
deposition in AD (Nitsch et al. 1996; Robert et al. 2001; Payton et al. 2003; Morse et al. 2004; Pakaski et al. 2005). These findings are in agreement with in vivo studies where chronic treatment with SSRI reduced Aβ plaques deposition in the cortex and the hippocampus of PS1APP transgenic mouse model of AD (Cirrito et al. 2011). Similarly, chronic treatment with SSRI reduced both the presence of Aβ plaques and tangles in 3xTg-AD mice (Nelson et al. 2007b).

L-tryptophan (TrP) is an essential amino acid that acts as 5-HT precursor (Cooper & Melcer 1961). Nutritional availability of TrP modulates 5-HT synthesis and availability in the peripheral and the brain (van der Stelt et al. 2004). Alteration in dietary TrP intake relative to other amino acids is a commonly used non-pharmacological approach to manipulate systemic TrP level and central 5-HT neurotransmission (Fadda 2000; van der Stelt et al. 2004; Cahir et al. 2007). Changes in dietary TrP content has been shown to alter 5-HT neurotransmitter levels in multiple brain regions including the hippocampus (van der Stelt et al. 2004; Jenkins et al. 2010).

Chapter 5 examines the effect of altered 5-HT neurotransmission (via chronic changes in dietary TrP intake) on 5-HT system including: (i) hippocampal SERT-IR fibre density and (ii) total number of 5-HT neurones in the dorsal and median raphe nuclei. In addition, chapter 5 also investigates the effect of altered dietary TrP intake on intraneuronal Aβ deposition in the hippocampus of the 3xTg-AD mouse model of AD.


All the experimental procedures for the above manuscript was carried out by H.N.N. including: daily measurement of the food intake, water intake and body weight of the mice, immunohistochemistry, optical density measurement of SERT-IR fibre density, total cell count of 5-HT neurones in the dorsal and
the median raphe nuclei as well as the optical density measurement of the intraneuronal Aβ in the hippocampus. In addition, H.N.N. also carried out all data analysis, prepared all the figures and the table and wrote the draft of the manuscript.

A. V. contributed to the writing of the manuscript.

J. J. R. A. perfused all the mice used for the study, coordinated the research, supervised the project and wrote the final draft of the manuscript.

Chapter 6
General discussion/conclusion
Finally in chapter 6, the main findings from the preceding chapters (chapter 3 – 5) are discussed altogether and compared with the current state of the literature followed by a short summary of the potential future research directions.

All the literature described in chapter 6 has been covered by H. N. N. and corrected by J. J. R. A.
Chapter 2

General Material and Methods
2.1. Animals

**In Chapter 3, Chapter 4 and Chapter 5**

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the Home Office license and were approved by the Animal Care and Ethical Committee at The University of Manchester (PCD 50/2506). All efforts were made to reduce the number of animals used by following the 3Rs (reduction, refinement and replacement).

The procedure for generating the triple transgenic (3xTg-AD) mouse model of AD has been described previously (Oddo *et al.* 2003a; Oddo *et al.* 2003b; Rodríguez *et al.* 2008; Rodríguez *et al.* 2009a; Rodríguez *et al.* 2009b) (see also chapter 1, section 1.2.3.). The human APP cDNA, harbouring the Swedish double (KM670/671NL, APP<sub>Swe</sub>) mutations and human four repeat tau, harbouring the P301L (tau<sub>P301L</sub>) mutation (both under the control of mouse Thy1.2 regulatory element), were co-microinjected into a single-cell embryo of a homozygous presenilin 1 (PS1<sub>M146V</sub>) knock-in mouse (Oddo *et al.* 2003b). Initial, Southern blot analysis showed that the APP<sub>Swe</sub> and tau<sub>P301L</sub> mutations were integrated in the same locus and confirmed that the two transgenes did not undergo independent assortment in subsequent generations (Oddo *et al.* 2003b). Therefore, 3xTg-AD mice are considered as a “single” transgenic line despite the fact that these mice carry three different transgenes (Oddo *et al.* 2003a; Oddo *et al.* 2003b). As described in chapter 1, co-integration of the APP<sub>Swe</sub> and tau<sub>P301L</sub> mutations at the same loci also has the advantage of minimising genetic interactions produced when crossing three independent lines of mice (Gotz *et al.* 2004a). The background of the PS1 knock-in mice is a hybrid 129/C57BL6. The non-transgenic (non-Tg) control mice used were also from the same strain and genetic background as the PS1 knock-in mice, but they harbour the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg control mice were obtained by crossing homozygous breeders. All mice were housed in the same-sex cage, kept in 12h light-dark cycles with free access to food and water.
2.2. Fixation and Tissue Processing

In Chapter 3, Chapter 4 and Chapter 5

Fixation:
Anaesthetisation and perfusion of mice were carried out by the licensee (José Julio Rodríguez Arellano, J. J. R. A.). Following perfusion, post-fixation, cutting of brain sections and all tissue processing steps were carried out by Harun Najib Noristani (H. N. N.).

1- Male 3xTg-AD and non-Tg control mice were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 2, 3, 6, 9, 12 and 18 months of age (J. J. R. A.).

2- Mice were perfused through the aortic arch with 3.75% acrolein (TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, Gillingham, UK) in 0.1 M phosphate buffer (PB) pH 7.4 (25ml), followed by single 2% paraformaldehyde (60 – 100ml) (J. J. R. A.). See chapter 2 supplementary materials for the preparation of perfusion solutions (section 2.S.1.).

3- Brains were then removed from the skull (H. N. N.).

4- The obtained brains were cut into 4 – 5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus (Rodríguez et al. 2008) (H. N. N.).

5- The brain sections were then post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1 M PB, pH 7.4 (H. N. N.).

6- Coronal sections of the brain were cut into 40 – 50μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK) (H. N. N.).

7- Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in a cryoprotectant solution (to prevent tissue freezing and any cold-induced damage to the brain sections during the storage period at -20 °C) containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4 (H. N. N.).
8- To define the anatomical position of the brain section according to bregma (a clear landmark visible on the skull, Fig. 2.1), a set of sections were counterstained with toluidine blue (H. N. N.). See chapter 2 supplementary materials for toluidine blue staining protocol (section 2.S.2.).

9- Coronal sections at levels -1.58 mm/-2.46 mm (hippocampus) and -4.36 mm/-4.96 mm (raphe nuclei) posterior to bregma (Fig. 2.1D – 2.1G), were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004) (H. N. N.).
Figure 2.1. Diagram showing the location of bregma in the mouse skull (A). Photographs of the mouse brain showing the location of the bregma (0) marked by blue lines and their respective anterior and posterior divisions in mm (B and C). Coronal slabs of tissue representing the dorsal hippocampus (D) and the raphe nuclei (E) corresponding to -1.94 mm and -4.60 mm posterior to bregma (F and G). Scale bars: 200μm (B – E).
A from (Brehar et al. 2008), B – E from (Rodríguez J.J. personal and unpublished photographs), F and G modified from (Paxinos & Franklin 2004).
2.3. Antibodies

*In Chapter 3, Chapter 4 and Chapter 5*

A polyclonal rabbit antibody against a peptide sequence corresponding to amino acids 602 – 622 of rat 5-HT transporter (SERT, Immunostar, Hudson, WI, USA) was used for the visualisation, localisation and determination of serotonergic fibres and terminals in the hippocampus. 5-HT neurones in the raphe nuclei were studied using a polyclonal rabbit antibody generated against the total 5-HT protein (Immunostar, Hudson, WI, USA). To detect intraneuronal Aβ aggregates and extracellular Aβ neuritic plaques, a monoclonal mouse antibody against the amino acid residues 1 – 16 of beta amyloid (Covance, Emeryville, CA, USA) was used. Finally, a polyclonal rabbit anti-S100 antiserum generated against the total S100 protein (DakoCytomation, Denmark; #Z 0311) was used to analyse the hippocampal astrocytes (see Table 2.1 for the list of the primary antibodies used and their respective dilutions). The specificity of the antibodies has been reported previously using both immunohistochemistry and western blots (Table 2.1).

*Table 2.1. Summary of primary antibodies used and their respective sources.*

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit anti serotonin (5-HT)</td>
<td>1:5000</td>
<td>Immunostar, USA, Catalogue No. 20080</td>
<td>(Liu et al. 2008)</td>
</tr>
<tr>
<td>Polyclonal rabbit anti 5-HT transporter (SERT)</td>
<td>1:2500</td>
<td>Immunostar, USA, Catalogue No. 24330</td>
<td>(Mamounas et al. 2000; Albright et al. 2007)</td>
</tr>
<tr>
<td>Monoclonal mouse anti beta amyloid (Aβ)</td>
<td>1:2000</td>
<td>Covance, USA, Catalogue No. SIG-39300</td>
<td>(Rodríguez et al. 2008; Rodríguez et al. 2009b; Olabarria et al. 2010; Olabarria et al. 2011)</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-S100β (S-100β)</td>
<td>1:5000</td>
<td>DakoCytomation, Denmark, Catalogue No. Z 0311</td>
<td>(Mbele et al. 2002; Brozzi et al. 2009)</td>
</tr>
</tbody>
</table>

To determine the specificity of the antibodies, omission of primary and/or secondary antibodies resulted in total absence of the target labelling (Fig. 2.2A, 2.2B, 2.2C, 2.3A, 2.3B, 2.3C). Furthermore, adsorption controls were carried out, using SERT- and 5-HT-specific peptides, which also showed no immunoreactivity (Fig. 2.2D, 2.3D).
Figure 2.2. Brightfield micrographs showing the control conditions used to determine the specificity of the SERT antibody. No labelling was observed in the dorsal hippocampus following omission of primary (A), secondary (B) as well as combined omission of both primary and secondary antibodies (C). In addition, the adsorption control using the SERT-specific peptide also showed no labelling (D). **Key:** DG: dentate gyrus, CA: cornu ammonis. Scale bars: 500μm (A – D).
From personal library.
Figure 2.3. Brightfield micrographs showing the control conditions used to determine the specificity of serotonin antibody. No labelling was observed in the dorsal raphe nucleus following omission of the primary (A), secondary (B) as well as combined omission of both primary and secondary antibodies (C). In addition, the adsorption control using the 5-HT-specific peptide also showed no labelling (D). Key: Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part. Scale bars: 500μm (A – D).

2.4. Immunohistochemistry - Peroxidase

In Chapter 3, Chapter 4 and Chapter 5

All immunohistochemical experiments were conducted by (H. N. N.). See chapter 2 supplementary materials for the detailed description and preparation of required solutions (section 2.S.3.) as well as the step-by-step protocol for the detailed peroxidase immunohistochemistry procedure (section 2.S.4.).

All mice brains from 3xTg-AD and non-Tg control groups were perfused and post-fixed at the same time in identical solutions to avoid variability. For immunohistochemistry, coronal brain sections at levels -1.58 mm/-2.46 mm (the
hippocampus) and -4.36 mm/-4.96 mm (the raphe nuclei) posterior to bregma were incubated for 30 minutes in a solution containing 30% methanol in 0.1 M PB and 3% hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, Sigma, Gillingham, UK). Brain sections were then thoroughly rinsed with 0.1 M PB for 5 minutes and placed in 1% sodium borohydride (Sigma, Gillingham, UK) dissolved in 0.1 M PB for 30 minutes. The brain sections were then washed with 0.1 M PB profusely and rinsed in 0.1 M trizma base saline (TS) for 10 minutes before incubating them in 0.5% bovine serum albumin (BSA, Sigma, Gillingham, UK) in 0.1 M TS and 0.25% Triton (Sigma, Gillingham, UK, x 100) for 30 minutes. All brain sections were incubated for 48 hours at room temperature in primary antibody solution (rabbit anti-SERT, 1:2500, rabbit anti-5-HT, 1:5000, Immunostar, Hudson, WI, USA and mouse anti-A\textsubscript{β}, 1:2000, Covance, USA). Brain sections were then thoroughly rinsed in 0.1 M TS for 30 minutes before incubating them in 1:400 dilutions of secondary antibodies (biotinylated donkey anti-rabbit IgG or biotinylated horse anti-mouse IgG, Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK) for 1 hour at room temperature (see Table 2.2 for the list of secondary antibodies used for peroxidase immunohistochemistry and their respective dilutions).

**Table 2.2. Summary of secondary antibodies used for peroxidase immunohistochemistry procedure and their respective sources.**

<table>
<thead>
<tr>
<th>Secondary Antibody (Peroxidase)</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Donkey anti-rabbit IgG</td>
<td>1:400</td>
<td>Jackson Immunoresearch, UK, Catalogue No. 711-065-152</td>
<td>(Noristani et al. 2010)</td>
</tr>
<tr>
<td>Biotinylated Horse anti-mouse IgG</td>
<td>1:400</td>
<td>Vector laboratorios, UK, Catalogue No. BA-2001</td>
<td>(Rodríguez et al. 2008)</td>
</tr>
</tbody>
</table>

Then brain sections were rinsed with 0.1 M TS for 30 minutes followed by incubation in avidin-biotin peroxidase complex (ABC, Vector Laboratories Ltd., Peterborough, UK) for another 30 minutes. The peroxidase reaction product was visualised by incubating the sections in a chromogen solution containing 0.022% of 3,3’diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003% H\textsubscript{2}O\textsubscript{2} for 6 minutes, as previously described (Rodríguez et al. 2008; Noristani et al. 2010). The chromogen reaction was stopped by rinsing the brain sections in 0.1 M TS for 6 minutes followed by 0.1 M PB for further 15 minutes. Brain sections were then
permanently mounted onto gelatinised slides and allowed to dry overnight. Dried brain sections were then further dehydrated in ascending concentrations of ethanol (50, 70, 80, 90, 95 and 100%) for 10 minutes each and finally in xylene for additional 30 minutes. Cover slips were applied using Entellan (Merck KGaA, Germany) and slides were left to dry overnight.

To minimise the methodological variability in any given experiment, brain sections of all 3xTg-AD and non-Tg control mice were processed at the same time using precisely the same experimental conditions. An intra-section coding system was used to allow identification of brain sections from each animal. See chapter 2 supplementary materials for a detailed description of intra-section coding (Fig. 2.S.1). This precaution was taken to avoid over-reaction, differences in chromogen reaction, saturation of immunoreactivity or changes in background staining levels. Coverslipped slides were coded by another lab member (Markel Olabarria, M. O.) to ensure blind analysis throughout the experiment. The codes were not revealed till the end of the experiment.

2.4.1. Optical Density (OD) Measurement

In Chapter 3 and Chapter 5

All optical density (OD) measurements were conducted by a single investigator (H. N. N.) who was blind to the subject number and group assignment. All slides were coded prior to OD analysis by another lab member (M. O.) and the code was not broken until all quantification was completed.

Immunohistochemical stains are “light absorbing dyes”. When a single stain is present the OD can be used as a measure of the relative amount of the labelled antigen relative to their anatomical location and surface area (Noristani et al. 2010). Using a computer-assisted imaging analysis (ImageJ 1.32j, NIH, USA), the expression and densities of serotonin transporter immunoreactive (SERT-IR) fibres and intraneuronal Aβ were analysed by measuring their OD (Fig. 2.4) (Cordero et al. 2005; Noristani et al. 2010). See chapter 2 supplementary materials for the detailed protocol of how to measure the OD using ImageJ (section 2.S.5).
Figure 2.4. Brightfield micrographs showing SERT (A, B) and Aβ (C, D) labelling within the dorsal hippocampus. The OD of SERT-positive fibres was measured in all hippocampal subfields and their different layers (B). Intraneuronal Aβ density was primarily measured in the pyramidal cell layer of the hippocampus (D). Non-specific section labelling was determined and measured from the corpus callosum (CC, E, F). Key: PCL: pyramidal cell layer, S.Or: stratum oriens, S.Rad: stratum radiatum, S.Mol: stratum lacunosum-moleculare, GCL: granule cell layer, ML: molecular layer, CC: corpus callosum. Scale bars: 500μm (A – F).

Immunohistochemically labelled brain sections with SERT and Aβ-specific antibodies from the dorsal hippocampus were selected for OD measurements (Fig. 2.4). The appropriate hippocampal level was identified according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004) and readings were obtained at bregma level -1.58 mm/-2.46 mm, as previously stated (section 2.4.). The intensity of the reaction products (OD) of SERT and Aβ were quantitatively
measured using a Nikon Eclipse 80i microscope coupled with a 8001 MicroFIRE camera and ImageJ 1.32j software.

In general, the procedure for measuring OD can be divided into 7 individual steps:
1- Identifying the region of interest (ROI), 2- Calibrating the optical density, 3- Maintaining a constant light intensity, 4- Acquiring and saving images, 5- Performing the subtraction of background staining, 6- Setting and determining the exact ROI area (to measure the OD) and 7- Measuring the OD.

2.4.1.1. Identifying the region of interest (ROI)
The size and the shape of the ROI had to be determined accurately to avoid the overlap between different layers and subfields of the hippocampus. For analysis of SERT-IR fibres, the borders of the hippocampal layer were determined according to anatomical boundaries, the location of the hippocampal fissure and anatomical division between different neuronal layers of the hippocampus as well as SERT labelling intensity (Lorente de Nó 1933; Witter & Amaral 2004; Noristani et al. 2010) (see also chapter 1, Fig. 1.12 for anatomical organisation of the hippocampus). Previous immunohistochemical studies have shown that the stratum lacunosum moleculare (S.Mol) encompasses the highest density of serotonergic fibres in the hippocampus (Vertes 1991; Vertes et al. 1999) (see also Fig. 2.4A and 2.4B). Moderate densities of serotonergic fibres have been reported in the stratum oriens (S.Or) and the stratum radiatum (S.Rad), whilst very low densities of serotonergic fibres are evident in the pyramidal cell layer (PCL) of the hippocampus (Vertes et al. 1999) (see also chapter 1, Fig. 1.24 for distribution of serotonergic fibres in different cell layers of the hippocampus). In the dentate gyrus (DG) of the hippocampus, the molecular layer (ML) displayed moderate density of serotonergic fibres that is considerably reduced in the hilus. Similar to the PCL, the granule cell layer (GCL) of DG displayed very low density of 5-HT projections (Fig. 2.4A).

Similarly, for analysis of Aβ in the hippocampus, the borders of the hippocampal layers were determined according to anatomical boundaries, the hippocampal fissure and the anatomical division between different neuronal layers of the
hippocampus as well as Aβ labelling (Lorente de Nó 1933; Witter & Amaral 2004; Noristani et al. 2010). Previous immunohistochemical studies in 3xTg-AD mice have shown that the Aβ immunoreactivity is only evident in the PCL of the hippocampus at 3 months of age (Oddo et al. 2003a; Oddo et al. 2003b; Mastrangelo & Bowers 2008) (see also Fig. 2.4B and 2.4C).

2.4.1.2. Calibrating the optical density

Before proceeding to the measurement of the real OD, the system was calibrated using a standard table (step-table, Fig. 2.5).

![Step-table](image)

**Figure 2.5.** Standard table used for calibrating the system before proceeding to the OD measurement.
*From (Rodríguez J.J. unpublished photograph).*

In order to adjust for the differences in the level of black and incident light, the respective OD of the individual band was measured from white to black using a rectangular tool without overlapping between adjacent bands (Fig. 2.5). Measured values ranged between 252.4 – 7.01, with the lower value representing the darker area, whilst the higher values represents area with no evident labelling (black, Fig. 2.6).

![Calibration table](image)

**Figure 2.6.** Standard OD values obtained following calibration of the system, which was used to obtain the calibration curve.
*From (Rodríguez J.J. unpublished photograph).*
In total 19 measurements were recorded for this calibration procedure, which generated a standard OD calibration curve (Fig. 2.7). This calibration curve was used to measure and determine the exact OD in all images analysed. All obtained OD values were within the capture calibration range (252.4 – 7.01).

**Figure 2.7.** Standard OD calibration curve obtained following calibration of the system. From (Rodríguez J.J. unpublished photograph).
2.4.1.3. Maintaining a constant light intensity

Optical artefacts such as incorrect colour balance and lamp voltage fluctuations can affect the signal recorded by the camera. To ensure the highest quality of images in terms of colour balance and saturation, the ND32, the ND32 and the NCB11 optical filters were used, which adjust light intensity. Alterations in light intensity can also affect the brightness of the image hence causing an effect on the measured mean OD. To exclude the experimental error such as light intensity, all images were taken at a constant light intensity and magnifications, keeping the same optical filters.

2.4.1.4. Acquiring and saving images

All images were taken using a Nikon Eclipse 80i microscope with a x4 objective (Fig. 2.4). All images were saved in “tif” format with a resolution of 1600 X 1200 pixels. These images were used to measure the OD.

2.4.1.5. Performing the subtraction of the background staining

The mean OD value of the background staining was measured in non-immunoreactive regions of the tissue, in every hippocampal section. The corpus callosum (CC) was selected as the control region, which displayed no labelling or minimal background staining for either SERT or Aβ immunoreactivity (Fig. 2.4E, 2.4F). All images used for the background staining were obtained in parallel with the specifically labelled areas using an identical light exposure and light intensity. The OD of control region was measured together with the experimental groups using the same calibration curve.

To analyse changes in SERT-IR fibre and intraneuronal Aβ densities against a constant control (OD\textsubscript{Constant Control}), 255 was divided by the control region (corpus callosum) and the obtained factor was multiplied by the OD obtained value from the regions of interest (ROI) in every given section (Noristani \textit{et al.} 2010). This was done to exclude any changes in the OD due to differences in immunoreactivity of control regions between different sections. Thus, the OD measurements were restricted to the values generated by the reactive tissue against a constant control (Noristani \textit{et al.} 2010; Olabarria \textit{et al.} 2011).

\[
\text{OD}_{\text{Constant Control}} = \left(\frac{255}{\text{CC}}\right) \times \text{ROI}
\]
Where CC (corpus callosum), ROI (region of interest) and OD (optical density).

The inverse optical density (IOD) was obtained by subtracting the obtained OD (OD_{Constant Control}) from the background measurement (255), as described in details below.

\[
\text{IOD} = 255 - \text{OD}_{\text{Constant Control}}
\]

Where OD (optical density) and IOD (inverse optical density).

2.4.1.6. Setting and determining the exact ROI area (to measure the OD)
SERT-IR fibre density of the complete CA1 subfield of the hippocampus and its different layers PCL, S.Or, S.Rad and S.Mol were measured independently (Fig. 2.4A, 2.4B). In the CA3 subfield, the SERT-IR fibre density was also measured in the stratum lucidum (S.Luc) (Fig. 2.4A, 2.4B). Similarly, SERT-IR fibre density of the DG and its different layers: GCL, ML and hilus were also measured individually (Fig. 2.4A, 2.4B). The density of intraneuronal Aβ was measured in the PCL of CA1, CA2 and CA3 subfields of the hippocampus, but not in GCL of DG, due to the lack of Aβ immunoreactivity in this region (Fig. 2.4C, 2.4D).

2.4.1.7. Measuring the OD
The measured OD values represent the mean values of all pixels in the region of interest (ROI). For RGB (colour) images, the mean OD was calculated automatically by converting each pixel to gray scale using the formula gray = (red + green + blue)/3, which was directly performed by the ImageJ programme. The OD readings were performed in both hemispheres and at least 6 readings per animals were obtained. Measurement of mean OD values were taken and averaged, after background subtraction, from each hippocampal layer in both the left and the right hemispheres of each brain slice. The results are shown as the inverse optical density (IOD/pixel).
2.4.2. Cell count of total 5-HT neurones in the dorsal and the median raphe nuclei

In Chapter 3 and Chapter 5

All counting of 5-HT neurones in the dorsal (DR) and in median raphe (MR) nuclei were conducted by a single investigator (H. N. N.) who was blind to the subject number and group assignment. All slides were coded prior to the counting of 5-HT neurones by another lab member (M. O.) and the codes were not broken until all 5-HT neuronal counts were completed.

To determine whether changes in hippocampal SERT-IR fibre density were due to alteration in 5-HT neurones, the total number of 5-HT immunoreactive (5-HT-IR) neurones in the DR and MR nuclei of 3xTg-AD mice and non-Tg controls were calculated. Every third 40µm thick coronal brain sections throughout the rostrocaudal extent of the DR nucleus, corresponding to bregma -4.36/-4.96 mm, was used to estimate the total number of 5-HT neurones in the DR and MR nuclei (Paxinos & Franklin 2004). The areas analysed for total 5-HT neuronal count included the dorsal raphe dorsal (DRD), the dorsal raphe ventral (DRV), the dorsal raphe interfascicular (DRI) and the dorsal raphe ventrolateral (DRVL) part as well as the MR and the para-median raphe (PMR) nuclei (see chapter 1, section 1.3.4.2. for detailed anatomical description of the raphe nuclei). The boundaries of areas in which 5-HT neurones were to be counted were clearly delineated (Fig. 2.8B), thus, counts were reproducible and counting 5-HT-IR neurones in every third brain sections constituted a true systematic sampling method (Vertes & Crane 1997) (see also Fig. 2.8).
Figure 2.8. Brightfield micrographs illustrating the 5-HT labelling (A, B) and toluidine blue staining (C), which matches the anatomical organisation of the different sub-divisions of the dorsal raphe nucleus. **Key:** Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part, DRVL: dorsal raphe ventrolateral part. Scale bars: 250μm (A–C). From personal library.

All 5-HT-IR neurones were intensely labelled against a light background making them easy to be identified with an equal chance of being counted (Vertes & Crane
1997) (Fig. 2.8). The main source of error in using this calculation method is the potential multiple counting of the same neurone in more than one section, as suggested previously (Vertes & Crane 1997). However, in this case, one has to consider that the maximum cell diameter of 5-HT neurones counted was approximately 25 – 30μm, whereas every third 40μm brain section was in total 120μm distant from the previously analysed brain section, making multiple counting of the same 5-HT neurone in adjacent brain sections highly unlikely (Vertes & Crane 1997; Noristani et al. 2010). To obtain a systematic random sampling of 5-HT-IR neurones, a sampling grid consisting of a counting frame was positioned over the DR and MR nuclei on each counted brain section. The number of 5-HT-IR neurones was determined blindly by a single observer (H. N. N.) using a 10 x 10 mm graticule under a Nikon Eclipse 80i microscope. All visible 5-HT-IR neurones were counted in every third 40μm thick coronal brain sections throughout the rostrocaudal extent of the different subdivisions of the DR nucleus (Paxinos & Franklin 2004). To define the DR and MR nuclei as well as their different sub-nuclei boundaries, adjacent brain sections were counterstained with toluidine blue (Fig. 2.8C). The estimated total number of 5-HT-IR neurones within different subdivisions of the raphe nuclei was calculated using the systematic section method according to Konigsmark equation (Konigsmark 1970; Vertes & Crane 1997) as described in details below. See chapter 2 supplementary materials for detailed description of Konigsmark equation and its use (section 2.S.6.).

\[
N_t \div n_s = V_t \div v_s
\]

\[
N_t = 3 \times \sum [(V_t \div v_s) \times n_s]
\]

Where: 
- \(N_t\) (total count): estimated number of total 5-HT neurones throughout rostrocaudal extent of the raphe nuclei corresponding to bregma -4.36/-4.96 mm (Paxinos & Franklin 2004).
- \(n_s\) (sample count): number of 5-HT-IR neurones counted in every third 40μm thick coronal brain sections throughout the rostrocaudal extent of the raphe nuclei.
- \(V_t\) (total volume): estimated total volume of the raphe nuclei corresponding to bregma -4.36/-4.96 mm (Paxinos & Franklin 2004) (range from 0.37mm\(^3\) – 0.72mm\(^3\)).
\( v_s \) (sample volume): volume (surface area \( \times \) section thickness) of the raphe nuclei obtained from every third 40\( \mu \)m thick coronal brain sections throughout the rostrocaudal extent of the raphe nuclei (range 0.23mm\(^3\) – 0.37mm\(^3\)).

Note that the \( N_i \) was obtained by multiplying the \( \sum [(V_i \div v_s) \times n_i] \) by 3 because every third 40\( \mu \)m thick coronal brain sections was counted in the experiment.

**2.5. Immunohistochemistry - Fluorescence**

_In Chapter 3 and Chapter 5_

All immunohistochemical experiments were conducted by (H. N. N.). See _chapter 2 supplementary materials_ for step-by-step protocol for fluorescence immunohistochemistry (section 2.S.7.).

For the detection and determination of SERT-IR fibres, S-100\( \beta \) astrocytes and their association with A\( \beta \) plaques, dual and triple indirect immunofluorescence labelling were used. To minimise the methodological variability, sections through the dorsal hippocampus containing both hemispheres of all animals were processed at the same time using precisely the same experimental conditions. Brain sections were incubated for 30 minutes in 30\% methanol in 0.1 M PB and 3\% H\( \text{}_2\)O\( \text{}_2 \) (Sigma, Gillingham, UK). These brain sections were then rinsed with 0.1 M PB for 5 minutes and placed in 1\% sodium borohydride (Sigma, Gillingham, UK) for 30 minutes. The sections were then washed with 0.1 M PB profusely before rinsing in 0.1 M TS for 10 minutes. Brain sections were then incubated in 0.5\% BSA (Sigma, Gillingham, UK) in 0.1 M TS and 0.25\% Triton (Sigma, Gillingham, UK, x 100) for 30 minutes.

For dual immunofluorescence labelling, brain sections were incubated for 48 hours at room temperature in a primary antibody cocktail containing: (1) rabbit anti-SERT, 1:2500, Immunostar, Hudson, WI, USA and (2) mouse anti-beta amyloid monoclonal antibody (A\( \beta \), 1:2000, Covance, Emeryville, CA), simultaneously. Subsequently, SERT-IR fibres and A\( \beta \) plaques were detected in a sequential manner on the same brain sections by incubation with their correspondent
fluorochrome-conjugated secondary antibodies (see Table 2.3 for the list of secondary antibodies used for fluorescence immunohistochemistry and their respective dilutions).

**Table 2.3. Summary of secondary antibodies used for fluorescence immunohistochemistry and their respective sources.**

<table>
<thead>
<tr>
<th>Secondary Antibody (Fluorescence)</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(FITC) conjugated goat anti-rabbit IgG</td>
<td>1:200</td>
<td>Invitrogen, Paisley, UK, Catalogue No. 111-096-144</td>
<td>(Noristani et al. 2010)</td>
</tr>
<tr>
<td>Rhodamine (TRITC)-conjugated goat anti-mouse</td>
<td>1:200</td>
<td>Invitrogen, Paisley, UK, Catalogue No. 115-026-075</td>
<td>(Olabarria et al. 2010)</td>
</tr>
<tr>
<td>Alexa 488 goat anti-rabbit</td>
<td>1:200</td>
<td>Invitrogen, Paisley, UK, Catalogue No.A11008</td>
<td>(Noristani et al. 2011)</td>
</tr>
<tr>
<td>Alexa 633 goat anti-rabbit</td>
<td>1:200</td>
<td>Invitrogen, UK, A21072</td>
<td>(Olabarria et al. 2010)</td>
</tr>
<tr>
<td>Alexa 595 goat anti-mouse</td>
<td>1:200</td>
<td>Invitrogen, Paisley, UK, Catalogue No. A11005</td>
<td>(Noristani et al. 2011)</td>
</tr>
</tbody>
</table>

For triple immunofluorescence labelling, brain sections were incubated for 48 hours at room temperature in a primary antibody cocktail containing: (1) rabbit anti-SERT, 1:2500, Immunostar, Hudson, WI, USA and (2) mouse anti-beta amyloid monoclonal antibody (Aβ, 1:2000, Covance, Emeryville, CA), simultaneously. Subsequently, each antigen was detected in a sequential manner on the same sections by incubation with their correspondent fluorochrome-conjugated secondary antibodies, sequentially. Then, these brain sections were washed in 0.1 M TS for 30 minutes and incubated in 0.5% BSA in 0.1 M TS and 0.25% Triton X-100 for 30 minutes. Subsequently, sections were incubated in the third primary antibody (rabbit anti-S-100β, 1:5000, DakoCytomation, Denmark) for 24 hours at room temperature. Brain sections were rinsed in 0.1 M TS for 30 minutes and incubated in 1:200 dilution of fluorochrome-conjugated secondary antibody (for 1 hour at room temperature and finally rinsed in 0.1M TS for 30 minutes.

Finally, in both double and triple immunofluorescence labelling, brain sections were rinsed with 0.1 M PB for 30 minutes and permanently mounted in an aqueous medium (Vectashield, Vector Laboratories Ltd., Peterborough, UK) and visualised using confocal scanning microscopy (Leica, SP2 inverted). Using confocal scanning microscopy the staining for SERT-IR fibres were observed throughout the
depth of the brain sections (40μm) in both non-Tg control and the 3xTg-AD mouse model of AD, with optical recorded sections taken at every 0.2μm between adjacent optical sections (Fig. 2.9). The obtained series of images were superimposed by the confocal software and converted to produce a flat 3-dimentional image of the samples with maximum resolutions.

**Figure 2.9.** Confocal images of SERT-IR fibres in the dorsal hippocampus of non-Tg control (A, C) and 3xTg-AD (B, D) animals. Note that the diagonal view of the sections (A and B) show SERT staining throughout the 40μm thickness of the brain sections in both non-Tg control (A, C) and the 3xTg-AD (B, D) mouse model of AD, confirming that there is no difference in antibody penetration between the two groups. Scale bars: 25μm (C and D).

*From personal library.*
2.6. Electron microscopy

In Chapter 4

All immunohistochemistry experiments for the electron microscopic studies were conducted by (H. N. N.). Flat embedding of immunostained brain sections and block preparation was done by (J. J. R. A.). See chapter 2 supplementary materials for step-by-step protocol for electron microscopy and processing of the brain sections for electron microscopy (section 2.5.8.). Ultra thin sectioning and electron microscopic imaging of the brain sections were conducted by Roger, S. Meadows (R. S. M.), who was blind to the animal number and groups. The obtained electron microscopic images were coded blindly by another member of the lab (M. O.) prior to the quantification of SERT-labelled profiles and the codes were not broken until all quantifications were completed. All counting of SERT-labelled profiles were conducted blindly by (H. N. N.) and were further verified by another more experienced investigator (J. J. R. A.), who were both blind to the animal number and group assignment, to assure an exact determination of labelled profiles.

Peroxidase immunohistochemistry combined with electron microscopy was used to visualise and analyse the ultrastructural changes in the hippocampal synaptic numerical density (N_v, #/μm^3) and their association with SERT-IR axons (SERT-Ax) and terminals (SERT-Te). In addition, the surface areas of SERT-Te were measured to determine changes in serotonergic terminal sizes between 3xTg-AD mice and age-matched non-Tg controls.

Following peroxidase immunohistochemistry using SERT-specific antibody, brain sections were rinsed and flattened in 0.1 M PB followed by post-fixation in 2% osmium tetroxide in 0.1 M PB for 1 hour (Pickel et al. 1984; Leranth & Pickel 1989; Rodríguez et al. 2001; Peddie et al. 2008b; Peddie et al. 2008a). The sections were then washed for 10 minutes in 0.1 M PB followed by a sequential dehydration through replacement with graded ethanol series (30, 50, 70 and 95%, 5 minutes each). Brain sections were further dehydrated using 100% ethanol (10 minutes) and propylene oxide (2 x 15 minutes). To start resin penetration, dehydrated brain sections were incubated in propylene oxide:EPON (1:1, Agar scientific Ltd., Stanstead, UK) overnight at room temperature and rotating. To complete resin impregnation, the propylene oxide:EPON was replaced with 100% EPON and the
brain sections were rotated for further 2 hours. Brain sections were then flat embedded between sheets of Aclar fluo halocarbon films (Agar scientific Ltd., Stanstead, UK) (Rodríguez et al. 2001; Peddie et al. 2008a; Peddie et al. 2008b). Polymerisation was carried out by incubating at 60°C overnight. Following polymerisation, the region of interest (CA1 subfield of the hippocampus) was selected and micro-dissected from the flat embedded brain sections and mounted on the tip of EPON blocks (Pickel et al. 1984; Rodríguez et al. 2001; Peddie et al. 2008a; Peddie et al. 2008b), keeping the same levels and coordinates between different animals (Fig. 2.10). All brains sections used for quantitative analysis were from the identical anatomical level corresponding to -2.06 mm posterior to Bregma (dorsal hippocampus) in both non-Tg control and 3xTg-AD animals (Fig. 2.10).

Figure 2.10. Brightfield micrographs illustrating the distribution of SERT-IR fibres in the dorsal hippocampus of 3 months old non-Tg control (A), the 3xTg-AD mouse model of AD (B) and their respective high magnification details of the CA1 subfield area of the hippocampus that was selected for the electron microscopic analysis (C, D). Hippocampal sections were selected at identical anatomical levels between different animals to ensure an exact and equivalent sampling, as illustrated by the red pyramids that were trimmed and further embedded (A and B). Key: **S.Or:** stratum oriens, **PCL:** pyramidal cell layer, **S.Rad:** stratum radiatum, **S.Mol:** stratum lacunosum moleculare, **DG:** dentate gyrus. Scale bars: 500μm (A and B), 100μm (C and D).

From (Noristani et al. 2011).

The EPON blocks were coded by another lab member (M. O.) prior to ultrathin
sectioning and the code was not broken until the quantification was completed. A diamond knife was used to cut serial ultra thin sections of these regions at a thickness ranging from 50 – 70nm. The series of ultra thin sections were collected on degreased copper mesh grids (200 mesh) and counterstained using the uranyl acetate and lead citrate method (Reynolds 1963) prior to their examination under the Philips FEI Tecnai 12 BioTwin (FEI, Eindhoven, The Netherlands) electron microscope. Random images of the areas of interest (blindly taken, R. S. M) were collected and the obtained negatives were digitalised on an Imacon Flextight 848 scanner (Imacon Inc. Hasselbald A/S, Copenhagen, Denmark). Each digitalised image represented a volume of 12.39µm³, for a total analysed CA1 volume of 396.48µm³ (in each animal) and 99.12µm³ per each CA1 layer (S.Or, PCL, S.Rad and S.Mol). See chapter 2 supplementary material for detailed description and calculation of surface area and volumes measurement of electron micrographs (section 2.S.9.).

2.6.1. Determination and nomenclature of neural profiles

In Chapter 4

The labelled profiles were classified as somata, dendrites, dendritic spines, unmyelinated axons, axon terminals and glia according to their morphological features, as described previously (Rodríguez et al. 2000; Rodríguez et al. 2001; Rodríguez et al. 2005; Peddie et al. 2008a; Peddie et al. 2008b) and originally defined by Peters et al. (Peters et al. 1991). The somatic profiles were recognised by the presence of the nucleus (Nu) and abundant cytoplasm organelles including the rough endoplasmic reticulum (rER), the smooth endoplasmic reticulum (sER) and the mitochondria, among others (Fig. 2.11A and 2.11B). Dendrites were identified by the presence of afferent axon terminals and/or content of the endoplasmic reticulum and the mitochondria (Fig. 2.11B) (Rodríguez et al. 2000). Dendritic profiles were differentiated from dendritic spines on the basis of their larger size and greater abundance of the mitochondria and other cellular organelles (Fig. 2.11B). In comparison to spines, dendrites also less frequently received input from axon terminals forming asymmetric excitatory-type synapses (Fig. 2.11B).
Figure 2.11. Electron micrographs showing illustrative images of somatic and dendritic profiles observed in the dorsal hippocampus. The somatic profiles were recognised by the presence of the nucleus (Nu) and abundant cytoplasmic organelles such as the rough endoplasmic reticulum (rER), the smooth endoplasmic reticulum (sER) and the mitochondria (M). Dendritic profiles (UD) were differentiated from dendritic spines (usp) due to their larger size and greater presence of M as well as other cellular organelles. In addition, UD were distinguished from usp due to lack of the rounded bulb that constituted the spine head. **Key:** Nu: nucleus, M: mitochondria, UD: unlabelled dendrites, rER: rough endoplasmic reticulum, sER: smooth endoplasmic reticulum, UT: unlabelled axon terminal, usp: unlabelled spine. Scale bars: 500nm (A and B).

Modified from (Noristani et al. 2011).

The dendritic spines identified referred exclusively to the rounded bulb that constituted the head and mainly received excitatory-type asymmetric synapses (Fig. 2.13B). The spine necks were usually <0.1μm in diameter and they were distinguished from small axons by the frequent presence of the spinous apparatus (Fig. 2.13B). Dendritic spines were usually larger in size compared to small axons or glial processes and had a more rounded bulbous shape (Fig. 2.13B). Unmyelinated axons were <0.2μm in diameter and contained microtubules and few small vesicles, if any.

Glial processes were characterised by their irregular profiles that in many cases were surrounding neighbouring neurones or synaptic elements. Astroglial profiles
were particularly evident around synaptic specialisation surrounding both pre- and post-synaptic terminals (Fig. 2.12). Any other profiles that were not clearly distinguished as belonging to any of the above categories were considered unidentified processes and were excluded from the analysis.

**Figure 2.12.** Electron micrographs illustrating glial processes and their associations with synaptic specialisations. Astroglial processes (blue) surrounds several asymmetrical synapses in the hippocampus, contacting both pre- and post-synaptic terminals (orange and green respectively, A). Asymmetric excitatory synapse of the hippocampus with the characteristic thin pre- and a thick post-synaptic density as well as rounded synaptic vesicles in the pre-synaptic terminal. One of the vesicles is fusioned with the plasma membrane likely liberating its vesicular content (B). **Key:** Pre-sy: pre-synaptic terminal, post-sy: post-synaptic terminal. Scale bars: 150nm (A), 100nm (B).

(Generous gift from Markel Olabarria).

2.6.1.1. Synaptic determination

Synapses were defined as either symmetric or asymmetric depending on their post-synaptic densities and synaptic vesicles composition (Fig. 2.13). Symmetric
synapses displayed thin pre- and post-synaptic densities with their axons containing pleomorphic (round and elongated) synaptic vesicles (Fig. 2.13A). Asymmetric synapses have only round synaptic vesicles in the axons and were characterised by the presence of a thin pre-synaptic density (sometimes barely visible) but with a thick and more prominent post-synaptic membrane specialisation (Rodríguez et al. 2000; Rodríguez et al. 2005) (Fig. 2.13B). Some of these asymmetric synapses displayed a notable discontinuity (>50 nm) in the post-synaptic density, as previously described (Rodríguez et al. 2000) and were, therefore, classified and characterised as perforated synapses (Fig. 2.13C). No measurement of the “synaptic area” was investigated in either non-Tg control or the 3xTg-AD mouse model of AD.
Figure 2.13. Electron micrographs illustrating the differential characteristic and morphological features of symmetrical (A), asymmetrical (B) and perforated (C) synapses. Symmetric synapses were characterised by their thin pre- and post-synaptic densities (A, open arrow) with axon terminals containing round (black line) and elongated (red line) synaptic vesicles. Asymmetric synapses display a thin pre- and a thick post-synaptic density (B, arrowhead); whilst perforated synapses are asymmetric synapses with a notable discontinuity (>50 nm) in the electron density of their post-synaptic junction (C, curved arrows), but in both cases with axons containing just round synaptic vesicles. Key: AT: axon terminal, SVs: synaptic vesicles, usp: unlabelled spine. Scale bars: 125 nm (A), 250 nm (B and C). From (Noristani et al. 2011).
2.6.1.2. Identifying SERT labelled profiles

SERT axon terminals (SERT-Te) were defined as being profiles between 0.2 – 1.5μm in diameter containing many small synaptic vesicles (SVs) some of them displaying electron-dense DAB reaction product (Fig. 2.14A, 2.14B). SERT-Te displayed two types of vesicles, namely: small synaptic vesicles containing 5-HT and large dense core vesicles (DCVs), which have been previously shown to contain 5-HT and characterise serotonergic terminals (Maley & Elde 1982; Nirenberg et al. 1995; Huang & Pickel 2002) (Fig. 2.14A, 2.14B). Occasionally, the labelling was directly associated with cytoplasmic organelles including DCVs (Fig. 2.14B) (Huang & Pickel 2002). Dense labelling for SERT was also observed in small unmyelinated axons (SERT-Ax), which were predominantly apposed to other small axons and/or terminals and in close vicinity to synaptic elements (Fig. 2.14C). SERT labelling was mainly absent in post-synaptic sites and was rarely observed in dendrites and glial profiles.
Figure 2.14. Electron micrographs showing the morphological features of SERT-terminals (A, B) and SERT-axon (C) in the CA1 subfield of the mouse hippocampus. SERT immunoreactivity in axon terminals (A and B) in the CA1 stratum lacunosum moleculare showing in some cases the characteristic dense core vesicles (DCVs) of serotonergic terminals (B) and forming asymmetric synapses (curved arrows). C: SERT plasmalemmal labelling in a small unmyelinated axon (SERT-Ax) in the CA1 stratum lacunosum moleculare, which is apposed to an unlabelled terminal (UT) and an unlabelled dendritic spine (usp). Key: M: mitochondria, UD: unlabelled dendrites, usp: unlabelled spine, UT: unlabelled axon terminal, UA: unlabelled axon. Scale bars: 500 nm (A – C). From (Noristani et al. 2011).
2.6.2. Ultrastructural numerical density of SERT profiles

In Chapter 4

The quantification of immunoreactive profiles, processed for electron microscopy, is significantly influenced by the penetration of the antibody into the tissue. To minimise artificial differences in labelling attributed to potential differences in the penetration of the antibody in the brain sections, ultrastructural analysis was entirely carried out at the tissue-EPON interface, where the greater densities of the immunoreactive profiles are seen (Pickel et al. 1992; Rodríguez et al. 2001). This constitutes the most superficial portions of the tissue that is in direct contact with the embedding plastic. This superficial layer was used to determine the numerical density ($N_v$, # labelled profiles /μm$^3$) of SERT-labelled profiles.

The peroxidase immunohistochemistry method was selected for quantification of SERT-labelled profiles instead of the immunogold labelling because the latter is less sensitive compared to peroxidase labelling (Cortese et al. 2009). Although gold labelling allows a more selective sub cellular localisation, it has reduced tissue penetration and limited diffusion that may result in an underestimation of the relative abundance of immunoreactive profiles (Leranth & Pickel 1989; Rodríguez et al. 1999; Cortese et al. 2009). Furthermore, immunogold labelling provides less reliable quantitative 3D estimation of the labelled profiles since it is primarily regarded as 2D (surface) method and is not reliably considered quantitative for 3D labelling (Cortese et al. 2009).

The $N_v$ of SERT-Te, SERT-Ax and synapses were determined according to the Cavalieri principle (Tang et al. 2001). See chapter 2 supplementary materials for detailed description of Cavalieri principle (section 2.S.10.).

$$N_v = \frac{\Sigma P}{t \times \alpha(p)}$$

Where $N_v$ is the numerical density, $\Sigma P$ is the number of SERT-Te, SERT-Ax or synapses counted, $t$ is the average ultra thin section thickness and $\alpha(p)$ is the corresponding surface area ($\mu$m$^2$).
2.6.3. Surface area measurement of SERT-Te profiles

In Chapter 4

To investigate possible changes in the size of serotonergic terminals between non-Tg control and 3xTg-AD mice, a morphometric analysis of SERT-Te was carried out by measuring the surface area of SERT-labelled terminals directly on electron micrographs, as previously described (Cohen et al. 1995; Cohen et al. 1997). SERT-Te surface area was measured in all hippocampal layers using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA). See chapter 2 supplementary materials for step-by-step procedure of surface area measurement using ImageJ (section 2.S.11.).

In general, the procedure for measuring surface area can be divided into 3 steps: 1- Identifying the labelled terminal, 2- Setting the scale and 3- Determining the exact surface area of the labelled terminal.

2.6.3.1. Identifying labelled terminals

The electron micrographs were coded blindly by another member of the lab (M. O.) before the surface area measurement and the codes were not broken till all quantifications were completed. All measurement of SERT-Te surface area were conducted blindly by (H. N. N.) and were further confirmed by another more experienced investigator (J. J. R. A.), who were both blind to the animal number and group assignment, to assure an exact determination of SERT-labelled terminal size.

As previously mentioned, SERT-Te were identified due to the presence of electron-dense DAB peroxidase product as well as the presence of small synaptic vesicles (see section 2.6.1.). Consistent with previous electron microscopic studies using SERT-specific antibodies, SERT labelling was predominantly evident along plasma membrane of serotonergic axons (Zhou et al. 1998; Pickel & Chan 1999).

2.6.3.2. Setting the scale

All electron micrographs obtained were of identical magnification each displaying the 2.3μm scale bars at the bottom of the micrographs (Fig. 2.15). This 2.3μm scale bars was used to set the scale in the ImageJ program.
2.6.3.3. Determining the exact surface area of the labelled terminal

The surface area of SERT-Te was determined by tracing the electron-dense DAB peroxidase product along axonal membrane in each series of electron micrographs used for stereological analysis. For each identified SERT-Te, the surface area was measured individually and averaged per total number of terminals in a given electron micrograph. The results are shown as average surface area of SERT-Te per animals (n = 3 per group) in each group.

Composite figures, adjusted for brightness, contrast and unsharpness were generated using Adobe Photoshop CS2 (Adobe Systems Inc. San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).
Figure 2.15. Electron micrographs showing morphological features of SERT-terminals used to determine the surface area of SERT-labelled terminals. Note that the white line at the bottom of the electron micrograph indicates the 2.3μm scale bar, which was present in all obtained electron micrographs and were used to set the scale before measuring SERT-Te surface area along plasma membrane. Scale bar: inset 0.5μm.
From personal library.
2.7. Statistical analysis

*In Chapter 3, Chapter 4 and Chapter 5*

Results are expressed as mean ± standard errors of the mean (S.E.M). A 2 way analysis of variance (ANOVA) was used to determine changes in SERT-IR fibre density and 5-HT neurones at different ages followed by the Tukey post hoc test (*Chapter 3*). Un-paired t-test was used to determine changes in SERT-IR fibres associated with Aβ plaques (*Chapter 3*), SERT-Te, SERT-Ax and synapses (*Chapter 4*), body weight, food intake, water intake, SERT-IR fibre density, 5-HT neurones and intraneuronal Aβ density (*Chapter 5*). Significance was accepted at \( p \leq 0.05 \). All data were analysed using the Minitab (Minitab 15 Software) and GraphPad Prism 4.0 (GraphPad Software, Inc. La Jolla, CA, USA) programmes.
Chapter 2S Supplementary Materials

2.S.1. Perfusion solutions

2% paraformaldehyde (PFA)
To prepare 1 litre of 2% PFA:

a) Heat 500mls of distilled water to reach 55°C
b) Add 20g of PFA powder to the heated double distilled water and stir well using a magnet stirrer
c) Add 2.5µl of NaOH to clear the solution

Make sure that the dissolved PFA is clear before filtering the solution using a Whatman filter paper!!!
d) Allow to cool for 20 minutes at room temperature
e) Add 480mls of 0.2M Phosphate buffer (PB), to reach a final concentration of 0.1M PB
f) Once cooled down, adjust the pH of the PFA solution to 7.4

Heparin
Add 1 vial of 10000U heparin into 300mls of 0.9% saline (9g NaCl/l)
Adjust the saline pH to 7.4

3.75% Acrolein
E.G. for 100mls
Add 3.75mls of Acrolein into 96.25 of 2% PFA solution.

Take extreme care when handing Acrolein, it is highly toxic!!!

Per individual mouse:
20mls of Heparin solution (to prevent blood clot formation).
Followed by 25mls of 3.75% Acrolein in 2% PFA and finally by 60 – 100mls of 2% PFA.
Remove the brains from the skull and post-fix for 2 hours in 2% PFA. Following the 2 hours post-fixation, cut the brains into 3 coronal slabs of tissue and further post-fix in 2% PFA for additional 22 hours. Replace the 2% PFA with 0.1M PB and
store in 4°C. Cut the brain into 40 – 50μm thickness coronal sections using a vibrating microtome and stored in cryoprotectant solution at -20°C till use.

2.S.2. Toluidine blue staining protocol

Solutions and reagents required for toluidine blue staining:

To prepare 100ml of 2% toluidine blue stock solution:
Weigh 2g of toluidine blue powder
Add 100 ml of double distilled water
Mix to dissolve using magnet stirrer (10 minutes)
Keep the stock solution in 4°C

To prepare 50ml of 1% NaCl:
Weigh 0.5g of NaCl
Add 50ml of double distilled water
Mix to dissolve (make this solution fresh each time).
Adjust the pH between 2.0 – 2.5 using HCl

To prepare toluidine blue working solution (pH 2.0-2.5):
Add 5ml of toluidine blue stock solution
Add 45 ml of 1% NaCl solution, pH 2.0 - 2.5
Mix well
Make this solution fresh and discard after use

Note that the pH should be around 2.3 and less than 2.5
A pH higher than 2.5 will make staining less contrast!!!

Procedure:

1. Stain brain sections in toluidine blue working solution (3 minutes)
2. Wash in distilled water, 3 changes (2 minutes each)
3. Dehydrate the stained brain sections quickly through 100% ethanol (30 seconds)

Note 10 dips each since stain fades quickly in alcohol!!!
4. Clear in xylene solution 2 changes (3 minutes each)
5. Coverslip with Entellan.
Material and Methods

Leave the coverslipped section to dry overnight, before removing the excess Entellan by using razor blade.

2.5.3. Solutions for immunohistochemistry

To prepare 1 litre of 0.1M PB

- Weigh 10.9g of sodium phosphate dibasic (HNa$_2$O$_4$)
- Weigh 3.2g of sodium phosphate monobasic (NaH$_2$PO$_4$)
- Add the HNa$_2$O$_4$ and the NaH$_2$PO$_4$ into 1 litre of double distilled water
- Stir well using magnet stirrer
- Adjust the pH to 7.4 using NaOH

To prepare 1 litre of 0.1M trizma base saline (TS)

- Weigh 12.1g of Tris
- Weigh 9.0g of NaCl
- Add the Tris and NaCl into 1 litre of double distilled water
- Stir well using magnet stirrer
- Adjust the pH to 7.6 using HCl

To prepare 100 ml of 0.1% bovine serum albumin (BSA) in 0.1M TS and 0.25% triton

- Weigh 0.1g of BSA powder
- Add the BSA into 100ml of 0.1M TS
- Add 250µl of Triton X-100

To prepare 500 ml 0.5% BSA in 0.1M TS and 0.25% triton

- Weigh 2.5g of BSA powder
- Add the BSA into 500ml of 0.1M TS
- Add 1,250µl of Triton X-100

2.5.4. Step by step protocol for immunohistochemistry – peroxidase

Select brain sections according to required anatomical level using mouse brain atlas.
Rinse the brain sections in 2 washes of 0.1M PB (5 minutes each), to remove the storage solution before starting immunohistochemistry procedures.
Day 1

- To block endogenous peroxidase from the final reaction product, incubate the brain sections in a cocktail solution containing 30% methanol in 0.1M PB and 3% H₂O₂ for 30 minutes.
- Rinse the sections with 0.1M PB for 5 minutes.
- Place the brain sections in a solution containing 1% sodium borohydride (1g in 100ml 0.1M PB) for 30 minutes. Squirt over tissue during incubation to ensure the brain sections are inside the dissolved sodium borohydride solution.
- Rinse the brain sections copiously with 0.1M PB until no bubbles remain in the crucibles.
- Rinse the brain sections in two separate washes of 0.1M TS (5 minutes each).
- Incubate the sections in a block containing 0.5% BSA in 0.1M TS and 0.25% triton for 30 minutes.
- Prepare the 1º antibody diluted in 2ml of 0.1% BSA in 0.1M TS and 0.25% triton using capped vials.
- Ball up the brain sections with a brush and transfer them from crucibles into the capped vials.
- Incubate whilst shaking for 48 hours at room temperature.

Day 3

- Transfer the brain sections back to crucibles and rinse in two washes of 0.1M TS (15 minutes each).
- Prepare the 2º antibody for peroxidase reaction (1:400) in capped vials, diluted in 2mls of 0.1% BSA in 0.1M TS and 0.25% triton.
- Transfer the brain sections to the capped vials containing the freshly prepared 2º antibody and incubate whilst shaking at room temperature for 1 hour.
- **During incubation in 2º antibody**, prepare the Avidin Biotin Complex (ABC) solution. Add 2 drops of solution A and 2 drop of solution B for every 10mls of 0.1% BSA in 0.1M TS and 0.25% triton and **immediately** vortex. Allow to stand for 30 minutes at room temperature prior to use, then aliquot into capped vials at 2mls each.
- Transfer the brain sections back to crucibles and rinse in two washes of 0.1M TS (15 minutes each).
Transfer brain sections back to the capped vials containing the ABC solution and incubate for 30 minutes at room temperature.

Transfer brain sections back to the crucibles and rinse in two washes of 0.1M TS (15 minutes each).

**During the washes in 0.1M TS**, prepare the 3,3’-diaminobenzidine (DAB) solution. Add 22mg of DAB for every 100mls of 0.1M TS whilst stirring. Immediately before to use, add 10µl of 30% H₂O₂ per every 100mls of DAB solution.

Following rinses incubate the brain sections in DAB, time depending on reaction strength or previous experiment, squirting solution over the tissue (ranging from 4 – 10 minutes).

Rinse the brain sections in two washes of 0.1M TS (3 minutes each) to stop the reaction. Dispose of DAB and 1st rinse of 0.1M TS by putting them into allocated waste containers.

Rinse the brain sections in three independent washes of 0.1M PB (5 minutes each).

For light microscopic examination (of peroxidase reaction), mount sections onto gelatinised slides and allow to dry overnight.

Dehydrate the mounted brain sections by using increasing concentrations of ethanol (50%, 60%, 70%, 80%, 90%, 95% and 100%, 10 minutes each) and finally into xylene for further 30 minutes.

Apply cover slips by using Entellan and remove all air bubbles.

Dry at lease overnight and clean off excess glue using razor blade.
Figure 2.S.1. Intra-section coding system used to identify brain sections from different animals following immunohistochemical staining. Schematic drawing of a brain section through the mouse brain at the level of the dorsal hippocampus corresponding to -2.06 mm posterior to Bregma (A) demonstrating the locations of the different subfield of the hippocampus (red). Representative brightfield micrograph of SERT-labelled brain sections through the dorsal hippocampus (B-G). Note that for intra-section coding, brain sections were either kept blank (B) or coded using a syringe and a needle as 1 top (represented by 1 hole on top of each hemisphere, C), 1 side (represented by 1 hole on the side of each hemisphere, D), 1 top 1 side (E), 2 top (F) and 2 side (G). Scale bars: 1 mm (B – G) (A) modified from (Paxinos & Franklin 2004), (B – G) from personal library.
2.S.5. Measuring optical density using ImageJ

Note that the ImageJ programme is freely available online and can be downloaded from (http://rsbweb.nih.gov/ij/download.html).

1.1 Open ImageJ:
File>
Open>
Desktop>
Graticules folder>
“Step tablet”

1.2 Select rectangular tool
- Measure each band on the step tablet without overlapping the adjacent bands (Fig. 2.S.2).

![Image of step tablet](image)

Figure 2.S.2. Standard table for calibrating the system before proceeding to the OD measurement.

- Analyse>
  Measure (or Ctrl + M)
- On approx band 10 adjust brightness to visualise remaining bands.
- Image>
  Adjust>
  Brightness/contrast
  Adjust maximum bar down until remaining bands are visible.

- Measure up to 19 bands in total (Fig. 2.S.3).
- Select global calibration to apply to all images.
- Save as calibration (experiment name).
- Select OK. This opens a graph. Save as graph and name it (Fig. 2.S.4).

Close graph and step tablet.
Measure OD of the samples.

Keeping a constant calibration density:
It is critical to keep a constant calibration density to prevent experimental variability when measuring the OD of images obtained at an identical condition.

1) Copy the measurements of standard OD table into an Excel files. Delete excess information and make sure that your values are in column A.

2) Copy column B from ‘Calibration for density’ file and paste into column B in the Excel file.

3) Save as the calibration file.

Next time when opening image J:

1) Open Step tablet:

   Analyze>
   Calibrate.

2) Open your saved calibration file in Excel.
3) Copy each column to the Calibration box.
4) Select Global calibration.
5) Click OK.
6) Close graph and Step tablet.
7) Measure the OD on the obtained images.
2.8.6. Konigsmark equation and its use

Konigsmark equation is a mathematical formula used to estimate the neuronal population in a given brain structure (Konigsmark 1970). There are mainly three different methods of using neuronal count data to estimate the total neuronal population of a structure. These include: (i) the total count method, (ii) the random section method and (iii) the systematic section method (Konigsmark 1970).

2.8.6.1. Total count method

The total count method is where all neurones in serial sections through the structure are counted (Konigsmark 1970). Although the total count method is the most accurate for estimating the total neuronal population in any given structure, there are two types of possible errors that are likely to occur when using the total count method including: (a) the counting error, where the counting neurones may be missed or counted twice by the investigator and (b) the split error, where the neurone counted is positioned near the section surface that may be split and appear for counting in two adjacent sections (Konigsmark 1970).

2.8.6.2. Random section method

The random section method is the most commonly used method of estimating the total number of neurones in a given brain structure. In the random section method the number of neurones are first determined per unit volume and multiplying this value by the total volume of the nuclei under investigation to estimate the total number of neurones. Most commonly, brain sections representing 5 – 10% of the brain structure is selected in the random selection method. Specific sections are chosen randomly to count neuronal numbers from the total number of sections throughout the structure (Konigsmark 1970). However, the random selection method has a major disadvantage because it assumes that the distribution of neurones remain stable throughout the structure. This is particularly important in neurodegenerative diseases where a specific sub-population of neurones are affected in a given brain structure, e.g. rather than a generalised neurodegeneration throughout the whole structure.
2.5.6.3. Systematic section method

The majority of structures in the nervous system are large in size, which makes it almost impossible to count all cells in serial sections. In addition, taking more than a certain percentage of the sample may not be necessary, when considering the added time and work involved in a total neuronal count. Systematic sample of known intervals throughout the structure is taken for systematic section method (Konigsmark 1970). Systematic section may be chosen as number from 1 to I where I is the intervals of the systematic sample. If sample K is selected then slides K, K + I, K + 2I, K + 3I…, are studied.

Unlike the total count method, the systematic section method does not include the split error, in which the unit located near the section surface may be cut and appear for counting in two adjacent sections. In addition, and unlike random section method, systematic section method takes into account when the distributions of neurones are different in a given structure.

Therefore, in chapter 3 and chapter 5 of this thesis, the estimated total number of 5-HT neurones in the DR and MR nuclei were obtained by using the systematic section method, in every third systematic brain sections throughout the rostrocaudal extent of the different subdivisions of DR nucleus between bregma -4.36 – 4.96 mm (Paxinos & Franklin 2004).
2.S.7. Step by step protocol for immunohistochemistry – Fluorescence

Select brain sections according to required anatomical level using the mouse brain atlas and rinse the obtained brain sections in 2 washes of 0.1M PB (5 minutes each), to remove the storage solution before starting immunohistochemistry.

Day 1
- To block endogenous peroxidase reaction, incubate the brain sections in a cocktail solution containing 30% methanol in 0.1M PB and 3% H₂O₂ for 30 minutes.
- Rinse the brain sections with 0.1M PB for 5 minutes.
- Place the brain sections in a solution containing 1% sodium borohydride (1g in 100ml 0.1M PB) for 30 minutes. Squirt over tissue during incubation to ensure that the brain sections are incubated inside the sodium borohydride solution.
- Rinse the brain sections copiously with 0.1M PB until no bubbles remain in the crucibles.
- Rinse brain sections in two independent washes of 0.1M TS (5 minutes each).
- Incubate the brain sections in a block containing 0.5% BSA in 0.1M TS and 0.25% triton for 30 minutes.
- Prepare the 1º antibody cocktail diluted in 2ml of 0.1% BSA in 0.1M TS and 0.25% triton using capped vials.
- Ball up the brain sections with a brush and transfer them from the crucibles into the capped vials.
- Incubate the brain sections in the 1º antibody cocktail whilst shaking for 48 hours at room temperature.

Day 3
- Transfer the brain sections tissue back to the crucibles and rinse in two washes of 0.1M TS (15 minutes each).

For fluorescence reaction
- Prepare 2º antibodies for fluorescence (1:200) in capped vials, diluted in 2mls of 0.1% BSA in 0.1M TS and 0.25% triton.
- Transfer the brain sections to the caped vials containing the 2º antibody.
- Incubate shaking for 1 hour at room temperature.
- Transfer the brain sections back to the crucibles and rinse in two washes of 0.1M TS (15 minutes each).

**Check the signal using a fluorescence microscopy.**

For dual labelling
- Incubate in the second 2° fluorescence antibody (1:200) in capped vials, diluted in 2mls of 0.1% BSA in 0.1M TS and 0.25% triton.
- Transfer the brain sections into caped vials containing the second 2° antibody as previously and incubate shaking at room temperature for 1 hour.
- Wash the brain sections in two separate washes of 0.1M TS (15 minutes each).
- Rinse the brain sections in 3 independent washes of 0.1M PB (5 minutes each).
- Mount the brain sections onto gelatinised slides and cover slip using fluorescence medium.
- Examine the brain sections using fluorescence microscopy.
2.S.8. Step by step protocol for electron microscopy processing

- Flatten brain sections in the 0.1M PB using Coor’s dishes.
- Remove the 0.1M PB using glass pipettes and replace it with 2% osmium tetroxide in 0.1M PB under the ventilating hood. Cover the section using a metal plate and incubate in 2% osmium tetroxide for 1 hour.
- Remove the 2% osmium tetroxide and rinse three times in 0.1M PB (3 minutes each).
  Note: after the removal of the osmium it needs to be inactivated by incubating it in 70% methanol before placing into a waste container!!!
- Dehydrate the brain section through replacement with graded ethanol series (5 minutes each):
  - 30% ethanol
  - 50% ethanol
  - 70% ethanol
  - 95% ethanol
  Note: make fresh ethanol solutions prior to use.
- To prepare 25ml of EPON:
  - 11ml Agar
  - 7.5ml DDSA
  - 6.75ml NMA
  - 262μl DMP-30
- Mix the prepared EPON and leave it on a rotator until needed.
- Transfer the brain sections into the capped vials and continue dehydration:
  - Two incubation of 100% ethanol (10 minutes each).
  - Two incubation of propylene oxide (15 minutes each).
  - Mixture of propylene oxide:EPON in 1:1 ratio overnight, at room temperature whilst rotating, to start resin impregnation.

Next Day
- Replace the 1:1 mixture of propylene oxide and EPON with 100% EPON and incubate for further 2 hours rotating to complete resin impregnation.
Material and Methods                                                                                                      Chapter 2

- Flat embed the brain sections between sheets of Aclar fluorhalocarbon film using as little EPON as possible and eliminating all air bubbles.
- Add weights and slowly oven roast at 60°C overnight to polymerise.

2.5.9. Calculation of surface area and volume measurements using electron micrographs

Each electron micrograph was 11.42µm x 15.50µm in size, which represented a surface area of 177µm² (Fig. 2.5.5). The volume for each electron micrograph was calculated by:

$$V = [ t \times \alpha(p) ]$$

Where V is the volume, t is the average ultra thin section thickness and \( \alpha(p) \) is the corresponding surface area e.g.

$$V = [ 70\text{nm} \times 177\mu\text{m}^2 ]$$
$$V = [ 0.07\mu\text{m}^2 \times 177\mu\text{m}^2 ] = 12.39\mu\text{m}^3$$

8 random sections were analysed for each hippocampal layers of the CA1 subfield including: S.Or, PCL, S.Rad and S.Mol:

$$V_{layer} = 8 \times 12.39\mu\text{m}^3 = 99.12\mu\text{m}^3$$

The total analysed CA1 volume was measured by adding the volume of the 4 layers analysed:

$$V_{total} = 4 \times 99.12\mu\text{m}^3 = 396.48\mu\text{m}^3$$
**Figure 2.S.5.** Electron micrograph showing an illustrative size (x and y axis) of an individual image used for determining the structure volume and quantitative analysis of SERT-Te, SERT-Ax and synapses within the hippocampus. Scale bar: 0.5μm (inset).

From personal library.
2.S.10. Cavalieri principle

The Cavalieri principle is a well-established unbiased stereological technique for estimating the volume of any structure under the investigation (Gundersen & Jensen 1987). The Cavalieri principle uses uniformly spaced sections cut throughout the structure and counts the number of points associated with a known area to estimate the total volume of the structure (Gundersen & Jensen 1987; Nyengaard & Gundersen 2006). There are three critical requirements for using the Cavalieri principle in practice namely: (i) that the position of the first section must be random, (ii) that the sections are positioned in parallel and (iii) that the thickness of each section is kept constant (Mayhew 1992; Nyengaard & Gundersen 2006). An essential characteristic of the Cavalieri principle is that all regions of the tissue under investigation have an equal chance of being sampled without any assumption being made about their size, shape or orientation. Following the random selection of the initial section from one end of the tissue block, a series of sections are selected for measurement that are spaced uniformly apart (Gundersen & Jensen 1987). The surface area from region of interest is measured in each individual section and multiplied by the section spacing to obtain an estimate of the three-dimensional (3D) volume for the object. Previous studies have suggested that extremely reliable estimates may be obtained with approximately 10 sections using the Cavalieri protocol (Royet 1991).

In chapter 4, the Cavalieri principle, an unbiased estimator of volume, was applied to the electron micrographs obtained using electron microscopy. Because selection of initial section is random rather than systematic sampling method throughout the hippocampal structure, the final values are indicated as number of profiles per unit volume of the hippocampal tissue (#/μ³) (see chapter 4).
2.5.11. Measuring area using ImageJ

Set Scale
Prepare the graticule image file in advance. The magnification must correspond to the picture being measured.

1. Open graticule image (Click File > Open).
2. Click the straight line icon.
3. Drag the measuring line across the graticule.
4. Click Analyse > Set scale.
5. Adjust known distance & unit for length.
6. Click the check box to activate global mode.
7. Click OK.

Measuring
1. After setting the scale open the image to be analysed (Click File > Open).
2. Click the polygon icon (3rd icon from left on toolbar).
3. Draw around the target area.
4. Click Analyse > Measure.
5. A numerical value is displayed in the results window.
6. Fill the area measured to make it easier to see where the area has been previously measured (Click Edit > Fill, or Edit > Draw).

Tips
Zoom in on the image with the magnifying glass icon. This will help to measure a small area and it doesn’t influence the magnification of the measuring area.
Chapter 3

Sero
tonin transporter changes in AD

Sero
tonin fibre sprouting and increase in serotonin transporter
immunoreactivity in the CA1 area of hippocampus in a
triple transgenic mouse model of Alzheimer’s disease.

3.1. ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease that deteriorates cognitive functions and associated brain regions such as the hippocampus, being the primary cause of dementia. Serotonin (5-HT) is widely present in the hippocampus, being an important neurotransmitter involved in learning and memory. Although recent evidence suggest alterations in 5-HT neurotransmission in AD, it is not clear how hippocampal 5-HT innervations are modified. In the chapter 3 of this thesis, the hippocampal 5-HT innervations was studied by analysing: (i) the expression, density and distribution of serotonin transporter immunoreactive (SERT-IR) fibres, (ii) the specific morphological characteristics of SERT-IR fibres and their relation to β-amyloid (Aβ) plaques and finally (iii) the total number of 5-HT neurones within the dorsal (DR) and median raphe (MR) nuclei in a triple transgenic (3xTg-AD) mouse model of AD. Quantitative light microscopy immunohistochemistry was used to compare 3xTg-AD and non-transgenic (non-Tg) mice of different ages (3, 6, 9, 12 and 18 months). 3xTg-AD mice showed a significant increase in SERT-IR fibre density within the hippocampus in a subfield, strata and age specific manner. The increase in SERT-IR fibres was specific to the CA1 stratum lacunosum moleculare. Increase in SERT-IR fibre density in 3xTg-AD mice was observed at 3 months (by 61%) and at 18 months (by 74%). However, no changes were found in the total number of raphe 5-HT neurones at any examined ages. These results indicate that the 3xTg-AD mouse model of AD display increased SERT-IR fibres sprouting, which may account for imbalanced serotonergic neurotransmission associated and/or related to cognitive impairment associated with AD.
3.2. INTRODUCTION

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative disease impairing cognition and memory (Braak et al. 1999; Garcia-Alloza et al. 2005). AD neuropathological hallmarks are β-amyloid (Aβ) plaques, neurofibrillary tangles (NFTs), neuronal death, synaptic loss and impaired synaptic plasticity (Braak et al. 1999; Scheff et al. 2006). Classically, the loss of cholinergic (ACh) neurones and the decrease in choline acetyltransferase (ChAT) activity were linked with AD (Birks & Melzer 2000). However, drugs enhancing the ACh system have modest efficacy, suggesting the involvement of other neurotransmitters (Birks & Melzer 2000) (see also chapter 1, section 1.1.1.6.).

Serotonin (5-HT) plays a critical role in cognition and memory (Schmitt et al. 2006; Evers et al. 2007). The dorsal (DR) and median (MR) raphe nuclei, which contain the majority of 5-HT neurones, widely project throughout the central nervous system (CNS) including the hippocampus (Vertes et al. 1999). As described in the introduction chapter (chapter 1, section 1.3.4.3.), 5-HT axons display different fibre morphologies depending on their nuclei of origin and projection sites, for review see (Kohler et al. 1982; Kosofsky & Molliver 1987; Molliver 1987; Mulligan & Tork 1988; Hensler 2006). 5-HT axons arising from the DR nucleus are represented by fine fibres (FF) with small fusiform or granular varicosities that are regularly spaced (Kosofsky & Molliver 1987). The MR nucleus gives rise to two different types of 5-HT axons that are either straight, non-varicose axons (stem axons, SA) or thick fibres with large and spherical varicosities that are irregularly spaced (known as beaded fibres, BF) (Kosofsky & Molliver 1987; Molliver 1987; Mulligan & Tork 1988; Bjarkam et al. 2005; Keuker et al. 2005; Hensler 2006).

Increasing evidence emphasises the involvement of 5-HT in AD (Tohgi et al. 1995; Meltzer et al. 1998a; Lai et al. 2002; Mowla et al. 2007; Truchot et al. 2007). AD patients have a 5-HT deficit and treatments with selective serotonin re-uptake inhibitors (SSRIs) or specific 5-HT receptor ligands improve cognitive functions (Porter et al. 2000; Mowla et al. 2007; Mossello et al. 2008; Terry et al. 2008). AD patients also show altered levels of 5-HT metabolite and 5-HT receptors within the cortex and the hippocampus (Meltzer et al. 1998a; Lai et al. 2002; Garcia-Alloza et
al. 2005; Kepe et al. 2006; Lorke et al. 2006; Bowen et al. 2008; Hasselbalch et al. 2008) and a decrease in 5-HT neurones and projections (Chen et al. 2000a; Thomas et al. 2006), which correlate with cognitive and behavioural alterations associated with AD (Lai et al. 2002; Garcia-Alloza et al. 2005). However, these studies are prone to misinterpretation due to contradictory results. Sprouting of 5-HT and non-5-HT fibres as well as increased 5-HT activity is observed following neurotoxic lesions and Aβ accumulation in rats and the APP23 transgenic mouse model of AD within the hippocampus and the cortex (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001; Verdurand et al. 2011). In contrast, the APP
swe/PS1ΔE9 transgenic mouse model of AD showed 5-HT fibre degeneration (Liu et al. 2008). Hence, the exact changes and alterations in 5-HT transmission associated with AD require further study.

Therefore, the use of animal models reproducing a complete AD pathology is fundamental for understanding 5-HT involvement in AD and the development of potential therapies (Rodríguez et al. 2008; Rodríguez et al. 2009a; Rodríguez et al. 2009b). Thus, by studying changes in hippocampal serotonergic innervations and raphe 5-HT neurones this chapter is aimed to determine the exact 5-HT alterations throughout AD progression using the triple transgenic (3xTg-AD) mouse model of AD, which expresses both Aβ plaques and NFTs with a spatio-temporal distribution that resembles AD brains and exhibits age-related cognition deficits (Oddo et al. 2003a; Oddo et al. 2003b; Clinton et al. 2007; Frazer et al. 2008) (see also chapter 1, section 1.2.3.).
3.3. MATERIAL AND METHODS

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

3.3.1. Animals
The procedures for generating 3xTg-AD mice have been described previously (Oddo et al. 2003a; Oddo et al. 2003b; Frazer et al. 2008). Briefly, human cDNA harbouring the Swedish APP mutation (KM670/671NL) and the human P301L four repeats mutated tau, were co-microinjected into a single-cell embryo of a homozygous PS1M146V knock-in mouse. The background of the PS1 knock-in mice is a hybrid 129/C57BL6. The non-transgenic (non-Tg) control mice used were from the same strain and genetic background as the PS1 knock-in mice, but they express the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg control mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12h light-dark cycles with free access to food and water.

3.3.2. Fixation and Tissue Processing
Male 3xTg-AD and their respective non-Tg controls were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg) at different ages (3, 6, 9, 12 and 18 months, n = 4 – 11). Mice were perfused through the aortic arch with 3.75% acrolein (25mls, TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, Gillingham, UK) and 0.1M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75mls). Brains were then removed and cut into 4 – 5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodríguez et al. 2008; Rodríguez et al. 2009a). The brain sections were post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1M PB, pH 7.4. Coronal sections of the brain were cut into 40 – 50μm thick slices using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at
pH 7.4. Coronal brain sections at levels -1.58 mm/-2.46 mm (hippocampus) and -4.36 mm/-4.96 mm (raphe nuclei) posterior to bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004).

3.3.3. Antibodies
A polyclonal rabbit antibody against a peptide sequence corresponding to amino acids 602 – 622 of rat 5-HT transporter (Immunostar, Hudson, WI, USA) was used for determination of serotonergic fibres in the hippocampus. 5-HT neurones in the raphe nuclei were studied using a polyclonal rabbit antibody antiserum generated against 5-HT (Immunostar, Hudson, WI, USA). In addition, a monoclonal mouse antibody against amino acid residues 1 – 16 of beta amyloid (Aβ, Covance, Emeryville, CA, USA) was used to detect Aβ plagues. The specificity of the antibodies has been reported previously using immunohistochemistry (Mamounas et al. 2000) and western blots (Albright et al. 2007). To determine the specificity of the antibodies adsorption controls were done using SERT and 5-HT peptides, respectively, which resulted in total absence of target labelling. Furthermore, omission of primary and/or secondary antibodies also showed no immunoreactivity (refer to material and methods chapter (chapter 2, Fig. 2.2 and Fig. 2.3) for negative control images of SERT and 5-HT immunoreactivity).

3.3.4. Immunohistochemistry
The selected brain sections were incubated for 30 minutes in 30% methanol in 0.1M PB and 3% hydrogen peroxide (H₂O₂, Sigma, UK). Brain sections were then rinsed with 0.1M PB for 5 minutes and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. The sections were then washed with PB profusely before rinsing in 0.1M Trizma base saline (TS) for 10 minutes. Brain sections were then incubated in 0.5% albumin bovine serum (BSA, Sigma, UK) in 0.1M TS and 0.25% Triton (Sigma, UK, x 100) for 30 minutes. Sections were then incubated for 48 hours at room temperature in primary antibody (rabbit anti-SERT, 1:2500 and rabbit anti-5-HT, 1:5000, Immunostar, Hudson, WI, USA). Brain sections were rinsed in 0.1M TS for 30 minutes and incubated in 1:400 dilutions of biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK) for 1 hour at room temperature. Sections were rinsed with 0.1M TS for 30
minutes followed by incubation for 30 minutes in avidin-biotin peroxidase complex (ABC, Vetor Laboratories Ltd., Peterborough, UK). The peroxidase reaction product was visualised by incubation in a solution containing 0.022% of 3,3’-diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003% H₂O₂ for 6 minutes, as described previously (Rodríguez et al. 2008; Rodríguez et al. 2009a). The reaction was stopped by rinsing the brain sections in 0.1M TS for 6 minutes followed by 0.1M PB for 15 minutes. Brain sections were permanently mounted onto gelatinised slides and allowed to dry overnight. Dried brain sections were then dehydrated in ascending concentrations of ethanol (50, 70, 80, 90, 95 and 100%) and finally in xylene. Coverslips were applied using Entellan (Merck KGaA, Germany) and slides were left to dry overnight.

For the detection and determination of serotonin transporter immunoreactive (SERT-IR) fibres and its relationship with Aβ senile plaques, a dual indirect immunofluorescence labelling was used. Brain sections were incubated for 48 hours at room temperature in primary antibody cocktail containing: (1) mouse anti-beta amyloid monoclonal antibody (Aβ, 1:2000, Covance, USA) and (2) rabbit anti-SERT polyclonal antibody (1:2500, Immunostar, Hudson, WI, USA) simultaneously. Subsequently, Aβ and SERT-IR fibres were detected in a sequential manner on the same brain sections by incubation with Rhodamine (TRITC)-conjugated goat anti-mouse and (FITC)-conjugated goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, brain sections were rinsed with 0.1M TS for 30 minutes and permanently mounted in an aqueous medium (Vectashield, Vetor Laboratories Ltd., Peterborough, UK).
3.3.5. Optical Density (OD) Measurement

Using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA), the expression and the density of SERT-IR fibres were analysed by measuring their optical density (OD), as described previously (Cordero et al. 2005) (see material and method chapter (chapter 2, section 2.4.1. for detailed description of OD measurement). Briefly, to exclude any experimental errors and/or bias, all images were taken at a constant light intensity. Optical filters (which adjust light intensity) were used to ensure the specificity of the signal recorded by the camera. The OD was calculated from a relative scale of intensity ranging from 0 – 255, with a measurement of 255 corresponding to the area with very low SERT-IR fibres and 0 corresponding to the densest area of labelling. The calibration density was kept constant for measuring all brain sections to avoid experimental variances. Non-specific OD in brain sections was measured from the corpus callosum (CC). SERT-IR fibre density of the complete CA1 subfield of the hippocampus and its different layers (pyramidal cell layer (PCL), stratum oriens (SO), stratum radiatum (S.Rad) and stratum lacunosum moleculare (S.Mol), were measured individually. In the case of CA3, due to its differential organisation and the presence of an additional stratum (the stratum lucidum, where the mossy fibres from the dentate gyrus arrive), SERT-IR fibre density was also measured independently. Similarly, SERT-IR fibre density of the dentate gyrus and its different layers (granule cell layer (GCL), molecular layer (ML) and hilus) were measured individually (see chapter 2, Fig. 2.4). To analyse the change in SERT-IR fibre density against constant control, 255 was divided by control region (corpus callosum) and the obtained factor was multiplied by the region of interest in every given section. Inverse optical density was obtained by subtracting from the obtained background level (255). Measurements of mean density were taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each brain section. The results are shown as inverse SERT-IR fibre density (IOD/pixel).

To determine SERT-IR fibre density in relation to Aβ plaques, confocal scanning microscopy (Leica SP5 upright) was used, recording section levels at every 0.2μm throughout the thickness of the section. The staining was observed throughout the depth of the brain section (40μm) recording optical sections at every 0.2μm (refer to
material and methods chapter (chapter 2, Fig. 2.9) for confocal images of SERT fibre immunoreactivity). No differences were observed in SERT antibody penetration (Pickel et al. 1992) and immunoreactivity throughout the thickness of the brain section between 3xTg-AD mice and the non-Tg controls; hence the changes in OD were used as measure of increased SERT-IR fibre density. Parallel confocal planes of the green channel (due to fluorescence FITC-conjugated secondary antibody) from the entire thickness of the section (40μm) were superimposed and SERT-IR fibre density was measured using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA).

To investigate SERT-IR fibres density in relation with Aβ plaques in 3xTg-AD mice, the OD measurement was analysed and compared in a 20 x 20μm square region of interest adjacent to Aβ plaque (around) and an identical region 20μm distant away from the Aβ plaque (away) in the same section (Spires et al. 2005) (see chapter 3 supplementary Fig. 1). Using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA), the mean gray value was calculated for each region of interest and the intensity of SERT-IR fibres were measured around and away from Aβ plaques independently, as previously described (Spires et al. 2005). The mean gray value of SERT-IR fibre intensity was obtained in each square region of 20 x 20μm adjacent to Aβ plaques and another 20 x 20μm region 20μm away from the same Aβ plaque in the same image. The 20μm distance between the around and the away from the Aβ plaques was chosen because previous study in the Tg2576 APP transgenic mouse model of AD had reported Aβ plaques-associated neurodegeneration including disrupted neurite process and reduced dendritic spine density within the 20μm distance from Aβ plaque edges (Spires et al. 2005) (see also chapter 3 supplementary Fig. 1). Therefore, all SERT-IR fibre within 20μm from Aβ plaque borders were considered as plaque-associated (around), whilst SERT-IR fibres with distant of more that 20μm were classified as not plaque-associated (away), as described previously (Spires et al. 2005).

3.3.6. Morphological analysis of hippocampal SERT fibres
A detailed morphological analysis was carried out in order to determine the different types of serotonergic fibres present within the CA1 subfield of the hippocampus in relation to their nuclei of origin. For each condition, at least 3 brain
sections from 12 animals were examined for quantification of SERT-IR fibres. Images were taken using Nikon Eclipse 80i Microscope with a 100x oil immersion objective. All visible SERT-IR fibres were analysed in 15 random images within the stratum lacunosum moleculare of CA1, each representing area of 10,800µm² with a total analysed surface area of 162,000µm². Therefore, the results are expressed as area density S_V (# of fibres/ mm²) (Rodríguez et al. 2008; Rodríguez et al. 2009a). SERT-IR fibres were classified as beaded fibres (BF), fine fibres (FF) and stem axons (SA) according to their origin from the raphe nuclei and their morphological features, as already described (Bjarkam et al. 2005; Keuker et al. 2005; Hensler 2006). BF, thick axons with irregularly spaced varicosities; FF, thin axons with typically small and evenly distributed varicosities; SA, large thick straight axons with no varicosities (Bjarkam et al. 2005; Keuker et al. 2005; Hensler 2006) (see also Fig. 3.3).

3.3.7. Cell count of 5-HT neurones in the raphe nuclei

To determine whether the change in hippocampal SERT-IR fibre density might be due to the loss of 5-HT neurones, the total number of 5-HT immunoreactive (5-HT-IR) neurones was estimated in the dorsal (DR) and the median raphe (MR) nuclei of 3xTg-AD mice and non-Tg controls. The areas analysed for 5-HT cell count included the dorsal raphe dorsal (DRD), the dorsal raphe ventral (DRV), the dorsal raphe interfascicular (DRI) and the dorsal raphe ventrolateral (DRVL) part as well as the MR and the para-median raphe (PMR) nuclei. The boundaries of areas in which 5-HT-positive neurones were to be counted were clearly delineated; thus, counts were reproducible and counting 5-HT stained neurones in every third brain section constituted a true random sample (Vertes & Crane 1997). All 5-HT-IR neurones were intensely labelled against light background, which made them easy to identify with equivalent chance of being counted (Vertes & Crane 1997). The main source of error in using this calculation is the potential multiple counting of the same neurones in more than one brain section, as suggested previously (Vertes & Crane 1997). However, in this case, one has to consider that the maximum cell diameter of 5-HT neurones counted was approximately 25 – 30µm, and every third 40µm section was 120µm distant from the adjacent one, making multiple counting of the same 5-HT neurone in adjacent brain sections extremely unlikely, as described previously (Vertes & Crane 1997). To obtain a systemic random
sampling of 5-HT-IR neurones, a sampling grid consisting of counting frame was positioned over the DR and the MR nuclei on each brain section. The number of 5-HT-IR neurones was determined blindly by a single observer using a 10 x 10 mm graticule. All visible 5-HT-IR neurones were counted in every third 40µm thickness coronal section throughout rostrocaudal extent of the different sub-divisions of the dorsal raphe nucleus corresponding to bregma -4.36/-4.96 mm (Paxinos & Franklin 2004). To define DR and MR as well as their sub-nuclei boundaries, adjacent brain sections were counterstained with toluidine blue (see chapter 2, Fig. 2.8). The estimated total 5-HT-IR neurones within different raphe nuclei were calculated according to Konigsmark equation (Konigsmark 1970), as described previously (Vertes & Crane 1997) (refer to supplementary section of the material and methods chapter (chapter 2, section 2.S.6.) for detailed description of the Konigsmark equation):

\[
N_t \div n_s = V_t \div v_s
\]

Where \(N_t\) (total count), \(n_s\) (sample count), \(V_t\) (total volume, range from 0.37mm\(^3\) – 0.72mm\(^3\)) and \(v_s\) (sample volume).

### 3.3.8. Statistical analysis

2 way analysis of variance (ANOVA) was used to determine changes in SERT-IR fibre density and 5-HT neurones at different ages followed by the Tukey post-hoc test. Un-paired t-test was used to determine changes in SERT-IR fibres associated with Aβ plaques. Significance was accepted at \(p \leq 0.05\). The data were analysed using Minitab (Minitab 15 Software). Results are expressed as mean ± standard errors of the mean (S.E.M.).
3.4. RESULTS

3.4.1. Distribution of hippocampal SERT fibres

In both, 3xTg-AD and non-Tg control mice SERT-IR fibres were heterogeneously distributed throughout the hippocampal formation (Fig. 3.1). SERT-IR fibres appeared mainly as fine and thick processes with numerous varicosities, which are characteristic of axonal profiles (Fig. 3.1E and 3.1F). The highest density of SERT-IR fibres were observed within the stratum lacunosum moleculare of CA1. Thick axons with large circular and irregularly spaced varicosity that are classified as beaded fibres (BF), accounted for 94% of total SERT-IR fibres in stratum lacunosum moleculare (Fig. 3.3B). Fine fibres (FF) accounted for less than 5% of total SERT-IR fibres, whereas large thick straight stem axons (SA) without varicosities were rare and accounted for the remaining percentage (Fig. 3.3B, 3.3D and 3.3F). This pattern of distribution of BF, FF and SA was consistent within all layers of the hippocampus. Strata radiatum and oriens exhibited moderate expression, whilst the lowest density of SERT-IR fibres was determined in the pyramidal cell layer (Fig. 3.1). In the dentate gyrus (DG), it was possible to observe a gradient in the density of SERT-IR fibres that was increasing from the inner to the outer molecular layer. However, this expression was not as marked as in the different CA subfields (Fig. 3.1A and 3.1B). The granule cell layer of the DG and the hilus showed a low to moderate SERT-IR fibre density with the majority of fibres exhibiting morphology of BFs (Fig. 3.1A and 3.1B).

3.4.2. Temporal changes of SERT-IR fibre density in the 3xTg-AD mouse model of AD

The global analysis showed no general changes in SERT-IR fibre density in the DG and CA subfields (Fig. 3.2A – 3.2C), except for CA1, which showed a clear but non-significant increase at 3 and 18 months ($F_{4, 77} = 4.451, 34\%, p = 0.083$ for 3 months and $34\%, p = 0.081$ for 18 months, respectively; Fig. 3.2C). A detailed quantitative analysis of individual CA1 layers revealed that this increase was due to a specific and significant increase in SERT-IR fibre density within the CA1 stratum lacunosum moleculare (Fig. 3.1B, 3.1D, 3.1F and 3.2D), which also exhibited the highest density of SERT-IR fibres in non-Tg control mice (Fig. 3.1A, 3.1C and 3.1E). Such increase in SERT-IR fibre density was also restricted to 3 and 18
months of age in 3xTg-AD mice compared to age-matched non-Tg controls (F_{4, 77} = 20.65, 61%, p = 0.0394 for 3 months and 74%, p = 0.0347 for 18 months, respectively; Fig. 3.2D). The CA1 stratum oriens and stratum radiatum also showed an evident but non-significant increase in SERT-IR fibres at 3 and 18 months (F_{4, 77} = 0.47, 30%, p = 0.77 for 3 months, 13%, p = 1.0 for 18 months in the stratum oriens and F_{4, 77} = 0.94, 19%, p = 0.98, for 3 months, 26%, p = 0.95 for 18 months in the stratum radiatum, respectively; see chapter 3 supplementary Fig. 2). Furthermore, neither in the DG nor in the CA3 subfield significant changes in SERT-IR fibres densities were observed (F_{4, 77} = 0.63, p = 0.997, p = 1.0, F_{4, 77} = 3.13, p = 0.945 and p = 1.0, Fig. 3.2A and 3.2B).
Figure 3.1. Brightfield micrographs showing SERT-IR fibres within the dorsal hippocampus of 3 months non-Tg control (A, C, E) and 3xTg-AD mice (B, D, F). Scale bars: A and B = 500µm, C and D = 100µm, E and F = 20µm. **Key:** DG: dentate gyrus, S.Rad: stratum radiatum, S.Mol: stratum lacunosum moleculare, f: hippocampal fissure. Adsorption control using SERT-specific peptide resulted in total absence of SERT-IR fibres (see Chapter 2, Fig. 2.2).
Figure 3.2. Bar graphs showing the age effect on SERT-IR fibre density within different hippocampal subfields at 3, 6, 9, 12 and 18 months between non-Tg control and 3xTg-AD group, dentate gyrus (A), CA3 (B), CA1 (C) and CA1 stratum lacunosum moleculare (D). Bars represent mean ± S.E.M. (n = 9 – 11). * p<0.05. 2 way ANOVA followed by Tukey post-hoc test. Key: IOD: inverse optical density.
3.4.3. Beaded and fine fibres increase in the 3xTg-AD mouse model of AD
At both 3 and 18 months of age significant increase in the density of BFs were found in 3xTg-AD mice compared to age-matched non-Tg controls ($F_{1, 180} = 50.85$, 18%, $p = 0.0309$ for 3 months and 43%, $p < 0.001$ for 18 months, respectively; Fig. 3.3A and 3.3B). Conversely, FFs density increased only at 18 months of age ($F_{1, 180} = 5.70$, 155%, $p = 0.0440$, Fig. 3.3C and 3.3D) in 3xTg-AD mice. No significant changes were observed in the density of SAs in either age group (Fig. 3.3E and 3.3F). These changes were not accompanied by the appearance of any age-related degenerative profiles such as swollen and tortuous varicosities, abnormal thickened axons, ballooned or spherical axon terminals (Phinney et al. 1999; Spires et al. 2005) (see also Fig. 3.3B, 3.3D and 3.3F).
Figure 3.3. Bar graphs showing the distribution of the different SERT-IR fibres within stratum lacunosum moleculare of CA1 subfield of the hippocampus (A, C and E). Bars represent mean ± S.E.M., * = p<0.05, *** = p<0.001 compared to age-matched control, un-paired t-test. Brightfield micrographs (B, D and F) illustrating the different fibre characteristics. Scale bars: B, D and F = 5µm. Key: BF: beaded fibres, FF: fine fibres, SA: stem axons.

3.4.4. The number of 5-HT neurones remains constant throughout ageing and in the 3xTg-AD mouse model of AD

5-HT immunoreactive (5-HT-IR) neurones were distributed throughout the different sub-divisions of both the DR (Fig. 3.4A – 3.4E) and the MR (Fig. 3.4F) nuclei. 5-HT-IR somatodendritic profiles were characterised by small rounded cell
bodies with sparse dendritic arborisations (Fig. 3.4C and 3.4D). The highest number of 5-HT-IR neurones was observed in the DR (Fig. 3.4A – 3.4F) nucleus. The 5-HT-IR neuronal population was also detected within both the MR and the PMR nuclei (Fig. 3.4F). The total number and the distribution of 5-HT-IR neurones showed no significant difference in either the DR and the MR nuclei between the 3xTg-AD and non-Tg control mice in any examined age group (F 4, 32 = 0.091, p = 0.767, F 4, 32 = 2.92, p = 0.097 respectively; Fig. 3.4E and 3.4F).

3.4.5. Increased SERT-IR fibre density is associated with Aβ plaques

Previous studies have shown that the extracellular accumulation of Aβ plaques within the hippocampus of 3xTg-AD mice starts between 9 and 12 months and increases with age being maximal at 18 months of age (Mastrangelo & Bowers 2008; Rodríguez et al. 2008). In 3xTg-AD mice, Aβ plaques are primarily localised in the CA1 subfield and more specifically within the stratum lacunosum moleculare (Fig. 3.5A). Quantitative analysis using immunofluorescence and confocal microscopy revealed a significant increase in overall SERT-IR fibre density in the CA1 stratum lacunosum moleculare of 18 months old 3xTg-AD mice compared to non-Tg control mice independent of the presence of Aβ plaques (measured by taking the average OD value of SERT-IR fibres around and away from Aβ plaques in 3xTg-AD mice compared to non-Tg control animals) (27.25%, p = 0.005, Fig. 3.5C). When analysing SERT-IR fibre density in relation to Aβ plaques, 3xTg-AD mice showed a significant increase in SERT-IR fibre density away from Aβ plaques compared to non-Tg control mouse (20.79%, p = 0.015, Fig. 3.5C). However, increased SERT-IR fibre density was much more marked in the vicinity of Aβ plaques compared to non-Tg control mice (33.67%, p = 0.003, Fig. 3.5B and 3.5C). Furthermore, in 3xTg-AD mice, SERT-IR fibre density adjacent to Aβ plaques was also significantly higher compared to SERT-IR fibre density away from Aβ plaques (10.7%, p = 0.029, Fig. 3.5B and 3.5C). No distorted and potentially degenerative SERT-IR fibres were observed either around or away from Aβ plaques (Fig. 3.5B).
Figure 3.4. Brightfield micrographs showing the distribution of serotonin-IR neurones within the dorsal and the median raphe nuclei of non-Tg control (A, C) and 3xTg-AD (B, D) at 18 months of age. Bar graphs showing the total number of 5-HT-IR neurones in the dorsal (E) and the median (F) raphe nuclei between non-Tg control and 3xTg-AD group. Bars represent mean ± S.E.M. (n = 4 – 5). Scale bars: A and B = 250µm, C and D = 75µm. Key: Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part. Adsorption control using serotonin-specific peptide resulted in total absence of 5-HT labelled neurones (see Chapter 2, Fig. 2.3).
Figure 3.5. Confocal micrographs showing the presence and distribution of Aβ plaques within the different subfields of the hippocampus (A). One can see that there is a major Aβ plaque accumulation within the S.Mo compared to the other areas (A). Dual confocal micrograph showing SERT-IR fibre sprouting adjacent to Aβ plaques (B). Scale bars: A = 100μm, B = 20μm (inset = 10 μm). Key: S.Mol: stratum lacunosum moleculare, S.Rad: stratum radiatum, DG: dentate gyrus. Bar graph showing the OD of SERT-IR fibres at 18 months 3xTg-AD compared to non-Tg control animals and the difference in SERT-IR fibre densities in 3xTg-AD animals between around and away from Aβ plaques (C). Bars represent mean ± S.E.M. (n = 4 – 5). * =p<0.05, ** =p<0.01 compared to non-Tg control, un-paired t-test and # =p<0.05 compared to SERT-IR fibre density away from Aβ plaques, paired t-test.
3.5. DISCUSSION

The main finding from chapter 3 is that the 3xTg-AD mouse model of AD displays age-dependent changes in serotonergic innervations in the hippocampus. Increased SERT-IR fibre density in 3xTg-AD mice starts at 3 months and does not appear again when compared to controls till 18 months of age. This increase in SERT-IR fibre density is region-specific being restricted to the stratum lacunosum moleculare of CA1, which in normal conditions is also known to host the highest density of SERT within the hippocampal formation (Vertes 1991; Vertes et al. 1999; Keuker et al. 2005). A detailed morphological analysis of SERT-IR fibres revealed no degenerative profiles of serotonergic axons (swollen and tortuous varicosities or abnormal thickened and ballooned spherical axon terminals) (Phinney et al. 1999; Spires et al. 2005) in both the 3xTg-AD and the non-Tg controls mice at any examined age. These results suggest that the observed increase in SERT-IR fibre density in 3xTg-AD mice is due to a specific outgrowth from undamaged axons, as described previously (Harkany et al. 2001) from both the DR and the MR nuclei because of sprouting of BFs (from the MR) and FFs (from the DR) (Bjarkam et al. 2005; Hensler 2006). This is specifically due to sprouting of SERT-IR fibres because (i) the increase in SERT-IR fibre density was not associated with an increase in the total number of 5-HT neurones in the DR and the MR nuclei (Fig. 3.4), which project to the hippocampus and also because (ii) there were increases in both SERT-IR fibre density (Fig. 3.1 and 3.2) as well as an increase in their area density ($S_v$, # of fibres per given area) of both BF and FFs in the hippocampus (Fig. 3.3).

These results are in broad agreement with previous studies, reporting that brain damage induced serotonergic fibres sprouting in different regions of the rodent brain, including the hippocampus (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001). This sprouting can be triggered during several pathological events. First, a direct damage to either 5-HT or non-5-HT fibres (induced by neurotoxins such as ibotenic acid and NMDA) can stimulate homotypic and heterotypic sprouting in the forebrain, the striatum and the hippocampus (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001). Second, it could be triggered by intracerebral injection of
Aβ_{1-42} (Harkany et al. 2001), which is the primary neurotoxin in AD pathology (Moreno et al. 2007). In fact, Aβ intracellular accumulation in 3xTg-AD mice initiates at an early age (3 months) that may induce damage to the affected neurones (Moreno et al. 2007; Rodríguez et al. 2008). Deposition of extracellular Aβ plaques in the 3x-Tg-AD mouse model of AD appears between 9 and 12 months and then progressively increases with advanced age, mainly targeting the CA1 hippocampal subfield (Oddo et al. 2003b; Rodríguez et al. 2008; Rodríguez et al. 2009a; Rodríguez et al. 2009b). Such build-up of extracellular plaques at a later age (18 months) may induce neurotoxic effects resulting in neuronal damage that in turn may also stimulate the observed sprouting of serotonergic fibres in 3xTg-AD mice (Fig 3.6A and 3.6B), which is in agreement with recent findings showing rapid serotonergic fibres sprouting within the basal forebrain, the cerebral cortex and occasionally the hippocampus as a trophic response to various agents such as S-100β protein (Zhou et al. 1995; Whitaker-Azmitia et al. 1997; Harkany et al. 2000b; Harkany et al. 2001).

Previous studies have suggested that increased appearance of BFs may be the start of a degenerative process of serotonergic fibres (Harkany et al. 2001). Contrary to the above suggestion, in this study the increase in BFs was observed at an early age (3 months) in 3xTg-AD mice without any pathological element presence and no apparent degeneration of fibres at older ages (between 6 – 18 months of age, Fig. 3.3A). In addition, no clear characteristics of degenerative profiles such as swollen and tortuous varicosities, abnormal thickened, ballooned and spherical terminal varicosities were observed in SERT-IR fibres at any examined ages, which are typically associated with degenerative serotonergic fibres (van Luijtenaar et al. 1988; van Luijtelaar et al. 1989; Steinbusch et al. 1990). However, one can not exclude that the pronounced increase in BF density at 18 months of age may reflect an early event before degeneration of SERT-IR fibres at more advanced age (24 months and beyond). Further morphological analysis of SERT-IR fibres at longer survival point (> 18 months of age) are required to confirm this possibility, which will be carried out at a later time in our laboratory.

Lack of SERT-IR fibre sprouting at 9 and 12 months in 3xTg-AD mice may be due to lower levels of extracellular Aβ plaque accumulation compared to 18 months (in
the CA1 subfield of the hippocampus). In agreement with this phenomenon, preliminary results from our laboratory suggest that SERT-IR fibre sprouting is further increased within the hippocampus at 24 months in 3xTg-AD mice, parallel to increased deposition of large extracellular Aβ plaques (H.N.N & J.J.R.A, unpublished observation). In addition, the finding of early (3 months) SERT fibre sprouting may indicate that this process could even start with the accumulation of intracellular Aβ, as we have previously shown (Rodríguez et al. 2008) (see also chapter 6 for detailed discussion and consideration of intraneuronal Aβ in inducing SERT-IR fibre sprouting).

Interestingly, injection of Aβ<sub>1-40</sub> peptide into the rat hippocampus triggered a transient increase in astrocytic 5-HT<sub>1A</sub> receptors that mediate S-100β release form astrocytes (Whitaker-Azmitia et al. 1990; Verdurand et al. 2011). A similar mechanism might be present in 3xTg-AD mice, where the evident intraneuronal Aβ aggregate at 3 months of age may induce a transient increase in astrocytic 5-HT<sub>1A</sub> receptors and in consequent promote S-100β protein release from astrocytes (see chapter 6, Fig. 6.1). The liberated S-100β protein acts as a neurotrophic factor that may be responsible for SERT-IR fibre sprouting. These data suggest that intraneuronal Aβ aggregates play a central role in S-100β release and SERT-IR fibre sprouting by stimulating an increase in astrocytic 5-HT<sub>1A</sub> receptor expression and functionality in 3xTg-AD mice. More recent results from our laboratory showed a decrease in intraneuronal Aβ density following high dietary intake of 5-HT precursor l-tryptophan (TrP), but no direct effect on SERT-IR fibre sprouting in 3xTg-AD mice (chapter 5, Fig. 5.3 and Fig. 5.4). However, it is important to note that intraneuronal Aβ density was only reduced by 17% following the chronic (1 month) increase in dietary TrP intake (chapter 5, Fig. 5.3). To prevent SERT-IR fibre sprouting in 3xTg-AD mice, further decrease in the density of intraneuronal Aβ may be needed. This may be achieved via longer administration of high TrP diet (see also chapter 6 for further discussion).

In line with involvement of S-100β in early SERT-IR fibre sprouting, latest findings from our laboratory showed a transient increase in the numerical density (N<sub>v</sub>, #/mm<sup>3</sup>) of S-100β-expressing astrocytes within the hippocampus in 3xTg-AD mice at 3 months of age (see chapter 3 supplementary Fig. 3). Increased S-100β-
expressing astrocytes were particularly more evident in the CA1 stratum lacunosum moleculare of CA1 subfield of the hippocampus, which also displayed pronounced increase in SERT-IR fibre density (chapter 3 supplementary Fig. 3). These findings are in agreement with a recent study in the transgenic mice over-expressing S-100β that also reported an early increase in SERT-IR fibres and S-100β-positive astrocytes in the dentate gyrus of the hippocampus at 10 weeks of age (Shapiro et al. 2010). In addition, S-100β over-expressing mice exhibit early increase in the maturation of hippocampal dendrite (1 month) (Whitaker-Azmitia et al. 1997) and impairment in hippocampus-dependent spatial memory at 3 months of age (Gerlai & Roder 1996) as happens also in 3xTg-AD mice (Oddo et al. 2003b; Clinton et al. 2007; Frazer et al. 2008).

Interestingly, the increased S-100β-expressing astrocytes are only present at 3 months of age, but not at 12, 18 and 24 months of age, suggesting that an alternative mechanism may be responsible for the observed SERT-IR sprouting at 18 months of age in 3xTg-AD mice (chapter 3 supplementary Fig. 3). Further studies are required to address the precise mechanism responsible for transient increase in S-100β-expressing astrocytes at 3 months of age.

Alternatively the early (3 months) increase in SERT-IR fibres could be due to over-expression of APP and tau mutations in 3xTg-AD mice (Oddo et al. 2003b; Clinton et al. 2007; Frazer et al. 2008), which could have an effect on the normal 5-HT system development. Serotonergic neurones emerge early during development of the brain and the spinal cord (Whitaker-Azmitia 2001). 5-HT neurones are detected between the embryonic days 11 – 12 (E11 – 12) in mice and rats (Whitaker-Azmitia 2001). The expression of SERT protein is initially detected at E12 (Zhou et al. 2000) that is then increased during the migration of 5-HT axons from the raphe nuclei between E20 and postnatal day 21 (P21) (Zhou et al. 2000). Such gradual increase in SERT expression is followed by significant decrease at P28 that is then maintained at this decreased level till adulthood (Whitaker-Azmitia 2001). It is possible that the early (3 months) increase in SERT-IR fibres in 3xTg-AD mice (which as justified can appear even earlier) may be due to an extended increase in the expression and maturation of serotonergic fibres during development and/or a delay in its postnatal down regulation. In support of the latter phenomenon, a recent
study in 3xTg-AD mice has found an increased number of cholinergic (ACh) neurones in the medial septum and diagonal band or Broca at 2 – 3 months of age, which the authors suggested may also be due to over-expression of APP and tau mutations in these mice (Perez et al. 2011). In addition, the APP_{swt}/PS1ΔE9 transgenic mouse model of AD displayed increased level of dopamine metabolite (3,4-ihydroxyphenylacetic acid, DOPAC) at 3 – 6 months compared to 10 – 12 months of age, suggesting a developmental and/or compensatory up-regulation of dopamine neurotransmission in the striatum (Perez et al. 2005). Taken together, these data suggest that over-expression of AD-related transgenes, specifically APP and PS1 can induce an effect on the development of different neurotransmitter (from glutamate to GABA) systems in transgenic mouse models of AD. Nevertheless, further studies during early development are required to uncover the effect of over-expression of AD-related transgenes upon the development of the brain.

The sprouting of serotonergic fibres can also be induced by trophic factors such as the brain derived neurotrophic factor (BDNF), which is highly present in the hippocampus (Luellen et al. 2007). BDNF-induced serotonergic axonal sprouting was demonstrated in the neocortex, the spinal cord and the hippocampus (Bregman et al. 1997; Mamounas et al. 2000; Grider et al. 2005). Increased BDNF level was observed following neurotoxic insults (p-chloroamphetamine lesion), being directly associated with 5-HT sprouting (Bregman et al. 1997; Grider et al. 2005). Transgenic mice with constitutive loss of BDNF expression displayed accelerated loss of serotonergic fibres, which was also associated with reduced extracellular 5-HT level in the hippocampus (Luellen et al. 2007). Furthermore, increased BDNF expression have been reported in different transgenic mouse models of AD including the APP23 (Burbach et al. 2004) and the more recently in 3xTg-AD mice (Rothman et al. 2012). Thus, a possible mechanism for the serotonergic fibres sprouting can be associated with abnormal levels of BDNF (Fig. 3.6B).

Extracellular build-up of plaques also stimulates glial reactivity in multiple regions in AD brains such as the entorhinal cortex, the neocortex and the hippocampus (Rodríguez et al. 2009b; Verdurand et al. 2011) (Fig. 3.6B). Increased release of neurotrophic factors such as S-100β by reactive astrocytes is therefore another
candidate mechanism for observed SERT-IR fibre sprouting in the hippocampal CA1 stratum lacunosum moleculare (Fig. 3.6B). Other studies in our laboratory have shown that astrocytes closely associated to Aβ neuritic plaques exhibit prominent astrogliosis compared to astrocytes located away from Aβ plaques (Olabarria et al. 2010). As shown by excitotoxic lesions, the astrogliosis, which results in the enhanced level of S-100β is also associated with large serotonergic fibres sprouting (Zhou et al. 1995). In addition, increased expression of S-100β has been reported in the temporal lobe and frontal cortex in of AD patients as well as in Down’s syndrome (DS) (Griffin et al. 1989). Furthermore, the gene for S-100β is located on chromosome 21, within the so called obligate region for DS, which also can exhibit AD type neuropathology (Griffin et al. 1989; Whitaker-Azmitia 2001).
Figure 3.6. Schematic cartoon illustrating the normal pattern of serotonergic fibres within the hippocampal CA1 and their relationship with neurones and glial cells (A) as well as the proposed hypothesis driving the observed serotonergic sprouting in AD (B). This sprouting is directly related with the presence of Aβ plaques and trophic and inflammatory factors secreted by neurones, astroglia and microglia, respectively (B). In addition, increased BDNF release from degenerative neurones may also contribute to the proposed serotonergic sprouting in AD.
The Aβ-induced neurotoxicity involves increased activation of glutamatergic system (Brorson et al. 1995; Miguel-Hidalgo et al. 2002) and impairment of calcium homeostasis (Brorson et al. 1995). Increased serotonergic input may help to counteract NMDA-induced neurotoxicity by inhibition of calcium influx and membrane hyperpolarisation, as suggested previously (Harkany et al. 2000b; Harkany et al. 2001). Such effect is mediated by activation of 5-HT1A and 5-HT1B receptors that are highly expressed in the hippocampal formation (Kepe et al. 2006; Peddie et al. 2008b). In support of the above phenomenon, a recent study by Verdurand and colleagues (2011) reported a transient increase in 5-HT neurotransmission and 5-HT1A receptor expression following intrahippocampal infusion of Aβ1-40 (Verdurand et al. 2011), suggesting that Aβ-induced neurotoxicity can trigger an increase in 5-HT system. Therefore, it is hypothesised that the observed sprouting of serotonergic fibres may be an intrinsic protective mechanism in response to Aβ-induced excitotoxic damage, glial response and subsequent release of S-100β from hypertrophic astrocytes and other inflammatory factors from activated microglia (Fig. 3.6B) (see also chapter 6 for further discussion on the role of astrocytes and microglia on SERT-IR fibre sprouting).

Contradictory findings have been reported on 5-HT1A receptor expression in AD brains (chapter 1, Table 1.11). Although few binding studies had reported AD-associated reduction in 5-HT1A receptors, others found no changes (chapter 1, Table 1.11). Interestingly, increased 5-HT1A receptor binding sites have been reported in the frontal cortex of AD brains (Lai et al. 2002) and in patients with mild cognitive impairment (Truchot et al. 2007; Truchot et al. 2008). This transient increase in hippocampal 5-HT1A receptors was also found in rats following injection of Aβ1-40 peptide (Verdurand et al. 2011). Altogether, these studies suggest that 5-HT1A receptors may mediate a potential neuro-protective role following activation by increased 5-HT input in AD. However, currently, there are no specific data on the expression of hippocampal 5-HT1A and 5-HT1B receptors in 3xTg-AD mice as well as their maintenance at advanced ages (18 months) and in particular their association with Aβ plaques. Nevertheless, the high basal levels of both receptors specially the 5-HT1A receptor and their putative role in synaptic plasticity and hippocampal neurogenesis make them a good candidate to play an active role in the cytoarchitectural reconstruction of AD damaged hippocampal
circuitry. Thus the exact and specific signalling mechanisms responsible for possible neuro-protective effects of increased 5-HT input in AD requires further studies; even if they are not the focus of this thesis (see chapter 6 for further discussion and limitations of the proposed neuro-protective role of 5-HT in the 3xTg-AD mouse model of AD).

On the other hand, the expression of APP and Aβ1-42 has been shown to promote neurite outgrowth. This potential neurotrophic effect of APP and Aβ1-42 has been demonstrated previously both in vitro (Salinero et al. 2000) and in vivo (Phinney et al. 1999). Transgenic mice with over expression of mutant APP showed aberrant sprouting of axons in the hippocampus (Phinney et al. 1999). These data suggest that Aβ deposition may be the ultimate cause for triggering 5-HT axonal sprouting, which underlie the observed increase in SERT-IR fibres in the CA1 stratum lacunosum moleculare at 18 months of age when the accumulation of Aβ plaques is high (Oddo et al. 2003b; Rodriguez et al. 2008; Rodríguez et al. 2009b). As mentioned earlier, the CA1 stratum lacunosum moleculare exhibits the highest density of SERT in the hippocampal formation compared to other hippocampal subfields including the stratum radiatum and the stratum oriens (Vertes 1991; Vertes et al. 1999; Keuker et al. 2005). Increased SERT-IR fibre sprouting occurs in BF originated form the MR nucleus that is the major type of serotonergic fibre innervating the hippocampal formation (Vertes 1991; Bjarkam et al. 2005). FF arising from the DR nucleus account for less than 5% of hippocampal SERT-IR fibres (Vertes 1991; Vertes et al. 1999), which also had shown increased sprouting in 3xTg-AD mice at 18 months.

Finally, altered 5-HT neurotransmission is intimately involved in the disturbance of mood disorder including clinical depression (Cannon et al. 2007). PET studies have consistently shown increased SERT density in drug-free depressed patients (Cannon et al. 2007). Depression is also considered as a risk factor for AD development and, in fact, 20 – 50% of AD patients exhibit depressive symptoms (Garcia-Alloza et al. 2005; Mossello et al. 2008). Therefore, increased SERT-IR fibre sprouting results in abnormal 5-HT neurotransmission, which might also induce behavioural alterations in 3xTg-AD mice leading to depression-like alterations. However, deep and specific behavioural tests are required to confirm
the onset and development of these behavioural alterations in 3xTg-AD mice in relation to their AD pathology.

In conclusion, this study demonstrates that 3xTg-AD mice exhibit an abnormal sprouting of serotonergic fibres within the stratum lacunosum moleculare of CA1 at 3 and 18 months. Increased sprouting occurs in different types of serotonergic fibres irrespective of their nuclei of origin. Lack of changes in 5-HT-IR neurones within the raphe nuclei support the heterotypic sprouting of serotonergic neurones within the hippocampus that is induced at the target region by damage to non-5-HT neurones. Altogether, these results suggest that the sprouting of serotonergic fibres in AD might act as a neuroprotective mechanism that defends the nervous system by counteracting Aβ-induced neurotoxicity. This in turn could be directly associated with either an increased re-uptake or enhanced synthesis of the neurotransmitter, which might have an effect in transmitter presence and homeostasis, hence contributing to cognitive deficit associated with AD.
Supplementary Figure 1. Confocal micrographs showing the presence of SERT-IR fibres in the hippocampus of non-Tg control (A) and 3xTg-AD mice (B, C). Optical density measurement was compared in a 20 x 20µm square region of interest adjacent to Aβ plaque (stained red, C) and an identical region 20µm distant from the Aβ plaque (white squares Ar/Aw, B). Note that in non-Tg control animals, SERT-IR fibre density was measured throughout the stratum lacunosum moleculare of the CA1 subfield of the hippocampus. In 3xTg-AD animals, increased SERT-IR fibre density was more evident adjacent to Aβ plaques. Key: Ar: around Aβ plaque, Aw: away from Aβ plaque. Scale bars: 25µm (A – C).
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Supplementary Figure 2. Bar graphs showing the age effect on SERT-IR fibre density within the CA1 stratum oriens (A) and the CA1 stratum radiatum (B) at 3, 6, 9, 12 and 18 months between non-Tg control and 3xTg-AD groups. Bars represent the mean ± S.E.M., (n = 9 – 11), 2 way ANOVA followed by Tukey post-hoc test. **Key:** IOD: inverse optical density.
Supplementary Figure 3. Bar graphs showing the age effect on $S$-100β-expressing astrocytes within the CA1 subfield of the hippocampus (A) and the CA1 stratum moleculare (B) at 3, 12, 18 and 24 months between non-Tg control and 3xTg-AD groups. Bars represent mean ± S.E.M., (n = 4 – 5), * $p < 0.05$, ** $p < 0.01$ un-paired t-test. Key: $N_v$: numerical density (#/mm$^3$).
Chapter 4

Ultrastructural serotonin transporter and synaptic modifications in AD

Increased hippocampal CA1 density of serotonergic terminals in a triple transgenic mouse model of Alzheimer’s disease: An ultrastructural study.

4.1. ABSTRACT

Alzheimer’s disease (AD) is a neurodegenerative pathology that deteriorates mnesic functions and associated brain regions including the hippocampus. Serotonin (5-HT) plays an important role in cognition. In fact, in the result section from chapter 3 it was demonstrated that the triple transgenic (3xTg-AD) mouse model of AD exhibits a biphasic increase in 5-HT transporter (SERT) fibre density in the CA1 stratum lacunosum moleculare (S. Mol) of the hippocampus at 3 and 18 months of age. Here in chapter 4, the ultrastructural localisation, distribution and the numerical density (Nv, #/mm³) of the hippocampal SERT axons (SERT-Ax), terminals (SERT-Te) and their relationship with SERT-IR fibre sprouting as well as synaptic Nv were analysed in compared to non-transgenic (non-Tg) control mice. The 3xTg-AD mouse model of AD showed a significant increase in SERT-Te Nv within the CA1 subfield of the hippocampus at both 3 (by 95%) and 18 months of age (by 144%). Pronounced increase in SERT-Te Nv was particularly evident in the CA1 S.Mol (by 227% at 3 and by 180% at 18 months). In addition, 3xTg-AD mice displayed reduced Nv of perforated axospinous synapses (PS) in the CA1 S. Mol (by 56% at 3 and by 52% at 18 months). No changes were observed in the Nv of symmetric synapses, asymmetrical synapses, SERT-Ax and other axons. These results suggest that concomitant SERT-Te Nv increase and PS reduction in 3xTg-AD mice may act as a compensatory mechanism in maintaining synaptic efficacy as a response to the AD-related cognitive impairment.
4.2. INTRODUCTION

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative disease that deteriorates memory and cognition (Braak et al. 1999). The Neuropathological hallmarks of AD include neuritic β-amyloid (Aβ) plaques, neurofibrillary tangles (NFTs), neuronal death and synaptic loss, which occur early in AD and is linked to impaired synaptic connectivity and plasticity (Selkoe 2002; Knight & Verkhratsky 2010).

Although, AD affects primarily the cholinergic (ACh) system (Birks et al. 2000), other neurotransmitter systems are also implicated such as the glutamatergic (Rupsingh et al. 2011) and the serotonergic (5-HT) systems (Mowla et al. 2007; Noristani et al. 2010) (see also chapter 1, section 1.1.1.6.). The majority of 5-HT neurones are located within the dorsal (DR) and median raphe (MR) nuclei projecting to numerous brain regions including the thalamus, the amygdala, the cortex and the hippocampus, where they play important role in mnescic and cognitive functions (Vertes et al. 1999; Schmitt et al. 2006). Patients with AD exhibit reduced 5-HT neurotransmission, which correlates with disease severity (Garcia-Alloza et al. 2005). Treatment with selective serotonin re-uptake inhibitors (SSRIs) and specific 5-HT receptor ligands improves cognition in AD patients (Mowla et al. 2007).

The degree and importance of changes in the 5-HT system associated with AD remains elusive in human studies, however, experiments on animal models indicate their pathological relevance. The APP_{swe}/PS1_{ΔE9} double transgenic mouse model of AD displayed degeneration of 5-HT fibres, whilst APP_{Sw, Ind} transgenic mice, over-expressing the APP Swiss/Indian double mutations, showed stable 5-HT fibre density in the parietal cortex and the hippocampus (Aucoin et al. 2005; Liu et al. 2008). Similarly, a more recent study in APP_{swe}/PS1_{ΔE9} mice revealed no alterations in 5-HT transporter binding sites (measured using [3H]-escitalopram radioligand) up to 11 months of age, despite pronounced Aβ accumulations in the cortex and the hippocampus (Holm et al. 2010). Interestingly, increased 5-HT fibres sprouting were reported following neurotoxin lesions and Aβ accumulation in the striatum and the hippocampus (Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001;
Verdurand et al. (2011) (Table 4.1). In addition, other transgenic mice (APP-23) showed aberrant non-5-HT hippocampal axonal sprouting, which was directly related to Aβ plaque deposition (Phinney et al. 1999). The serotonin transporter (SERT) is expressed in serotonergic axons and axonal terminals, being critical for 5-HT re-uptake (Huang & Pickel 2002). In chapter 3 it was reported that there is an increase in hippocampal SERT-immunoreactive (SERT-IR) fibre density in the triple transgenic (3xTg-AD) mouse model of AD (Noristani et al. 2010), which mimics closely the spatio-temporal pathology and mnesic alterations of AD brains (Oddo et al. 2003b). Interestingly, the increase in SERT-IR fibre density was more evident in the proximity to Aβ plaques, suggesting that aberrant SERT-IR axonal sprouting and Aβ deposition are closely linked during the development of AD-related neuropathology (Noristani et al. 2010).

Studies of synaptic density in AD patients and transgenic mouse models of AD also revealed inconsistent results. Whereas post-mortem studies showed a steady decrease in synapses (DeKosky & Scheff 1990; Scheff et al. 2006), different transgenic mouse models exhibited either increase, decrease or no overall alterations in synaptic density (Buttini et al. 2002; King & Arendash 2002), see also (chapter 4, supplementary Table 1). Indeed, 3xTg-AD mice showed no changes in total synapse density (Yao et al. 2005), although the numerical density (Nv, #/µm³) of perforated axospinous synapses in the hippocampus seemed to be reduced (Bertoni-Freddari et al. 2008).

In this sense, and as it was already introduced in chapter 2 (material and methods), two types of synapses called asymmetric (also known as Gray’s type I) and symmetric (also knows as Gray’ type II) synapses (Palay 1956) are identified in the brain based on their ultrastructural parameters including: the shape of pre-synaptic vesicles and the presence of post-synaptic densities (Gray 1959; Colonnier 1968). Asymmetric synapses mainly have spherical pre-synaptic vesicles and a clear post-synaptic density, whilst symmetrical synapses contain pre-synaptic vesicles that are flattened or elongated without an obvious post-synaptic density (see chapter 4, supplementary Fig. 1).
In addition, asymmetrical synapses are the more predominant type of synapses in the brain and are associated with excitatory neurotransmitters such as glutamate (Gray 1959; Colonnier 1968). Symmetrical synapses are commonly linked with inhibitory neurotransmitter such as γ-aminobutyric acid (GABA) (Beaulieu & Somogyi 1990; Barbaresi et al. 1997). Perforated synapses are asymmetric synapses that have a distinct morphology characterised by a notable discontinuity (> 50nm) in their post-synaptic densities (Rodriguez et al. 2000) (see also chapter 4, supplementary Fig. 1C). Similar to other asymmetric synapses, perforated synapses are associated with excitatory glutamate neurotransmitter in the brain and are critical for spatial and working memory function in the hippocampus and are linked with plasticity processes (Geinisman et al. 1986a; Meshul et al. 1994; Rodriguez et al. 2001; Nicholson et al. 2004; Peddie et al. 2008a; Peddie et al. 2008b).

In chapter 3 it was demonstrated that 3xTg-AD mice displayed an increase in SERT-IR fibre density in the CA1 stratum lacunosum moleculare of the hippocampus (Noristani et al. 2010). This increase in SERT-IR fibre density initially appeared at 3 months, which is a parallel process associated with the evident intraneuronal accumulation of Aβ (Oddo et al. 2003b; Noristani et al. 2010). However, SERT-IR fibre density between 6, 9 and 12 months of age, was comparable to non-transgenic (non-Tg) control mice, whilst there was a late sprouting recurrence at 18 months, concomitant with the build-up of large extracellular Aβ neuritic plaques in 3xTg-AD mice (Oddo et al. 2003b; Noristani et al. 2010). The current chapter (chapter 4) further extends and expands previous findings from chapter 3, by analysing the ultrastructural changes in hippocampal SERT axons (SERT-Ax), terminals (SERT-Te) and their association with synaptic Nv and connectivity in the hippocampus of 3xTg-AD mice compared to non-Tg controls, by using ultrastructural peroxidase immunohistochemistry, at these two ostensibly relevant key points in either the start-up of anomalous intra-neuronal Aβ accumulation (3 months) and the consolidation of Aβ neuropil plaque formation and aggregation (18 months).
Table 4.1. Summary of studies on serotonergic system alterations in animal models of AD.

<table>
<thead>
<tr>
<th>AD Model</th>
<th>Neuropathology</th>
<th>Brain Region</th>
<th>5-HT alteration (age)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP&lt;sup&gt;cys&lt;/sup&gt;PS1&lt;sup&gt;ΔE9&lt;/sup&gt;</td>
<td>Plaques</td>
<td>C, A, H</td>
<td>5-HT fibre degeneration (12 and 18 months)</td>
<td>(Liu et al. 2008)</td>
</tr>
<tr>
<td>APP&lt;sup&gt;cys&lt;/sup&gt;PS1&lt;sup&gt;ΔE9&lt;/sup&gt;</td>
<td>Plaques</td>
<td>FC, PFC, H</td>
<td>No change (4, 8 and 11 months)</td>
<td>(Holm et al. 2010)</td>
</tr>
<tr>
<td>hAPP&lt;sup&gt;cys&lt;/sup&gt;/PS1 ΔE9</td>
<td>Plaques</td>
<td>H</td>
<td>No change (6, 12/14 and 18 months)</td>
<td>(Aucoin et al. 2005)</td>
</tr>
<tr>
<td>Intrastriatal ibotenic acid injected rat model</td>
<td>Excitotoxic lesion</td>
<td>SI</td>
<td>Vigorous sprouting of 5-HT fibres</td>
<td>(Zhou et al. 1995)</td>
</tr>
<tr>
<td>MBN injected NMDA rat model</td>
<td>Increased APP expression</td>
<td>NBM</td>
<td>Abundant sprouting of 5-HT fibres within damaged area</td>
<td>(Harkany et al. 2000b)</td>
</tr>
<tr>
<td>MBN injected Aβ&lt;sub&gt;1–42&lt;/sub&gt; rat model</td>
<td>Cholinergic lesion</td>
<td>NBM</td>
<td>5-HT fibre sprouting</td>
<td>(Harkany et al. 2001)</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td>Plaques and Tangles</td>
<td>H</td>
<td>65% increased SERT-IR fibre density (3 and 18 months)</td>
<td>(Noristani et al. 2010)</td>
</tr>
<tr>
<td>Intrahippocampal Aβ&lt;sub&gt;1–40&lt;/sub&gt; injected rat model</td>
<td>Aggregated amyloid material</td>
<td>H</td>
<td>Increased 5-HT activity within the vicinity of injection site</td>
<td>(Verdurand et al. 2011)</td>
</tr>
</tbody>
</table>

Key: C: Cortex, A: amygdala, H: hippocampus, ST: striatum, NBM: nucleus basalis magnocellularis, NMDA: N-methyl-D-aspartate, FC: frontal cortex, PFC: prefrontal cortex. Note that the reference highlighted in yellow represents a single study that reported degeneration of 5-HT fibres, highlighted in green are studies that reported no alterations in 5-HT fibres, whilst highlighted in red are studies reported 5-HT fibre sprouting as well as a single study that reported increased 5-HT activity in the vicinity of Aβ<sub>1–40</sub> injection site.
4.3. MATERIALS AND METHODS

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

4.3.1. Animals

The procedures for generating 3xTg-AD mice have been described previously (Oddo et al. 2003b; Noristani et al. 2010). In brief, human cDNA harbouring the Swedish APP mutation (KM670/671NL) and the human P301L four repeats mutated tau, were co-microinjected into a single-cell embryo of a homozygous PS1M146V knock-in mouse. The background of the PS1 knock-in mice is a hybrid 129/C57BL6. The non-Tg control mice used were also from the same strain and genetic background as the PS1 knock-in mice, but they harbour the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12h light-dark cycles with free access to food and water.

4.3.2. Fixation and Tissue Processing

Male 3xTg-AD and their respective non-Tg control mice were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 3 and 18 months of age (n = 3). Mice were perfused through the aortic arch with 3.75% acrolein (TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, Gillingham, UK) and 0.1M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde. Brains were then removed and cut into 4 – 5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as previously described (Noristani et al. 2010). These slabs of brain tissues were then post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1M PB, pH 7.4. Coronal sections of the brain were cut into 40 – 50μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome sections at level - 2.06 mm posterior to Bregma (dorsal hippocampus), were selected for
immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004). To optimise the penetration of immunoreagents, brain sections were freeze-thawed, as described previously (Rodríguez et al. 2000). For this procedure the brain sections were (i) incubated in a cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB, pH 7.4, (ii) rapidly immersed in Chlorodifluoromethane, followed by liquid nitrogen and (iii) placed in 0.1M PB at room temperature to thaw the tissue. Then the brain sections were rinsed in 0.1M PB, followed by 0.1M Trizma base saline (TS), pH 7.6.

4.3.3. Antibodies

A polyclonal rabbit antibody against a synthetic peptide sequence corresponding to amino acids 602 – 622 of the rat 5HT transporter (Immunostar, Hudson, WI, USA) was used for the determination of SERT-positive axons (SERT-Ax) and terminals (SERT-Te) in the hippocampus. For the identification of Aβ plaques, a monoclonal mouse antiserum monoclonal antibody against amino acid residues 1 – 16 of beta amyloid (Aβ, Covance, Emeryville, CA, USA) was used. The specificity of the antibody has been reported previously using immunohistochemistry (Noristani et al. 2010) and western blots (Albright et al. 2007). To further determine the specificity of the antibody, adsorption controls were done using SERT-specific peptide, which resulted in total absence of target labelling. Furthermore, omission of primary and/or secondary antibody also showed no immunoreactivity (refer to material and methods chapter (chapter 2, Fig. 2.2) for negative control images of SERT immunoreactivity).

4.3.4. Immunohistochemistry

The selected brain sections were incubated for 30 minutes in 30% methanol in 0.1M PB and 3% hydrogen peroxide (H₂O₂, Sigma, UK). Brain sections were then rinsed with 0.1M PB for 5 minutes and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. The sections were then washed with PB profusely before rinsing them in 0.1M TS for 10 minutes. Brain sections were then incubated in 0.5% bovine serum albumin (BSA, Sigma, UK) in 0.1M TS and 0.25% Triton (Sigma, UK, x 100) for 30 minutes. The sections were incubated for 48 hours at room temperature in the primary antibody (rabbit anti-SERT, 1:2500, Immunostar, Hudson, WI, USA). The brain sections were rinsed in 0.1M TS for 30 minutes and
incubated in 1:400 dilutions of biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Strattech Scientific Ltd., Soham, UK) for 1 hour at room temperature. Brain sections were then rinsed with 0.1M TS for 30 minutes followed by incubation for 30 minutes in avidin-biotin peroxidase complex (ABC, Vector Laboratories Ltd., Peterborough, UK). The peroxidase reaction product was visualised by incubation of the brain sections in a solution containing 0.022% of 3,3’diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003% H₂O₂ for 6 minutes, as previously described (Noristani et al. 2010). The reaction was stopped by rinsing the brain sections in 0.1M TS for 6 minutes followed by 0.1M PB for 15 minutes.

For the detection and determination of SERT-IR fibres and their relationship with Aβ neuritic plaques, dual indirect immunofluorescence labelling was used. The brain sections were incubated for 48 hours at room temperature in a primary antibody cocktail containing: (i) mouse anti-beta amyloid monoclonal antibody (Aβ, 1:2000, Covance, USA) and (ii) rabbit anti-SERT polyclonal antibody (1:2500, Immunostar, Hudson, WI, USA) simultaneously. Subsequently, Aβ plaques and SERT-IR fibres were detected in a sequential manner on the same brain sections by incubation with Alexa 595 goat anti-mouse and Alexa 488 goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, brain sections were rinsed with 0.1M PB for 30 minutes and permanently mounted in an aqueous medium (Vectashield, Vetor Laboratories Ltd., Peterborough, UK).

4.3.5. Electron microscopy
Following the DAB treatment (immunohistochemistry), brain sections were rinsed and flattened in 0.1M PB followed by post-fixation in 2% osmium tetroxide in 0.1M PB for 1 hour (Pickel et al. 1984; Leranth & Pickel 1989; Rodríguez et al. 2001; Peddie et al. 2008b; Peddie et al. 2008a). The brain sections were then washed for 10 minutes in 0.1M PB followed by sequential dehydration through replacement with increasing concentration of ethanol series (30, 50, 70 and 95%, 5 minutes each). The brain sections were further dehydrated using 100% ethanol (10 minutes) and propylene oxide (2 x 15 minutes). To start the resin penetration, dehydrated brain sections were incubated in propylene oxide:EPON (1:1, Agar scientific Ltd., Stanstead, UK) overnight at room temperature and rotating. To
complete the resin impregnation, the propylene oxide:EPON was replaced with 100% EPON and the brain sections were rotated for further 2 hours. Brain sections were then flat embedded between sheets of Aclar fluo halocarbon films (Agar scientific Ltd., Stanstead, UK) (Pickel et al. 1984; Leranth & Pickel 1989; Rodríguez et al. 2001; Peddie et al. 2008a; Peddie et al. 2008b). Polymerisation was carried out by incubating at 60°C overnight. Following polymerisation, the region of interest (CA1 subfield of the hippocampus) was selected and micro-dissected from the flat embedded tissue and mounted on the tip of EPON blocks (Pickel et al. 1984; Rodríguez et al. 2001; Peddie et al. 2008a; Peddie et al. 2008b), keeping the same levels and coordinates between different mice (Fig. 2.10). All brain sections used for quantitative analysis were from the identical anatomical level corresponding to -2.06 mm posterior to Bregma (dorsal hippocampus) in both non-Tg control and 3xTg-AD groups (Fig. 4.1). A diamond knife was used to cut serial ultra thin sections of these regions at a thickness ranging from 50 – 70 nm. The series of sections were collected on degreased copper mesh grids (200 mesh) and counterstained using the uranyl acetate and lead citrate method (Reynolds 1963) prior to their examination under the Philips FEI Tecnai 12 BioTwin (FEI, Eindhoven, The Netherlands) electron microscope. Random images of the areas of interest (blindly taken) were collected and the negatives digitalised on an Imacon Flextight 848 scanner (Imacon Inc. Hasselbald A/S, Copenhagen, Denmark) each one representing a volume of 12.39µm$^3$, for a total analysed CA1 volume of 396.48µm$^3$ (in each animal) and 99.12µm$^3$ per each CA1 layer (S.Or, PCL, S.Rad and S.Mol). See chapter 2 supplementary material for detailed description and calculation of surface area and volumes measurement of electron micrographs (chapter 2, section 2.S.9.).
Figure 4.1. Brightfield micrographs illustrating the distribution of SERT-IR fibres in the dorsal hippocampus of 3 months old non-Tg control (A) and 3xTg-AD mice (B) and their respective high magnification details of the CA1 subfield area of the hippocampus that was selected for the electron microscopic analysis (C, D). Hippocampal sections were selected at identical anatomical levels between the different animals to ensure an exact and equivalent sampling. Scale bars: 500μm (A, B), 100μm (C, D). Key: S.Or: stratum oriens, PCL: pyramidal cell layer, S.Rad: stratum radiatum, S.Mol: stratum lacunosum moleculare, DG: dentate gyrus. Adsorption control using SERT-specific peptide resulted in total absence of SERT-IR fibres (see Chapter 2, Fig. 2.2)

4.3.6. Labelling profiles and Nomenclature

The labelled profiles were classified as somata, dendrites, dendritic spines, unmyelinated axons, axon terminals and glia, according to their morphological features, as described previously (Rodríguez et al. 2000; Peddie et al. 2008b) and defined by Peters et al. (Peters et al. 1991). See also (chapter 2, section 2.6.1.) for detailed description of neuronal profiles using electron microscopy. Synapses were defined as either symmetric when having thin pre- and post-synaptic densities or asymmetric when having a thin pre- and a thick post-synaptic membrane specialisation (Rodríguez et al. 2000). Perforated synapses were defined as those asymmetric synapses with a notable discontinuity (> 50nm) in the electron density of their post-synaptic junctions, as previously described (Rodríguez et al. 2000) (see also chapter 4, supplementary Fig. 1).
4.3.7. Numerical density

To determine the numerical density (\(N_v\), # of labelled profiles/\(\mu m^3\)), the ultrastructural analysis was entirely carried out on the most superficial portions of the tissue in contact with the embedding plastic, from both sides of the vibratome brain section, to minimise artificial differences in labelling attributed to potential differences in the penetration of reagents, as described previously (Rodríguez et al. 2000). Regions used for this analysis were chosen randomly by a single investigator without any information about the genotype or the age of the animal (see also chapter 2, for detailed description of blind analysis). The \(N_v\) of SERT-Te, SERT-Ax and synapses were determined, on serial ultra thin sections, according to the Cavalieri principle, as described elsewhere (Tang et al. 2001) (refer to supplementary section of the material and methods chapter (chapter 2, section 2.S.10), for detailed description of the Cavalieri principle).

\[
N_v = \frac{\sum P}{[t \times \alpha(p)]}
\]

Where \(N_v\) is the numerical density, \(\sum P\) is the number of SERT-Te, SERT-Ax or synapses counted, \(t\) is the average ultra thin section thickness and \(\alpha(p)\) is the corresponding surface area (\(\mu m^2\)).

3 vibratome sections from each of the 12 animals (\(n = 3\) per group) were examined for ultrastructural quantification of SERT-immunolabelled profiles. The \(N_v\) of labelled profiles were determined and identified by the presence of amorphous electron-dense DAB reaction product (Fig. 4.3 – 4.5). This method was selected over immunogold labelling because the latter is less sensitive compared to the peroxidase labelling (Cortese et al. 2009). Although gold labelling allows a more selective sub-cellular localisation, it has reduced tissue penetration and limited diffusion that may result in an underestimation of the relative abundance of the immunoreactive profiles (Rodríguez et al. 2000; Cortese et al. 2009). Furthermore, immunogold labelling provides less reliable quantitative 3D estimation of the labelled profiles since it is primarily regarded as a 2D (surface) method and is not reliably considered quantitative for 3D labelling (Cortese et al. 2009).
In total 17,993 neuronal profiles were identified with 592 (3.3%) containing peroxidase labelling for SERT (Fig. 4.3 - 4.5). Altogether, 11,108 synapses were identified over a corresponding total volume of 4,757.76μm³. See chapter 2, supplementary materials for detailed calculation of the surface area and volume measurements using electron micrographs (section 2.S.9.).

In this chapter, the term “terminal area” is used to describe the surface area of SERT-labelled terminals as an indication of terminal size, measured directly on electron micrographs. A previous study using peroxidase immunocytochemistry against TPH (the rate limiting enzyme for 5-HT synthesis) combined with electron microscopy, showed stable size of serotonergic terminals (measured using surface area of TPH-immunolabelled terminals directly on electron micrograph) in multiple brain regions including the entorhinal cortex, the frontoparietal cortex and the hippocampus of rats (Cohen et al. 1995). However, it must be emphasised that such surface area measurement of labelled terminals only provide information about the “potential to release and uptake” rather than the actual level of neurotransmitter being released in these terminals. They can, however, provide some information about the capacity of these terminals, for example just as the size of the physical plant in a factory does not tell one about production levels, but the capacity to produce. The surface area of all SERT-Te was measured in all hippocampal layers using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA), (refer to chapter 2, section 2.6.3. for detailed surface area measurement of SERT-Te using ImageJ). No measurement of the “synaptic area” was investigated in either non-Tg control or in the 3xTg-AD mouse model of AD.

Finally, it has been previously shown that the majority of SERT-immunolabelled terminals are widely distributed in the hippocampus with only few terminals displaying synaptic specialisations (Cohen et al. 1995). To uncover the possible changes in serotonergic terminals with synaptic specialisations, SERT-Te with synaptic structure present adjacent to SERT-labelled terminals (referred here as SERT-Te⁺ synapses) was investigated directly in all studied electron micrographs.
Composite figures, adjusted for brightness, contrast and sharpness, were generated using Adobe Photoshop CS2 (Adobe Systems Inc. San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).

4.3.8. Statistical analysis

Results are expressed as mean ± standard errors of the mean (S.E.M.). Unpaired student t-test was applied to determine differences in the $N_v$ of SERT-Te, SERT-Ax and synapses between 3xTg-AD mice and non-Tg controls at 3 and 18 months of age. Significance was accepted at $p \leq 0.05$. The data were analysed using GraphPad Prism 4.0 (GraphPad Software, Inc. La Jolla, CA, USA).
4.4. RESULTS

At light microscope level, in both 3xTg-AD and non-Tg control mice, serotonin transporter-immunoreactive (SERT-IR) fibres were heterogeneously distributed throughout the hippocampal formation (Fig. 4.1, 4.2A and 4.2B). SERT-IR fibres appeared mainly as fine and thick processes with numerous varicosities, which are characteristic of axonal profiles (Fig. 4.1C, 4.1D, 4.2A and 4.2B). The highest densities of SERT-IR fibres are evident in the stratum lacunosum moleculare of the CA1 subfield of the hippocampus (Fig. 4.1C, 4.1D, 4.2A and 4.2B). The stratum radiatum and oriens exhibited moderate expression, whilst the lowest densities of SERT-IR fibres are present in the pyramidal cell layer. The 3xTg-AD mouse model of AD showed increased density of SERT-IR fibres in the stratum lacunosum moleculare of the CA1 subfield exclusively at 3 and 18 months compared to age-match non-Tg controls (Fig. 4.1C, 4.1D, 4.2A and 4.2B), as shown by augmented SERT-IR fibre optical and area density, which corresponded to the previously described findings during the progression of AD (Noristani et al. 2010). Increased SERT-IR fibre density in 3xTg-AD mice was even more evident in the proximity of Aβ plaques (Fig. 4.2D – 4.2F, see also chapter 4, supplementary Fig. 3)

4.4.1. Ultrastructural distribution of hippocampal SERT profiles

SERT EM analysis confirmed that the majority of SERT-IR profiles were small axons and axon terminals and/or varicosities with different size and morphological features according to their raphe nuclei origin (Fig. 4.3A, 4.3B, 4.3D – 4.3F, 4.4C – 4.4F) (Noristani et al. 2010). SERT-IR profiles were identified by the presence of amorphous electron-dense DAB reaction product that was mainly associated with the plasma membrane of axons and terminals (Fig. 4.3D – 4.3F, 4.4C – 4.4F) with or without association with the synaptic specialisation (Fig. 4.3A, 4.3D – 4.3F). From the 17,993 axonal profiles analysed, 592 (3.3%) contained peroxidase labelling for SERT. The majority of SERT-IR profiles consisted of morphologically heterogeneous population of axonal terminals and/or varicosities (SERT-Te) that made up 81% of the SERT contained profiles (n = 479 out of 592, Fig. 4.3A, 4.3B, 4.3D – 4.3F, 4.4C – 4.4F, 4.5B – 4.5E). Occasionally, SERT labelling was directly associated with cytoplasmic organelles, including the dense core vesicles (DCVs), which are characteristic of serotonergic terminals (Fig. 4.3F) (Huang & Pickel
2002). Dense labelling for SERT was also observed in small unmyelinated axons (SERT-Ax), which were predominantly apposed to other small axons and/or terminals (Fig. 4.3C). SERT-Ax comprised 17% of the total SERT-IR profiles (n = 104 out of 592) and was seldomly in contact with dendritic profiles. SERT labelling was absent in post-synaptic sites and was rarely observed in glial profiles. From the 479 immunolabelled SERT-Te identified in the current study, only 64 (13%) showed synaptic specialisation (referred here as SERT+ synapses) mostly represented by asymmetrical synapses with dendritic spines and dendrites (Fig. 4.3A and 4.3B). SERT-Te area varied in size from 0.12 to 1.01µm² (mean 0.2 ± 0.01µm²). The main post-synaptic target of SERT-Te were unlabelled dendrites onto which these terminals established asymmetrical synapses, which sometimes localised in a close vicinity to glutamatergic excitatory synapses (Fig. 4.3A, 4.4C – 4.4F, 4.5B – 4.5E).
**Figure 4.2.** Brightfield micrographs showing SERT-IR fibres in the dorsal hippocampus of 18 months old non-Tg control (A) and 3xTg-AD mice (B). Dual confocal micrographs showing SERT-IR fibres in the dorsal hippocampus of 18 months old non-Tg control (C) and a 3xTg-AD (D), the latter containing Aβ plaques (E) showing SERT-IR fibres specific sprouting adjacent to Aβ plaques (straight arrows) compared to regions with no Aβ plaques (curved arrows) within the CA1 subfield of the hippocampus (F, merged image). Scale bars: 100μm (A and B), 25μm (C, D, E and F). **Key:** S.Rad: stratum radiatum, S.Mol: stratum lacunosum moleculare, f: hippocampal fissure. Note. The above figure was kept because it is the original version published in the journal of Cell Death and Disease. For more convincing examples of SERT fibre sprouting adjacent to Aβ plaques please see chapter 4, supplementary Fig. 3.
Figure 4.3. Electron micrographs showing morphological features of SERT axon terminals (SERT-Te) and SERT small axons (SERT-Ax) in the hippocampal CA1 subfield of 18 months old 3xTg-AD (A, B), 3 months old non-Tg control (C, E) and 18 months old non-Tg control (D, F). Peroxidase labelling for SERT-Te is seen within multiple terminals (T1-3) in the pyramidal cell layer of the hippocampus. One of them (SERT-T1) is forming an asymmetrical synapse (curved arrow) with an unlabelled dendrite (UD) (A). Immunoperoxidase labelling of two SERT-Te (T1-2) in the CA1 stratum lacunosum moleculare, establishing two asymmetrical synaptic contacts (curved arrows) with an unlabelled dendrite (UD) (B). SERT plasmalemmal labelling in a small unmyelinated axon (SERT-Ax) in the CA1 stratum lacunosum moleculare, which is apposed to an unlabelled terminal (UT) and an unlabelled dendritic spine (usp) (C). SERT immunoreactivity in small (D) and large axon terminals (E – F) in the CA1 stratum lacunosum moleculare showing in some cases the characteristic dense core vesicles (DCVs) of serotonergic terminals (F) and forming asymmetric synapses (curved arrows). Scale bars: 500 nm (A – F). Key: S.Py: pyramidal cell layer, S.Mol: stratum lacunosum moleculare, M: mitochondria, rER: rough endoplasmic reticulum, Nu: nucleus, UD: unlabelled dendrites, usp: unlabelled spine, UT: unlabelled axon terminal, UA: unlabelled axon.

4.4.2. Temporal changes in SERT-Te numerical density and size in the 3xTg-AD mouse model of AD

An increase in SERT-Te Nv was observed in the CA1 subfield of the hippocampus in a strata-specific manner (Fig. 4.4A and 4.4B). The global analysis showed a significant increase in SERT-Te Nv at 3 and 18 months in 3xTg-AD mice compared to non-Tg controls (95%, p = 0.0436 and 148%, p = 0.0064, respectively; Fig. 4.4A). A more detailed quantitative analysis of CA1 individual strata revealed that
the increased SERT-Te Nv was restricted to the CA1 stratum lacunosum moleculare, which also exhibited the highest density of SERT-IR fibres within the hippocampal formation in basal conditions (Fig. 4.1C, 4.1D, 4.2A and 4.2B). This increase also appeared at the same ages and were almost double the values compared to age-matched non-Tg control mice (227%, p = 0.0148 at 3 months and 180%, p = 0.0329 at 18 months, Fig. 4.4B). SERT-Te Nv showed no alterations in either stratum oriens or stratum radiatum between 3xTg-AD and non-Tg control groups at any examined ages (p = 0.5659, p = 0.1948 for stratum oriens and p = 0.0571, p = 0.2607 for stratum radiatum at 3 and 18 months, respectively; see chapter 4, supplementary Fig. 2). The increase in SERT-Te Nv was associated with a generalised increase in their size as measured by determining their surface area, which was age specific (by 108%, p = 0.0264 at 3 months and by 89%, p = 0.0474, at 18 months, Fig. 4.5A – 4.5E) but not strata specific. In the stratum oriens 3xTg-mice showed an age-associated decrease in SERT-Te Nv in the CA1 subfield of the hippocampus (48% decrease, p = 0.0463, chapter 4, supplementary Fig. 2A). No age-associated changes were observed in the SERT-Te size in 3xTg-AD mice between 3 and 18 months of age. However, non-Tg control mice showed an age-associated decrease in SERT-Te Nv in the CA1 subfield of the hippocampus (39% decrease, p = 0.0413) and CA1 stratum radiatum (54% decrease, p = 0.0353, chapter 4, supplementary Fig. 2A), although their size remained stable (Fig. 4.4A, 4.5A). In addition, SERT-Ax Nv and their size remained stable in 3x-Tg-AD and non-Tg control in all strata and examined ages.

4.4.3. Reduced Nv of perforated synapses in the hippocampus of the 3xTg-AD mouse model of AD

In parallel to the peroxidase labelling done to analyse the SERT labelled profiles, the same material was used to characterise the Nv of hippocampal synapses that were not labelled with SERT (referred in chapter 4 as SERT⁻ synapses). The SERT⁻ synaptic elements and post-synaptic densities were heterogeneously distributed throughout the different hippocampal layers (Fig. 4.3B, 4.3E, 4.4C – 4.4F, 4.5B – 4.5E) as revealed by the use of serial EM sections, which allowed us to distinguish and quantify the different types of synapses according to their post-synaptic densities and synaptic vesicles composition (see chapter 4, supplementary Fig. 1). Symmetric synapses have thin pre- and post-synaptic densities with axons
containing pleomorphic (round and elongated) synaptic vesicles (see Chapter 4, supplementary Fig. 1A), whilst asymmetric synapses displayed round synaptic vesicles in the axons and were characterised by the presence of a thin pre-synaptic density with a thick and more prominent post-synaptic membrane specialisation (Rodríguez et al. 2000; Rodríguez et al. 2005) (Chapter 4, supplementary Fig. 1B). Some of these asymmetric synapses presented a notable discontinuity (> 50nm) in the electron density of their post-synaptic junctions, as previously described (Rodríguez et al. 2000), and were considered as perforated synapses (Chapter 4, supplementary Fig. 1C). The majority of SERT synapses were asymmetric being characterised by a thin pre- and a thick post-synaptic membrane specialisation (Fig. 4.3D and 4.3E). In total, 11,108 SERT synapses were identified of which 10,889 (98.03%) were asymmetrical type between axons and dendritic spines, the remaining 219 (2%) were symmetrical synapses mainly established onto dendrites and dendritic shafts (Fig. 4.3A). Only 863 (7.9%) of the asymmetric synapses were identified as perforated.
Figure 4.4. Bar graphs showing the age effect on SERT-Te numerical density \(N_v\) within overall CA1 subfield of the hippocampus (A) and stratum moleculare of the CA1 (B) at 3 and 18 months in non-Tg control and 3xTg-AD. Bars represent mean ± S.E.M., * = p<0.05, ** = p<0.01 compared to age-matched non-Tg control, # = p<0.05 compared to 3 months non-Tg control, un-paired t-test. Representative electron micrographs illustrating SERT-Te \(N_v\) in the CA1 stratum moleculare subfield of the hippocampus at 3 and 18 months in non-Tg control (C and E) and 3xTg-AD mice (D and F). Scale bars: 500 nm (C – F). **Key:** S.Mol: stratum lacunosum moleculare, M: mitochondria.
Figure 4.5. Bar graphs showing the age effect on SERT-Te surface area in the CA1 subfield of the hippocampus (A) at 3 and 18 months in non-Tg control and 3xTg-AD animals. Bars represent mean ± S.E.M., * = p<0.05, un-paired t-test. Representative electron micrographs of the SERT-Te surface area in the CA1 subfield of the hippocampus in 3xTg-AD animals (C and E) compared to non-Tg control mice (B and D). Scale bars: 500 nm (B – E). Key: S.Rad: stratum radiatum, S.Mol: stratum lacunosum moleculare, M: mitochondria.
Although, global analysis of the hippocampal CA1 area showed no differences in the \( N_v \) of total, symmetric and asymmetric SERT\(^-\) synapses, there was a significant decrease in the \( N_v \) of perforated axospinous synapses in 3xTg-AD mice compared to non-Tg controls (Fig. 4.6C). The analysis of the whole hippocampus revealed a significant decrease in perforated axospinous \( N_v \) in 3xTg-AD mice at 3 but not at 18 months of age (64% loss, \( p = 0.0056 \) and 39% loss, \( p = 0.1453 \)). The layer specific analysis also revealed that the stratum lacunosum moleculare exhibits significant deficit in the \( N_v \) of perforated axospinous synapses (Fig. 4.7C) at 3 and 18 months compared to age-matched non-Tg control (56% loss, \( p = 0.0260 \) and 50% loss, \( p = 0.0394 \)). Furthermore, the non-Tg control group also showed an age-associated decrease in perforated axospinous \( N_v \) both in the CA1 subfield of the hippocampus and in the stratum lacunosum moleculare of the CA1 (38% loss, \( p = 0.0260 \) and 34% loss, \( p = 0.0070 \), Fig. 4.6C and 4.7C), this was not the case for \( N_v \) of other types of SERT\(^-\) synapses.

However, no changes were found in the synaptic \( N_v \) that was specifically established by SERT-labelled (SERT\(^+\) synapses) profiles when comparing 3xTg-AD mice to non-Tg controls either in the whole CA1 subfield or the CA1 stratum lacunosum moleculare (\( p = 0.4796 \), \( p = 0.3739 \) at 3 months and \( p = 0.1529 \), \( p = 0.5185 \) at 18 months, respectively; Fig. 4.6D and 4.7D). SERT\(^+\) synapses were mainly asymmetric synapses with dendritic spines and dendrites. Ultrastructural analysis of SERT-Te\(^+\) synaptic specialisation also showed no difference in SERT-Te\(^+\) axo-spine and axo-dendritic synapses between 3xTg-AD and non-Tg control mice.
Figure 4.6. Bar graphs showing the age effect on unlabelled (SERT) total (A), asymmetrical (B), and perforated synapses (C) as well as the specific synaptic numerical density ($N_v$) of SERT labelled (SERT+) synapses (D) in the CA1 of the hippocampus at 3 and 18 months in non-Tg control and the 3xTg-AD mouse model of AD.

Bars represent mean ± S.E.M., ($n = 3$), ** = $p<0.01$ compared to age-matched non-Tg controls, # = $p<0.05$, compared to 3 month non-Tg controls, un-paired t-test.
Figure 4.7. Bar graphs showing the age effect on unlabelled (SERT) total (A), asymmetrical (B), and perforated synapses (C) as well as the specific synaptic numerical density ($N_v$) of SERT labelled (SERT+) synapses (D) at 3 and 18 months in non-Tg control and the 3xTg-AD mouse model of AD in the CA1 stratum lacunosum moleculare of the hippocampus. Bars represent mean ± S.E.M., (n = 3), * = p<0.05 compared to age-matched non-Tg controls, ## = p<0.01 compared to 3 month non-Tg controls, unpaired t-test.
4.5. DISCUSSION

4.5.1. Age dependent and CA1 strata specific increase in SERT terminals
The main finding of this study is that the 3xTg-AD mouse model of AD displays an age-dependent increase in the Nv of SERT-immunoreactive terminals. Global quantitative analysis of the hippocampal CA1 subfield revealed that the Nv of SERT-Te was increased at 3 and 18 months in 3xTg-AD mice when compared to age-matched non-Tg controls. The increase in SERT-Te Nv was more evident within the stratum lacunosum moleculare of the CA1 area, which in normal conditions is also known to host the highest density of serotonergic projections in the hippocampal formation (Vertes et al. 1999). In addition to the increased Nv, the size of SERT-Te was also increased in the CA1 subfield of the hippocampus. No changes were observed in SERT-Ax Nv. These data suggest that there is an age-dependent increase in hippocampal serotonergic input that is closely associated with the development of AD pathology in 3xTg-AD mice. Furthermore, the finding of increased SERT-Te Nv confirms the previous hypothesis of SERT-IR fibres sprouting in the CA1 stratum lacunosum moleculare of the hippocampus (as shown by increased optical and area density of SERT-IR fibres) at 3 and 18 months in 3xTg-AD mice compared to age-matched non-Tg controls (Noristani et al. 2010) (chapter 3).

Serotonergic fibre sprouting is generally defined as fibre outgrowth reflected by increase in size and diameter of axons, appearance of new spindle varicosities with no sign of damaged SERT axons (Zhou et al. 1995; Harkany et al. 2000b; Noristani et al. 2010). Serotonergic fibre sprouting may act as a compensatory mechanism by maintaining an overall stable hippocampal circuitry and synaptic connectivity. Thus, these results provide the first ultrastructural evidence for increased serotonergic input within the hippocampus of 3xTg-AD mice. Previously, increased 5-HT fibre sprouting was found following acute brain damage such as Aβ, ibotenic acid and NMDA injections in rats (Table 4.1) (Zhou et al. 1995; Harkany et al. 2000b; Harkany et al. 2001; Verdurand et al. 2011). In addition, this 5-HT fibre sprouting phenomenon has been reported in mouse models of other neurodegenerative disorders including the Menkes' disease (Martin et al. 1994) and amyotrophic lateral sclerosis (ALS) (Bose & Vacca-Galloway 1999). Furthermore,
a vigorous 5-HT fibre sprouting has also been found in primate model of Parkinson’s disease (PD) following selective lesion of the dopaminergic neurones (achieve via intracarotid injection of a DA neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) (Gaspar et al. 1993). Thus, it is evident that 5-HT fibre sprouting is closely linked with brain damage and neurodegenerative diseases.

In 3xTg-AD mice, evident intraneuronal Aβ accumulation starts at 3 months that may also cause early damage to the affected neurones (Wirths et al. 2004). Extracellular deposition of Aβ1-42 contributes to plaque formation, which in 3xTg-AD mice begins between 9 and 12 months of age and consolidates at 18 months, primarily targeting the CA1 hippocampal subfield and in particular the stratum lacunosum moleculare (Oddo et al. 2003b; Noristani et al. 2010). The 3xTg-AD mouse model of AD exhibit an age-related increase in extracellular build-up of Aβ plaques that continues into advanced age (Oddo et al. 2003b). Indeed, previous studies including our recent studies in the same cohort of animals confirmed that 3xTg-AD mice exhibit extensive Aβ and tau pathology at 18 months of age (Oddo et al. 2003b; Noristani et al. 2010) (see also chapter 4, Fig. 4.2E, supplementary Fig. 3). Such massive build-up and consolidation of Aβ plaques at a later age (18 months) may induce neurotoxic effects resulting in neuronal damage that in turn may also stimulate an increase in SERT-Te in 3xTg-AD mice (Noristani et al. 2010). Lack of SERT-IR fibre sprouting at earlier ages (6 to 12 months) in 3xTg-AD mice may be due to lower level of extracellular Aβ plaque deposition in the CA1 subfield of the hippocampus, as well as due to a less compromised synaptic connectivity compared to 18 months of age (Noristani et al. 2010). In agreement with this phenomenon, a recent study in the APPswe/PS1ΔE9 double transgenic mouse model of AD with severe Aβ pathology showed no alteration in cortical and hippocampal SERT density up to 11 months of age (Holm et al. 2010). In addition, our preliminary results suggest that SERT-IR fibre sprouting is further increased in the hippocampus at 24 months of age in 3xTg-AD mice concomitant with formation of large extracellular Aβ plaques (H.N.N & J.J.R.A, unpublished observation). Furthermore, the finding of early increase in SERT-Te (3 months) may indicate that this process could even start with the accumulation of intracellular Aβ1-42 (Oddo et al. 2003b), which in turn might account for an initial protective response to restore hippocampal functionality (Noristani et al. 2010).
(See also chapter 6 for detailed discussion and consideration of intraneuronal Aβ in inducing SERT-IR fibre sprouting).

The neurotoxic effect of Aβ involves increased activation of the glutamatergic neurotransmission and impairment of calcium homeostasis (Miguel-Hidalgo et al. 2002). Increased 5-HT input may help to counteract neurotoxicity by inhibiting calcium influx and inducing membrane hyperpolarisation (Harkany et al. 2000b; Harkany et al. 2001). This effect is mediated by activation of 5-HT1A and 5-HT1B receptors that are highly expressed in the hippocampal formation (Ogren et al. 2008; Peddie et al. 2008b). In support of the above hypothesis, a recent study by Verdurand and colleagues (2011) reported a transient increase in 5-HT neurotransmitter level and 5-HT1A receptors expression following intrahippocampal infusion of Aβ (Verdurand et al. 2011), suggesting that Aβ-induced damage may trigger an increase in 5-HT neurotransmission. Increased expression of hippocampal 5-HT1A receptors was also reported in patients with mild cognitive impairment who exhibit greater risk of developing AD (Truchot et al. 2007). Therefore, an increased 5-HT input may represent an intrinsic protective mechanism in response to Aβ-induced excitotoxic damage (Noristani et al. 2010). In fact, increased SERT-Te area in 3xTg-AD mice suggests that these terminals have greater “potentials to release and uptake” 5-HT neurotransmitter, thus enhancing the synaptic strength.

A pronounced increase in SERT-Te Nv was observed specifically in the CA1 stratum lacunosum moleculare. Such layer-specific effect may be due to the highest density of 5-HT projections in the stratum lacunosum moleculare compared to other hippocampal layers (Vertes et al. 1999). In addition, and as mentioned earlier, in 3xTg-AD mice excess extracellular Aβ plaques are particularly evident in the stratum lacunosum moleculare (Noristani et al. 2010). These combined phenomena may account not only for the SERT-IR fibres sprouting but may also directly affect the synaptic density and connectivity in the hippocampus.

As mentioned earlier, the majority of SERT-Te lacked clear synaptic junctions, with only 13% of terminals showing recognisable synaptic junctions (referred here as SERT-Te+ synapses, Fig. 4.6D and 4.7D). These findings are in agreement with
previous studies in the rat hippocampus and the cortex, which reported the incidence of serotonergic terminals with synaptic specialisation between 12 – 22%, whilst the majority of serotonergic terminals are non-junctional (Seguela et al. 1989; Oleskevich et al. 1991; Cohen et al. 1995; Miner et al. 2000), suggesting that 5-HT neurotransmission mainly occurs via volumetric transmission. Unlike the increase in SERT-Te N_v, no changes were observed in SERT-Te^+ synapses between 3xTg-AD mice and non-Tg controls at any of the examined ages (Fig. 4.6D and 4.7D). Given the low incidence of SERT-Te^+ synapses in the hippocampus, the fact that there is a stable SERT-Te^+ synaptic N_v between 3xTg-AD and non-Tg control mice would have minimal effects on the total 5-HT neurotransmission in the hippocampus and its interpretation.

4.5.2. AD associated changes in synaptic density
Contrary to the accepted phenomenon linking AD to synaptic loss, this study found no overall deficit in synaptic N_v in the hippocampal CA1 subfield (Scheff et al. 2006; Knight & Verkhratsky 2010). These results are in agreement with previous studies, which, by employing either qualitative or semi-quantitative light immunohistochemistry, reported no overall changes in synaptic associated proteins such as synaptophysin in the hippocampus and in the cortex of multiple transgenic mouse models of AD including the APP23, PDAPP, PSAPP, APP_Sw and more recently in 3xTg-AD mice (see chapter 4, supplementary Table 1). Similarly, no changes in the total N_v of synapses were observed in the pyramidal layer of the hippocampal CA1 subfield in 3xTg-AD mice at 13 months of age (Bertoni-Freddari et al. 2008). The current study further extends and clarifies the previous report by Bertoni-Freddari and colleagues (2008) by analysing all the hippocampal strata in 3xTg-AD mice up to 18 months of age. Although no changes were found in the overall synaptic N_v there was a significant decrease in the N_v of perforated axospinous synapses in the CA1 area of the hippocampus and more specifically in the CA1 stratum lacunosum moleculare that was evident at an early age (3 months, 56%) and continued into a more advanced age (18 months, 52%).

The present study also shown that perforated axospinous synapses are specifically affected during ageing in the non-Tg control mice (Fig. 4.6C and 4.7C) and the 3xTg-AD mice (chapter 4, supplementary Fig. 2). The age-related decrease is
consistent with previous results in aged rats that found reduced hippocampal perforated synapses as well as a decrease in the size of post-synaptic density (Geinisman et al. 1986b; Nicholson et al. 2004), although others have reported no changes in the total number of all perforated and non-perforated axospinous synapses (Geinisman et al. 2004). One factor that may account for this discrepancy includes variations in hippocampal volume (Geinisman et al. 2004). Conflicting results have been reported in relation to hippocampal volume alterations in different transgenic models of AD (Schmitz et al. 2004; Delatour et al. 2006; Valla et al. 2006; Oberg et al. 2008; Maheswaran et al. 2009). Previous studies in single and double transgenic mouse models of AD including the PS1, the APP/PS-1 and the PSAPP mice showed no alterations in hippocampal volume using longitudinal in vivo MRI scanning (Delatour et al. 2006) or Cavalieri’s principal for the estimation of total hippocampal volume (Schmitz et al. 2004; Valla et al. 2006). Contrary, Oberg et al. (2008) reported reduced hippocampal volume in the APP/PS1 double transgenic mouse model of AD using in vivo longitudinal MRI scanning (Oberg et al. 2008). On the other hand, Maheswaran and colleagues (2009) have demonstrated increased hippocampal volume in the TASTPM mouse model of AD with severe amyloidosis, which could be associated with accumulation of Aβ plaques and reactive glial cells (Maheswaran et al. 2009). In the current study an equal hippocampal volume was analysed between 3xTg-AD mice and the non-Tg controls, which reliably eradicates the effect of possible volume alterations on synaptic Nv (Coggeshall & Lekan 1996; Geinisman et al. 2004). In addition, ultra thin section series were cut at a constant thickness (see material and methods) between the two groups to avoid the bearing effect of section thickness on synaptic Nv (Coggeshall & Lekan 1996). Furthermore, the criteria used for synapse determination included not only the presence of post-synaptic density but also the occurrence of the adjacent pre-synaptic element containing the synaptic vesicles, which provides a reliable and un-biased estimation of synaptic Nv compared to that of just profile count (Coggeshall & Lekan 1996). Finally, the present findings are consistent with a recent electron microscopic study that also reported reduced Nv of perforated synapses in 3xTg-AD mice, when quantifying synapses per unit volume (Bertoni-Freddari et al. 2008).

Perforated axospinous synapses in the hippocampus play an important role in
spatial and working memory (Geinisman et al. 1986a; Nicholson et al. 2004). Increased perforated synapses have been linked with the induction of long-term potentiation (LTP) and improved memory performance in rodents (Stewart et al. 2005). Reduced perforated synapses shows good correlation with deterioration of cognitive function including learning and memory (Geinisman et al. 1986b; Nicholson et al. 2004). Given the pivotal role of perforated axospinous synapses in cognitive function, the reduced N_v of perforated synapses found in the current study may reflect the impaired synaptic plasticity and subsequent deterioration of learning and memory processes in 3xTg-AD mice (Oddo et al. 2003b; Bertoni-Freddari et al. 2008). In this direction, post-mortem studies on human tissue have consistently reported a decrease in synaptic density in the cortex and in the hippocampus (DeKosky & Scheff 1990; Scheff et al. 2006). In transgenic models, which exhibit plaque pathology, both an increase and a decrease in synaptic density was found in the neocortex and in the hippocampus (Buttini et al. 2002; King & Arendash 2002). Transgenic models with tangle pathology have also showed reduced hippocampal synaptic density (Yoshiyama et al. 2007), see also (chapter 4, supplementary Table 1). Different factors may account for these controversies including differences in (i) transgenic models (ii) strains, (iii) age, (iv) brain area studied and (v) methods used to quantify synaptic density (Coggeshall & Lekan 1996).

In conclusion, the present study suggest that SERT fibre sprouting is directly related with an increase in the N_v and the surface area of SERT-Te that may act as a compensatory mechanism in maintaining overall synaptic efficacy. In addition, this increase in serotonergic input may be an intrinsic neuro-protective response to counteract Aβ-induced neurotoxicity and altered hippocampal glutamatergic circuitry, as revealed by the decrease in asymmetric perforated N_v, accounting for the early behavioural alterations and later establishment of severe and permanent cognitive and mnesic impairments in AD. In fact, increased 5-HT neurotransmission via chronic treatment with SSRI (paroxetine) improved memory performance and reduced the development of Aβ and tau pathologies in 3xTg-AD mice (Nelson et al. 2007b). Thus, increasing 5-HT neurotransmission in AD may provide a better therapeutic approach not only to improve behavioural abnormalities, but also to interfere with the underlying neuropathology associated with the disease.
Supplementary Figure 1. Electron micrographs showing the differential characteristic and morphological features of symmetrical (A), asymmetrical (B) and perforated (C) synapses. Symmetric synapses are characterised by their thin pre- and post-synaptic densities (A, open arrow) with axons containing round (black line) and elongated (red line) synaptic vesicles. Asymmetric synapses display a thin pre- and a thick post-synaptic density (B, arrowhead) whilst perforated synapses are asymmetric synapses with a notable discontinuity (> 50nm) in the electron density of the post-synaptic junctions (C, curved arrows), but in both cases with axons containing just round synaptic vesicles. Scale bars: 125 nm (A), 250 nm (B and C). Key: AT: axon terminal, SVs: synaptic vesicles, usp: unlabelled spine.
Supplementary Figure 2. Bar graphs showing the age effect on SERT-Te numerical density (Nv, #/µm³) within the CA1 stratum oriens (A) and the CA1 stratum radiatum (B) of the hippocampus at 3 and 18 months in non-Tg control and 3xTg-AD. Bars represent mean ± S.E.M., # = p<0.05 compared to the same genotype at 3 months of age, un-paired t-test.
Supplementary Figure 3. Dual confocal micrographs showing SERT-IR fibres in the dorsal hippocampus of 18 months old non-Tg control (A) and a 3xTg-AD mouse at 18 months of age (B), the latter containing Aβ plaques (C) showing SERT-IR fibres specific sprouting adjacent to Aβ plaques (straight arrows) compared to regions with no Aβ plaques (curved arrows) within the CA1 subfield of the hippocampus in an 18 month 3xTg-AD mouse (D, merged image). Scale bars: 20μm A – D.
From personal library.
**Supplementary Table 1.** Summary of changes in synaptic density in different transgenic mouse models of AD.

<table>
<thead>
<tr>
<th>Transgenic mouse Model</th>
<th>Neuropathology</th>
<th>Age (months)</th>
<th>Method used</th>
<th>Brain Area Investigated</th>
<th>Change in Synaptic Density vs control (%)</th>
<th>Reference</th>
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<td>FC, H</td>
<td>Reduced SYN</td>
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<td>3 and 18</td>
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<td>H</td>
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<td>(Noristani et al. 2011)</td>
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</table>

**Key:** SYN-IR: synaptophysin immunoreactivity, NeoC: neocortex, H: hippocampus, FC: frontal cortex, EM: electron microscopy, PS: perforated synapse. Note that highlighted in yellow are studies that reported decrease in synaptophysin immunoreactivity, highlighted in green are studies reported no changes, whilst highlighted in red are studies that reported increase in synaptophysin immunoreactivity. In addition, highlighted in blue are electron microscopic studies that reported reduced perforated synapse in the 3xTg-AD mouse model of AD.
Chapter 5

Tryptophan as a potential AD recovery path

High L-tryptophan diet reduces CA1 hippocampal intraneuronal β-amyloid accumulation but does not affect serotonergic fibre sprouting in the triple transgenic mouse model of Alzheimer’s disease.

5.1. ABSTRACT

Alzheimer’s disease (AD) is a progressive neurodegenerative disease that impairs mnesic functions via the accumulation of neuritic β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) in specific brain regions associated with learning and memory including the hippocampus. Aβ deposition starts inside the neurone and is then secreted to the extracellular space contributing to the build-up of neuritic plaques. The raphe nuclei gives rise to serotonin (5-HT) projections that are extensively present in the hippocampus, being a key neurotransmitter involved in learning and memory. Results from chapters 3 and 4 reported increased 5-HT transporter (SERT) fibres and terminals in the hippocampal CA1 subfield in an AD triple transgenic (3xTg-AD) mouse model, which occurs concomitant with intraneuronal Aβ deposition. L-tryptophan (TrP) is an essential amino acid that acts as 5-HT precursor. The current study analysed the effect of chronic (1 month) food diets consisting of low, normal and high TrP contents (LTrP, NTrP, HTrP, 0.04, 0.20 and 0.40g/100g) on CA1 intraneuronal Aβ deposition, SERT fibre density and 5-HT neurones in the dorsal (DR) and median raphe (MR) nuclei. The obtained result shows that 3xTg-AD mice fed with HTrP diet displayed reduced intraneuronal Aβ density (17% decrease) compared to 3xTg-AD mice in NTrP diet. In non-transgenic (non-Tg) control animals, HTrP diet induced an increase in SERT fibre density (by 35%) that was more evident in the stratum lacunosum moleculare (by 48%) compared to non-Tg mice in LTrP diet. 3x-Tg-AD animals showed increased SERT fibre density in the CA1 stratum lacunosum moleculare compared to diet-matched non-Tg control animals, irrespective of dietary TrP content (by 104% for LTrP, by 74% for NTrP and by 35% for HTrP diet). No differences were observed in the total 5-HT neurones in the DR and the MR nuclei between the 3xTg-AD and non-Tg control mice in all three diet groups. These results show for the first time that increased 5-HT neurotransmission via dietary TrP intake reduces AD-related neuropathology, suggesting that enhanced 5-HT neurotransmission may be effective not only in addressing behavioural abnormalities, as shown by the clear SERT fibre sprouting, but also in reducing AD-related neuropathology.
5.2. INTRODUCTION

Alzheimer’s disease (AD) is a devastating neurodegenerative disease that deteriorates learning, memory and cognition (Braak et al. 1999). Patients with AD also exhibit behavioural disturbances including agitation, irritability, anxiety, delusion and depression (Lyketsos & Olin 2002). The neuropathological hallmarks of AD include neuritic β-amyloid (Aβ) plaques, neurofibrillary tangles (NFTs), neuronal and synaptic loss (Selkoe 2002). Aβ is produced via the amyloidogenic processing of amyloid precursor protein (APP) that involves enzymatic cleavage of the protein by β and γ-secretases (Hardy & Allsop 1991). Aβ is firstly accumulated inside neurones followed by extracellular neuropil deposition in the form of neuritic plaques (Masters et al. 1985; Kang et al. 1987). Increasing evidence suggests that intraneuronal accumulation of Aβ initiates the neurodegenerative process in AD brains (Rodríguez et al. 2008; Wirths et al. 2009; Umeda et al. 2011), for review see (Wirths et al. 2004; Gimenez-Llort et al. 2007; Gouras et al. 2010).

In addition to impaired cholinergic (ACh) neurotransmission (Birks & Melzer 2000), AD also affects other neurotransmitter systems including glutamate (Miguel-Hidalgo et al. 2002) and serotonin (5-HT) (Mowla et al. 2007; Mossello et al. 2008; Noristani et al. 2010) (see also chapter 1, section 1.1.1.6.). The dorsal (DR) and median raphe (MR) nuclei encompass the majority of 5-HT neurones that project to multiple brain regions including the brain stem, the thalamus, the cortex and the hippocampus; therefore playing an important role in memory and cognition (Vertes 1991; Vertes et al. 1999; Schmitt et al. 2006; Evers et al. 2007). In fact, AD patients show reduced 5-HT neurotransmission, which correlate with the disease severity (Garcia-Alloza et al. 2005; Mossello et al. 2008). Indeed, treatment with selective serotonin re-uptake inhibitors (SSIRs) increases 5-HT neurotransmission and not only improves cognition in AD patients but also reduces behavioural disturbances associated with AD (Mowla et al. 2007; Mossello et al. 2008; Rozzini et al. 2010).

L-tryptophan (TrP) is an essential amino acid that acts as 5-HT precursor (Cooper & Melcer 1961); therefore 5-HT synthesis and its availability is influenced by TrP
Alteration in dietary TrP intake is frequently used as a non-invasive method to manipulate systemic TrP levels and therefore central 5-HT neurotransmission (Fadda 2000; van der Stelt et al. 2004; Cahir et al. 2007). Changes in dietary TrP content alter basal extracellular 5-HT levels in multiple brain regions including the hippocampus (van der Stelt et al. 2004; Jenkins et al. 2010). In addition, reduced TrP intake impairs learning and memory not only in animals (Nomura 1992; Lieben et al. 2004; Uchida et al. 2007; Jenkins et al. 2010), but also in healthy human volunteers (Riedel et al. 1999; Schmitt et al. 2000; Merens et al. 2008; Sambeth et al. 2009), for review see (Mendelsohn et al. 2009; Silber & Schmitt 2010). In patients with AD, reduced TrP intake deteriorates cognitive function (Porter et al. 2000; Newhouse et al. 2002; Porter et al. 2003). Therefore, chronic increase in 5-HT neurotransmission (by oral administration of TrP) is associated with improved memory acquisition, consolidation and storage in rodents (Haider et al. 2006; Khaliq et al. 2006; Haider et al. 2007), whilst daily TrP injections also improved spatial memory in aged rats (Levkovitz et al. 1994; Richter-Levin & Segal 1996).

In vitro studies have shown that application of 5-HT promotes the non-amyloidogenic processing of APP metabolite (APPs) (Nitsch et al. 1996; Robert et al. 2001). It has also been shown that increased 5-HT neurotransmission (via treatment with SSRI) reduced APP translation and lowered the pathogenic Aβ peptide secretion hence potentially preventing Aβ deposition in AD (Payton et al. 2003; Morse et al. 2004; Pakaski et al. 2005). These findings are supported by an in vivo study in which chronic treatment with SSRI reduced both the presence of Aβ plaques and NFTs in the triple transgenic (3xTg-AD) mouse model of AD (Nelson et al. 2007b), which mimics the spatio-temporal neuropathology and mnesic alterations of AD brains (Oddo et al. 2003a; Oddo et al. 2003b).

On line with this, a biphasic increase in serotonin transporter-immunoreactive (SERT-IR) fibres and terminals (SERT-Te) were reported in chapters 3 and 4, which take place concomitant with the development of AD-related neuropathology in 3xTg-AD mice (Noristani et al. 2010; Noristani et al. 2011). The initial increase in hippocampal SERT-IR fibre density appeared at 3 months of age, which occurs in parallel with the evident intraneuronal accumulation of Aβ in 3xTg-AD mice.
(Mastrangelo & Bowers 2008; Rodríguez et al. 2008; Noristani et al. 2010); whilst there was a late sprouting recurrence at 18 months, concomitant with the build-up of large extracellular neuritic plaques in 3xTg-AD mice. However, the relationship between altered 5-HT neurotransmitter levels and serotonergic fibre density is not clearly understood. In addition, it remains to be determined how altered 5-HT neurotransmission, via increased dietary TrP intake, may affect AD-related neuropathology.

The current chapter analysed the chronic (1 month) effect of increasing/reducing dietary TrP intake on hippocampal intraneuronal Aβ accumulation in 3xTg-AD mice, which actually is one of the best representative model of the disease (Rodríguez et al. 2008; Rodríguez et al. 2009b). Furthermore, it aimed to examine the real effect of altered TrP diet on hippocampal SERT-IR fibre density and the total number of 5-HT neurones in the DR and MR nuclei, which could uncover new therapeutic approaches in this devastating disease.
5.3. MATERIALS AND METHODS

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

5.3.1. Animals

The procedures for generating 3xTg-AD mice have been described previously (Oddo et al. 2003a; Oddo et al. 2003b; Rodríguez et al. 2008; Rodríguez et al. 2009a; Rodríguez et al. 2009b). Briefly, human cDNA harbouring the Swedish APP mutation (KM670/671NL) and the human P301L four repeats mutated tau, were co-microinjected into a single-cell embryo of a homozygous PS1M146V knock-in mouse. The background of the PS1 knock-in mouse is a hybrid 129/C57BL6. The non-transgenic (non-Tg) control mice used were also from the same strain and genetic background as the PS1 knock-in mice, but they harbour the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12h light-dark cycles with free access to food and water independent of the diet.

5.3.2. Diets

Following birth and weaning period (P21) the animals were housed in same-sex groups of at least 4 mice in standard laboratory housing environment until 2 months of age, having free access to standard rodent chow and water. At 2 months of age, male non-Tg control (n = 15) and 3xTg-AD (n = 21) mice were weighted and randomly assigned to three dietary conditions consisting of low, normal and high TrP contents (LTrP, NTrP, HTrP n = 5 for non-Tg control and n = 7 for 3xTg-AD mice in each diet condition, Fig. 5.1). All TrP diets were manufactured and purchased from the Special Diets Services Ltd. (SDS, UK). The diets were prepared in pellet forms and were isocaloric. Table 5.1 indicates the composition of different amino acids in all three TrP diets used in the current study. NTrP diet contained 0.20g TrP/100 g of the food, whilst the TrP contents in the LTrP and the HTrP diets were 0.04 and 0.40g TrP/100g, respectively (20 and 200% of the NTrP diet).
Except TrP, other amino acid contents were identical between the three experimental diets (see Table 5.1). Mice had free access to their respective diets and water during the 30 days of the experiment, allowing them to acclimate to their respective diets, thus minimising any metabolic disequilibrium due to diet alterations. Body weight, food intake and water intake was measured daily throughout the experiment. All animals were perfused at 3 months of age (Fig. 5.1).

The 1 month period of dietary TrP intake was chosen because previous studies have shown that 1 month of TrP intake is sufficient to induce significant changes in 5-HT neurotransmission in multiple brain regions including the hippocampus (Haider et al. 2006; Cahir et al. 2007; Haider et al. 2007; Jenkins et al. 2010). In addition, 3 months of age was chosen because previous studies including our own have shown that in 3xTg-AD mice, intraneuronal accumulation of Aβ initiates at this age (Mastrangelo & Bowers 2008; Rodríguez et al. 2008). Intraneuronal accumulation of Aβ is associated with deterioration of cognitive function including long-term synaptic plasticity and memory retention in the 3xTg-AD mouse model of AD (Oddo et al. 2003b; Billings et al. 2005). Furthermore, earlier studies in chapters 3 and 4 have shown increased hippocampal SERT-IR fibres and terminals (SERT-Te) density in 3xTg-AD mice at 3 months of age (Noristani et al. 2010; Noristani et al. 2011).

![Experimental design](image)

**Figure 5.1.** Experimental design. Following the weaning period (P21) male 3xTg-AD and non-transgenic (non-Tg) control mice were housed in standard laboratory housing environment until 2 months of age. At 2 months of age mice were randomly assigned into three dietary conditions consisting of LTrP, NTrP and HTrP contents (0.04, 0.20 and 0.40g/100g) for 1-month period. All animals were sacrificed by perfusion at 3 months of age. **Key:** LTrP: low TrP diet, NTrP: normal TrP diet, HTrP: high TrP diet.
Table 5.1. Amino acid composition (g/100g) of low, normal and high TrP diets.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>LTrP Diet</th>
<th>NTrP Diet</th>
<th>HTrP Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
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<td>1.19</td>
<td>1.19</td>
</tr>
<tr>
<td>Lysine</td>
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<tr>
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<td>0.39</td>
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<tr>
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<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
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<td>0.78</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>0.80</td>
<td>0.80</td>
</tr>
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<td>1.18</td>
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<tr>
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</tr>
<tr>
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<td>0.79</td>
<td>0.79</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>Glycine</td>
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<tr>
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<td>2.97</td>
<td>2.97</td>
</tr>
<tr>
<td>Energy (kcal/kg)</td>
<td>3840.28</td>
<td>3839.51</td>
<td>3838.54</td>
</tr>
</tbody>
</table>

5.3.3. Fixation and Tissue Processing

Male 3xTg-AD and non-Tg control mice were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 3 months of age. To investigate the level of intraneuronal Aβ in 3xTg-AD mice before TrP diet experiment, another group of male 3xTg-AD were perfused at 2 months of age (n = 7). All non-Tg control and 3xTg-AD mice were perfused through the aortic arch with 3.75% acrolein (TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, UK) and 0.1M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde. Brains were then removed and cut into 4 – 5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus, as described previously (Rodríguez et al. 2008). These slaps of brain tissues were post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1M PB, pH 7.4. Coronal sections of the brain were cut into 40 – 50μm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal brain sections at levels -1.58
mm/-2.46 mm (hippocampus) and -4.36 mm/-4.96 mm (raphe nuclei) posterior to bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004).

5.3.4. Antibodies
A polyclonal rabbit antibody raised against a synthetic peptide sequence corresponding to amino acids 602 – 622 of the rat 5HT transporter (Immunostar, Hudson, WI, USA) was used for the determination of SERT-IR fibre density in the hippocampus. 5-HT neurones in the raphe nuclei were studied using a polyclonal rabbit antibody antiserum generated against 5-HT (Immunostar, USA). Monoclonal mouse antibody against amino acid residues 1 – 16 of beta amyloid (Aβ, Covance, Emeryville, CA, USA) was used to detect intraneuronal Aβ accumulation in the hippocampus. The specificity of the antibodies has been reported previously using immunohistochemistry (Mamounas et al. 2000; Rodríguez et al. 2008; Rodríguez et al. 2009b; Noristani et al. 2010; Olabarria et al. 2010) and western blots (Albright et al. 2007). To determine the specificity of the antibodies, adsorption controls were done using SERT and serotonin-specific peptides, respectively, which resulted in total absence of target labelling. Furthermore, omission of primary and/or secondary antibodies also showed no immunoreactivity (refer to material and methods chapter (chapter 2, Fig. 2.2 and Fig. 2.3) for negative control images of SERT and 5-HT immunoreactivity).

5.3.5. Immunohistochemistry
The selected brain sections were incubated for 30 minutes in 30% methanol in 0.1M PB and 3% hydrogen peroxide (H₂O₂, Sigma, Gillingham, UK). Brain sections were then rinsed with 0.1M PB for 5 minutes and placed in 1% sodium borohydride (Aldrich, UK) for 30 minutes. The brain sections were washed with PB profusely before rinsing in 0.1M TS for 10 minutes. Brain sections were then incubated in 0.5% bovine serum albumin (BSA, Sigma, Gillingham, UK) in 0.1M TS and 0.25% Triton (Sigma, Gillingham, UK, x 100) for 30 minutes. Brains sections were incubated for 48 hours at room temperature in primary antibody (rabbit anti-SERT, 1:2500, rabbit anti-5-HT, 1:5000, Immunostar, Hudson, WI, USA and mouse anti-Aβ, 1:2000, Covance, USA). The brain sections were rinsed in 0.1M TS for 30 minutes and incubated in 1:400 dilutions of biotinylated donkey
anti-rabbit IgG (Jackson Immunoresearch, Stratech Scientific Ltd., Soham, UK) for 1 hour at room temperature. Brain sections were then rinsed with 0.1M TS for 30 minutes followed by incubation for 30 minutes in avidin-biotin peroxidase complex (ABC, Vector Laboratories Ltd., Peterborough, UK). The peroxidase reaction product was visualised by incubating the brain sections in a solution containing 0.022% of 3,3’-diaminobenzidine (DAB, Aldrich, Gillingham, UK) and 0.003% H$_2$O$_2$ for 6 minutes, as previously described (Rodríguez et al. 2008; Rodríguez et al. 2009a; Noristani et al. 2010). The reaction was stopped by rinsing the brain sections in 0.1M TS for 6 minutes followed by 0.1M PB for 15 minutes. Brain sections were permanently mounted onto gelatinised slides and allowed to dry overnight. Brain sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and finally xylene. Coverslips were applied using Entellan (Merck KGaA, Germany) and slides were left to dry overnight.

5.3.6. Optical Density (OD) Measurement
Using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA), the expression and density of SERT-IR fibres and intraneuronal Aβ was analysed by measuring their optical density (OD), as we have described previously (Cordero et al. 2005; Noristani et al. 2010) (see chapter 2, section 2.4.1. for detailed description of OD measurement). In brief and to exclude any experimental errors and/or bias, all images were taken at a constant light intensity. Optical filters (which adjust light intensity) were used to ensure the specificity of the signal recorded by the camera. The OD was calculated from a relative scale of intensity ranging from 0 – 255, with a measurement of 255 corresponding to the area with very low SERT-IR fibres and 0 corresponding to the densest area of SERT labelling, as previously described (Noristani et al. 2010). The calibration density was kept constant for measuring all brain sections to avoid experimental variability. Non-specific OD in brain sections was measured from the corpus callosum. SERT-IR fibre density of the complete CA1 subfield of the hippocampus and its different layers (pyramidal cell layer (PCL), stratum oriens (S.Or), stratum radiatum (S.Rad) and stratum lacunosum-moleculare (S.Mol), except CA3 where stratum lucidum, was also studied, were measured independently. Similarly, SERT-IR fibre density of the dentate gyrus and its different layers (granule cell layer (GCL), molecular layer (ML) and hilus) were measured individually (see chapter 2, Fig. 2.4). The
density of intraneuronal Aβ was measured in the pyramidal cell layer of CA1, CA2 and CA3 subfield of the hippocampus. To analyse the change in SERT-IR fibre density and intraneuronal Aβ against a constant control, 255 was divided by control region (corpus callosum) and the obtained factor was multiplied by the region of interest in every given section as described previously (Noristani et al. 2010). Inverse optical density (IOD) was obtained by subtracting from the obtained background level (255). Measurements of the mean density were taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each slice. The results are shown as inverse optical density (IOD/pixel).

5.3.7. Cell count of 5-HT neurones in the raphe nuclei
To determine whether alteration in dietary TrP intake have an effect on 5-HT neurone density, the total number of 5-HT-immunoreactive (5-HT-IR) neurones was estimated in the dorsal (DR) and median raphe (MR) nuclei of 3xTg-AD and non-Tg control mice. The areas analysed for 5-HT cell count included the dorsal raphe dorsal (DRD), the dorsal raphe ventral (DRV), the dorsal raphe interfascicular part (DRI), the MR and the para-median raphe (PMR) nuclei. The boundaries of areas in which 5-HT-IR neurones were to be counted were clearly delineated, thus, counts were reproducible and counting 5-HT stained neurones in every third section constituted a true random sample (Vertes & Crane 1997). All 5-HT-IR neurones were intensely labelled against light background that made them easy to identify with equal chance of being counted (Vertes & Crane 1997). The main source of error in using this calculation is the potential multiple counting of the same profile in more than one brain section, as previously suggested (Vertes & Crane 1997; Noristani et al. 2010). However, in this case, one has to consider that the maximum cell diameter of the 5-HT neurones counted was approximately 25 – 30μm and every third 40μm section was 120μm distant from the adjacent one, making multiple counting of the same cell profile in adjacent brain sections highly unlikely, as described previously (Vertes & Crane 1997; Noristani et al. 2010). To obtain a systemic random sampling of 5-HT-IR neurones a sampling grid, consisting of counting frame, was positioned over the DR and the MR nuclei on each brain section. The number of 5-HT-IR neurones was determined blindly by a
single observer (H.N.N) using a 10 x 10 mm graticule. All visible 5-HT-IR neurones were counted in every third 40µm thickness coronal brain sections throughout the rostrocaudal extent of the different sub-divisions of the raphe nuclei corresponding to bregma -4.36/-4.96 mm (Paxinos & Franklin 2004). To define DR and MR as well as their sub-nuclei boundaries, adjacent brain sections were counterstained with toluidine blue (see chapter 2, Fig. 2.8). The estimated total 5-HT-IR neurones within the raphe nuclei were calculated according to Konigsmark equation (Konigsmark 1970), as described previously (Vertes & Crane 1997; Noristani et al. 2010) (refer to supplementary section of the material and methods chapter (chapter 2, section 2.S.6.) for detailed description of the Konigsmark equation):

\[ N_t \div n_s = V_t \div v_s \]

Where \( N_t \) (total count), \( n_s \) (sample count), \( V_t \) (total volume, range from 0.55 mm\(^3\) – 0.65 mm\(^3\)) and \( v_s \) (sample volume).

Composite figures, adjusted for brightness, contrast and unsharpness, were generated using Adobe Photoshop CS2 (Adobe Systems Inc. San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).

### 5.3.8. Statistical analysis

Results are expressed as mean ± standard errors of the mean (S.E.M.). Individual body weight was measured daily, average food and water intake was measured by dividing the total amount consumed per number of mice per group. At 2 months of age, the 3xTg-AD mice showed significant increase in body weight (by 11%, \( p = 0.0288 \)) and food intake (by 10%, \( p = 0.0097 \)) compared to age-matched non-Tg control group. To avoid the possible effect of differences in body weight on food and water intake, food and water consumption was analysed as percentage of body weight between 3xTg-AD and non-Tg control mice (Fig. 5.2). Un-paired t-test was used to determine changes in body weight, food intake, water intake, as well as for the differences in SERT-IR fibre density, 5-HT neurones and intraneuronal Aβ density. Significance was accepted at \( p \leq 0.05 \). All obtained data were analysed using GraphPad Prism 4.0 (GraphPad Software, Inc. La Jolla, CA, USA).
5.4. RESULTS

5.4.1. Dietary TrP intake-associated changes in body weight gain, food and water intake

Mice fed with LTrP diet showed reduced body weight gain irrespective of their genotype, when compared to animals on NTrP and/or HTrP diets (Fig. 5.2A). Both non-Tg control and 3xTg-AD mice showed significant reduction in their body weight gain from 1 week of LTrP diet compared to animals in NTrP diet (5%, p = 0.0273 for non-Tg and 6%, p < 0.0001 for 3xTg-AD, respectively; Fig. 5.2A). LTrP diet-induced decrease in body weight was sustained during all the experimental period in both non-Tg control and 3xTg-AD groups compared to NTrP diet group (12%, p = 0.0115 for non-Tg and 10.5%, p = 0.0037 for 3xTg-AD, respectively; Fig. 5.2A). Non-Tg control and 3xTg-AD mice fed with HTrP diet showed no differences in their body weight gain compared to same genotype animals in NTrP diet group (Fig. 5.2A).

Despite the significant decrease in body weight gain, non-Tg control animals showed significant increase in food intake after 3 weeks of LTrP diet (103%, p = 0.0069, Fig 5.2B). LTrP-induced increase in food consumption was maintained during all the experimental period in non-Tg control mice compared to non-Tg control mice fed with NTrP diet (67%, p = 0.0015, Fig. 5.2B). Non-Tg control mice fed with HTrP diet showed no difference in food consumption compared to non-Tg mice in NTrP diet group (Fig. 5.2B). No difference in food intake was observed in 3xTg-AD mice fed with either LTrP or HTrP diets compared to 3xTg-AD mice in NTrP diet.

In addition to increased food intake, non-Tg control mice also showed significant increase in water consumption after 1 week of LTrP diet (107%, p < 0.0001, Fig. 5.2C). Increased water intake was continuously evident in non-Tg control mice fed with LTrP diet during all experimental period compared to non-Tg mice in NTrP diet group (294%, p < 0.0001). Similarly, 3xTg-AD mice fed with LTrP diet also showed significant increase in water intake from 2 weeks of LTrP diet (55%, p = 0.0215), which was maintained during all experimental period compared to 3xTg-AD mice in NTrP diet (46%, p = 0.0370, Fig. 5.2C). Both non-Tg control and
3xTg-AD animals fed with HTrP diet showed no differences in water intake compared to same genotype animals in NTrP group (Fig. 5.2C).

When comparing water intake between the two genotypes, 3xTg-AD animals fed with NTrP and HTrP diets showed significant increase in water intake starting after 1 week of the diet (79%, p = 0.0031 for NTrP and 79%, p = 0.0316 for HTrP, respectively; Fig. 5.2C). This increase in water intake was sustained during all the experimental period (106%, p = 0.0011 for NTrP and 124%, p = 0.0033 for HTrP, respectively; Fig. 5.2C).

Overall during the 1-month period, non-Tg control mice fed with LTrP diet group consumed significantly more food (25%, p = 0.0422, chapter 5 supplementary Fig. 1A) and water (271%, p < 0.0001, chapter 5 supplementary Fig. 1B) compared to non-Tg mice fed with NTrP diet. On the other hand, 3xTg-AD mice fed with LTrP diet showed a non-significant increase (6%, p = 0.4211, chapter 5 supplementary Fig. 1A) in food consumption but a significant increase in water consumption (48%, p = 0.0076, chapter 5 supplementary Fig. 1B) compared to 3xTg-AD mice in NTrP diet.
**Figure 5.2.** Line graphs showing the TrP diet effect on weekly body weight gain (A), food intake (B) and water intake (C). *, ** and *** = $p<0.05$, $p<0.01$ and $p<0.001$ non-Tg fed with low TrP diet compared to non-Tg group fed with normal TrP diet. *, ** and *** = $p<0.05$, $p<0.01$ and $p<0.001$ 3xTg-AD fed with low TrP diet compared to 3xTg-AD fed with normal TrP diet. #, ##, ### = $p<0.05$, $p<0.01$ and $p<0.001$ 3xTg-AD fed with normal TrP diet compared to diet-matched non-Tg. #, ##, ### = $p<0.05$ and $p<0.01$ 3xTg-AD fed with high TrP diet compared to diet-matched non-Tg. # = $p<0.05$ 3xTg-AD fed with low TrP diet compared to diet-matched non-Tg, un-paired t-test.
5.4.2. Dietary TrP intake-associated changes on intraneuronal Aβ density in the hippocampus

Immunohistochemical analysis of brain from 2 and 3 months old 3xTg-AD animals confirmed the presence of intraneuronal Aβ in the hippocampus (Fig. 5.3), the cortex and the amygdala, whereas no Aβ pathology was detected in non-Tg control mice. In the hippocampus, 3xTg-AD mice showed intraneuronal Aβ immunoreactivity in the pyramidal cell layer within CA1, CA2 and CA3 subfields, but with no apparent presence in the dentate gyrus (Fig. 5.3A, 5.3B and 5.3C). It was possible to observe a gradient in the density of intraneuronal Aβ in different subfields of the hippocampus that was reducing from CA1 to CA2 and CA3 subfields (Fig. 5.3A – 5.3H). There was an age-related increase in intraneuronal Aβ density in the hippocampus.

Quantitative analysis revealed that 3 months old 3xTg-AD mice fed with LTrP and NTrP diets for 1 month showed a significant increase in intraneuronal Aβ accumulation and optical density compared to 3xTg-AD mice before the start of the diet (2 months of age, 18%, p = 0.0371 for LTrP and 27%, p = 0.0006 for NTrP diets, respectively; Fig. 5.3G and 5.3H). However, 3xTg-AD mice fed with HTrP diet showed significant decrease in intraneuronal Aβ density in CA1 pyramidal cell layer compared to age-matched 3xTg-AD mice in NTrP diet (17% decrease, p = 0.0460, Fig. 5.3G), which is equivalent to 3xTg-AD mice at 2 months of age (p = 0.6197, Fig. 5.3C, 5.3F and 5.3G). However, no significant differences were observed in intraneuronal Aβ density in either the CA3 subfield of the hippocampus or the amygdala between 3xTg-AD mice fed with HTrP and NTrP diets (chapter 5, supplementary Fig. 2). On the other hand, 3xTg-AD mice fed with LTrP diet showed no significant difference in intraneuronal Aβ density compared to 3xTg-AD mice in NTrP diet in any of the studied brain regions (see Fig. 5.3 and chapter 5, supplementary Fig. 2).

5.4.3. Dietary TrP intake-associated changes on hippocampal serotonergic fibre density in non-Tg control animals

In both non-Tg control and 3xTg-AD mice SERT-IR fibres were heterogeneously distributed throughout the hippocampal formation (Fig. 5.4). SERT-IR fibres appear mainly as fine and thick processes with numerous varicosities, which are
characteristic of axonal profiles (Fig. 5.4A – 5.4F). The highest density of SERT-IR was evident in the S.Mol with S.Rad and S.Or expressing moderate levels, whilst PCL of the hippocampus displayed very low SERT-IR fibre density (Fig. 5.4A – 5.4F). Qualitative analysis showed a general increase in hippocampal SERT-IR fibre density in non-Tg control mice fed with HTrP compared to animals in LTrP/NTrP diets (Fig. 5.4A, 5.4B and 5.4C).

However, quantitative analysis in non-Tg control mice revealed no significant differences in SERT-IR fibre density between LTrP and NTrP diet groups. Comparisons between non-Tg control mice in NTrP and HTrP diet groups revealed a non-significant increase in SERT-IR fibre density in the CA1 subfield of the hippocampus and in the S.Mol (19.6 vs 25.5, 30%, p = 0.0662 for CA1 subfield and 25.6 vs 33.6, 31%, p = 0.0916 for S.Mol, respectively; Fig. 5.4G and 5.4H). However, when comparing between HTrP and LTrP diets, non-Tg control mice fed with HTrP diet showed significant increase in SERT-IR fibre density in the CA1 subfield and the S.Mol compared to non-Tg control mice in LTrP diet (18.8 vs 25.5, 35%, p = 0.0417, for CA1 subfield and 26.6 vs 33.6, 48%, p = 0.0253, for S.Mol, respectively; Fig. 5.4H and 5.4G).

Unlike non-Tg control group, 3xTg-AD mice showed stable SERT-IR fibre density irrespective of dietary TrP contents (Fig. 5.4D, 5.4E and 5.4F). Quantitative analysis revealed no differences in SERT-IR fibres density between 3xTg-AD mice fed with LTrP diet compared to NTrP/HTrP diets (Fig. 5.4G and 5.4H).

Comparison between the two genotypes revealed significant increase in SERT-IR fibre density in CA1 subfield of the hippocampus in 3xTg-AD mice compared to age-matched non-Tg controls in LTrP and NTrP but not HTrP diet group (18.8 vs 28.9, 54%, p = 0.0234 for LTrP, 19.6 vs 29.8, 52%, p = 0.0188 for NTrP and 25.5 vs 29.4, 15%, p = 0.0998% for HTrP diet, Fig. 5.4G). However, 3xTg-AD mice exhibit significant increase in SERT-IR fibre density in the CA1 S.Mol compared to non-Tg control, irrespective of dietary TrP contents (22.6 vs 46.1, 104%, p = 0.0046 for LTrP, 25.6 vs 44.5, 74%, p = 0.0108 for NTrP and 33.6 vs 45.4, 35%, p = 0.0060 for HTrP diet, Fig. 5.4H).
Figure 5.3. Brightfield micrographs showing the distribution of intraneuronal Aβ in the pyramidal cell layer of the hippocampus in the 3xTg-AD mouse model of AD at 2 months of age at the start of the experiment (A, E) and at 3 month old animals fed with LTrP (B, F), NTrP (C, G) and HTrP (D, H) diets for 1 month period. I: Bar graph showing the effect of altered dietary TrP intake on intraneuronal Aβ density in the hippocampal CA1 pyramidal cell layer in 3xTg-AD animals fed with LTrP, NTrP and HTrP diets. Bar represent mean ± S.E.M. of inverse optical density readings (n = 5 – 7). **, *** = p<0.01, p<0.001 compared to 2 months 3xTg-AD, un-paired t-test. Scale bars: A – C = 500µm, D – F = 100µm. Key: IOD: inverse optical density, LTrP: low TrP diet, NTrP: normal TrP diet, HTrP: high TrP diet.
Figure 5.4. Brightfield micrographs showing SERT-IR fibres within the dorsal hippocampus of 3 months non-Tg control (A, B, C) and 3xTg-AD mice (D, E, F) fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets for 1 month period. Bar graphs showing the effect of altered dietary TrP intake on SERT-IR fibre density within the CA1 subfield of the hippocampus (G) and CA1 stratum lacunosum moleculare (H) between non-Tg control and 3xTg-AD groups. Bars represent mean ± S.E.M. of inverse optical density readings ($n = 5–7$). #, ##, = $p<0.05$, $p<0.01$ compared to diet-match non-Tg, = $p<0.05$ compared to non-Tg mice fed with LTrP diet, un-paired t-test. Scale bars: A – F = 250µm, Insets = 100µm. Key: S.Or: stratum oriens, PCL: pyramidal cell layer, S.Rad: stratum radiatum, S.Mol: stratum lacunosum moleculare, IOD: inverse optical density, LTrP: low TrP diet, NTrP: normal TrP diet, HTrP: high TrP diet. Adsorption control using SERT-specific peptide resulted in total absence of SERT-IR fibres (see Chapter 2, Fig. 2.2).

5.4.4. Dietary TrP does not affect the total number of 5-HT neurones in the dorsal and the median raphe nuclei

5-HT immunoreactive (5-HT-IR) neurones were distributed throughout the different sub-divisions of both the DR (Fig. 5.5A – 5.5G) and MR (Fig. 5.5H) nuclei. 5-HT-IR somatodendritic profiles were characterised by small rounded cell bodies with sparse dendritic arborisations (Fig. 5.5A – 5.5E insets). 5-HT-IR
neurones were observed within different sub-nuclei of the DR including the DRD, the DRV and the DRI (Fig. 5.5A – 5.5F). The 5-HT-IR neuronal population were also detected within both the MR and the PMR nuclei (Fig. 5.5H). Altered dietary TrP intake had no effect on 5-HT neurone morphology in the 3xTg-AD and non-Tg mice (Fig. 5.5A – 5.5F insets). The distribution and total number of 5-HT-IR neurones also showed no significant differences in either the DR or the MR nuclei between the 3xTg-AD and non-Tg mice in LTrP, NTrP and HTrP diet groups (Fig. 5.5G, 5.5H).
Figure 5.5. Brightfield micrographs showing the distribution of serotonin-IR neurones within the dorsal raphe nucleus of non-Tg control (A, B, C) and 3xTg-AD mice (D, E, F) fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets for 1 month period. Bar graphs showing the effect of altered dietary TrP intake on the total number of 5-HT-IR neurones in the dorsal (G) and the median (H) raphe nuclei between non-Tg control and 3xTg-AD group. Bars represent mean ± S.E.M., (n = 5 – 7). Scale bar: A – F = 500µm, insets = 100µm. Key: Aq: aqueduct, DRD: dorsal raphe dorsal, DRV: dorsal raphe ventral, DRI: dorsal raphe interfascicular part, LTrP: low TrP diet, NTrP: normal TrP diet, HTrP: high TrP diet. Adsorption control using serotonin-specific peptide resulted in total absence of 5-HT labelled neurones (see Chapter 2, Fig. 2.3).
5.5. DISCUSSION

The present study demonstrates, for the first time in the 3xTg-AD mouse model of AD, that increase in dietary intake of L-tryptophan (TrP) reduced Aβ neuropathology in the hippocampus, whilst having no effect on hippocampal serotonergic fibre density and total 5-HT neurones in the DR and MR nuclei.

5.5.1. LTrP diet reduces body weight gain whilst increasing food and water consumption

Young 3xTg-AD mice weigh significantly more compared to age-matched non-Tg control mice. This observation is consistent with previous reports, which also showed higher body weight in 3xTg-AD mice at 2 months (Knight et al. 2010) and 4 months of age compared to non-Tg control mice (Julien et al. 2010; Arsenault et al. 2011). The underlying mechanism responsible for increased body weight is not clear, although possible factors may include accelerated growth and an increase in the deposition of fat tissues (adiposity) that remains to be determined (Knight et al. 2010).

LTrP diet reduced body weight gain in both non-Tg control and 3xTg-AD groups compared to mice fed with NTrP diet (Fig. 5.2A). These findings are in agreement with previous studies in normal rats (Gonzalez-Burgos et al. 1998; Orozco-Suarez et al. 2003; Cahir et al. 2007; Jenkins et al. 2010), mice (Kantak et al. 1980; De Marte & Enesco 1986) and chickens (Carew et al. 1983). LTrP diet decreases plasma level of TrP (De Marte & Enesco 1985) that may in turn reduce the level of protein synthesis necessary for normal growth, as suggested previously (Kantak et al. 1980; De Marte & Enesco 1986; Gonzalez-Burgos et al. 1998). In addition, deficiency in dietary TrP intake also alters thyroid and growth hormone levels that is associated with reduced bone growth and body weight gain in chickens (Carew et al. 1983).

Chronic LTrP diet induced an increase in food and water consumption (Fig. 5.2B, 5.2C). Repeated intraperitoneal injection of TrP suppressed food intake in non-fasted mice (Coskun et al. 2006) and rats (Ju & Tsai 1995) as well as food-deprived and stressed rats (Amer et al. 2004). On the other hand, depletion of 5-HT synthesis
(via intraventricular injection of 5-HT neurotoxin, PCPA) increased food consumption in rats (Breisch et al. 1976). In addition, an increase in 5-HT level (via intraperitoneal injection of SSRI, sertraline) suppressed food intake in rats (Simansky & Vaidya 1990). Furthermore, preventing 5-HT metabolism (via administration of MAO-A inhibitor, clorgyline) reduced food intake in mice, rats and hamsters (Feldman 1988). As a possible mechanism, the LTrP diet-induced increase in food intake may be due to reduced TrP level in plasma and central 5-HT neurotransmission, which has an inhibitory effect on food consumption (Lam et al. 2010).

Others also reported increased water intake in mice following chronic decrease in dietary TrP intake (Kantak et al. 1980). Increasing evidence suggest that 5-HT exerts a negative effect on drinking behaviour (Castro et al. 2001) via activation of multiple 5-HT receptors (Reis et al. 1990; De Castro-e-Silva et al. 1997; Castro et al. 2002; de Arruda Camargo et al. 2010). Therefore, LTrP diet-induced increase in water intake may be due to reduced central 5-HT availability and activation of 5-HT receptors that exert a specific inhibitory effect on water intake.

5.5.2. Increased dietary TrP intake reduces intraneuronal Aβ in the CA1 subfield of the hippocampus

Previous studies, including our own, have demonstrated the presence of intraneuronal Aβ in the hippocampus of 3xTg-AD mice from 2 months of age (Rodríguez et al. 2008), which increases with advanced age (Mastrangelo & Bowers 2008). Such intraneuronal accumulation of Aβ in the hippocampus and the amygdala correlates with the earliest cognitive impairment (deficit in long-term memory retention) (Billings et al. 2005) as well as neurogenic impairment (Rodríguez et al. 2008) in 3xTg-AD mice. Immunotherapy and subsequent clearance of intraneuronal Aβ is associated with rescuing hippocampal-dependent memory function in 3xTg-AD mice (Billings et al. 2005).

Increased TrP intake enhances 5-HT neurotransmission in multiple brain regions including the hippocampus, which is associated with improved cognition in rodents (Haider et al. 2006; Haider et al. 2007). Clinical studies also have shown beneficial
effects of selective serotonin re-uptake inhibitors (SSRIs) not only in improving memory and cognitive functions but also in reducing behavioural abnormalities in AD patients (Schneider et al. 1991; Taragano et al. 1997; Mowla et al. 2007; Mossello et al. 2008; Rozzini et al. 2010). Chronic increase in dietary TrP intake reduced (by 17%) intraneuronal Aβ density in 3xTg-AD mice compared to age-matched 3xTg-AD animals fed with NTrP diet (Fig. 5.3G). These findings are in general agreement with a previous study in 3xTg-AD mice, where increasing 5-HT neurotransmission (via chronic treatment with SSRI, paroxetine) reduced Aβ and tau neuropathology (Nelson et al. 2007b). Interestingly, increased dietary TrP intake increases extracellular 5-HT and induces antidepressant effect similar to SSRI administration in rats (van der Stelt et al. 2004).

Aβ is an insoluble neurotoxic protein produced by the enzymatic cleavage of APP, through the amyloidogenic pathway involving β- and α-secretases (Hardy & Allsop 1991). Proteolytic cleavage by β-secretase generates slightly shortened sAPP-β molecule and a 99-residue portion (C99), which further undergoes cleavage by γ-secretases resulting in neurotoxic accumulation of insoluble Aβ peptides (Esler & Wolfe 2001). On the other hand, the non-amyloidogenic processing of APP involves α-secretase, which results in secretion of soluble form of APP (sAPPα) and an 83-residue portion (C83) that undergoes further enzymatic cleavage by γ-secretase, generating two soluble proteins namely: p3 and p6 (Esler & Wolfe 2001) see also (Fig. 5.6).

Although the underlying mechanisms involved in 5-HT-mediated decrease in intraneuronal Aβ accumulation has not been addressed in vivo, in vitro experiments suggest that 5-HT stimulates the non-amyloidogenic processing of APP metabolite (APPS) by stimulating sAPPα cellular release (Nitsch et al. 1996; Robert et al. 2001) (see also Fig. 5.6). In addition, paroxetine reduced APP translation and lowered pathogenic Aβ peptide secretion in vitro (Payton et al. 2003; Morse et al. 2004). Administration of citalopram (another commonly prescribed SSRI) and imipramine (a tricyclic anti-depressant) facilitates the soluble form of APP (APPS) secretion in vitro and may potentially prevent the accumulation of insoluble Aβ in AD brains (Pakaski et al. 2005). These findings are in agreement with a recent in
vivo study where acute administration of multiple SSRIs including fluoxetine, desvenlafaxine and citalopram reduced Aβ level in the brain interstitial fluid (measured using in vivo microdialysis) in the PS1APP transgenic mouse model of AD, which exhibit severe Aβ neuropathology (Cirrito et al. 2011). In addition, chronic treatment with SSRIs (citalopram and paroxetine) reduced Aβ plaque burden in the cortex and the hippocampus of the PA1APP and the 3xTg-AD transgenic mice, respectively (Nelson et al. 2007b; Cirrito et al. 2011); whilst in the latter model this reduction was also accompanied by NFT reduction and improved memory performance (Nelson et al. 2007b). Furthermore, a recent positron emission tomography (PET) clinical study had reported that exposure to SSRI antidepressants (for at least 5 years) reduced Aβ plaque load in cognitively normal individuals (Cirrito et al. 2011). Administration of 5-HT2A/2C agonist dextrofenfluramine (DEXNOR) increased APPS in the CSF, whilst reducing Aβ secretion in primary basal forebrain neuronal culture of guinea pigs (Arjona et al. 2002). Enhanced 5-HT neurotransmission following an increased dietary TrP intake may alleviate AD-related neuropathology by stimulating the non-amyloidogenic processing of APP, where the secreted protein is no longer available for the amyloidogenic pathway mediated by β- and γ-secretase cleavage (Fig. 5.6), as previously suggested for treatment with SSRI (Arjona et al. 2002; Pakaski et al. 2005). Another possible mechanism responsible for HTTrP diet-induced decrease in intraneuronal Aβ density could be due to inhibition of β-secretase activity as a result of increased central 5-HT neurotransmission (Fig. 5.6). In supported of this phenomenon, a recent study by Takahashi and Miyazawa (2011) have reported that 5-HT derivatives may inhibit β-secretase hence possibly reducing the amyloidogenic accumulation of Aβ in AD (Takahashi & Miyazawa 2011).
Figure 5.6. Amyloid precursor protein (APP) processing in AD. Non-amyloidogenic processing of APP via α-secretase results in secretion of soluble form of APP (sAPPα) and an 83-residue portion (C83) that undergoes further enzymatic cleavage by γ-secretase, generating two soluble proteins namely: p3 and p6 (left). On the other hand, the amyloidogenic processing of APP involves proteolytic cleavage by β-secretase resulting in the secretion of the slightly shortened sAPP-β molecule and the retention of a 99-residue portion (C99). Similar to C83, the C99 fragment undergoes cleavage by γ-secretase resulting in neurotoxic accumulation of insoluble Aβ peptides. Scale bars: 50µm (A – D).
5.5.3. Increased dietary TrP intake increases serotonergic fibre density in non-Tg control but has no effect on 3xTg-AD mice

Chronic increase in dietary TrP intake induced a significant increase in hippocampal SERT-IR fibre density in non-Tg control mice compared to animals fed with LTrP diet (Fig. 5.4). However, the effect of reducing dietary TrP content on hippocampal SERT-IR fibre density was less evident between LTrP and NTrP diets. Several mechanisms may be responsible for lack of change in SERT-IR fibre density between LTrP and NTrP diet groups including (i) differences in TrP contents between LTrP and NTrP diets and (ii) relatively later start of dietary experiment in relation to the brain development.

The minimum TrP level required for serotonergic fibre development is 0.01% (0.01g in 100g of diet) (Bell & John 1981). The LTrP diet used in the current study contained 0.02%, which is double the minimum required amount for the normal 5-HT fibre development (see Table 5.1). To induce an effect on SERT-IR fibre density, greater differences in TrP content might be required between the two diets. In support of this phenomenon, SERT-IR fibre density was significantly increased in non-Tg control mice fed with HTrP diet compared to LTrP diet groups, which has 10 times more TrP content (Fig. 5.4). In addition, the TrP diet experiment was started at 2 months of age, when the developmental process for 5-HT projections has already taken place (Lauder 1990; Bruning et al. 1997).

Increased hippocampal SERT-IR fibre density was observed in 3 months old 3xTg-AD mice compared to age-matched non-Tg control groups, irrespective of dietary TrP contents (Fig. 5.4). This observation is consistent with previous studies in chapters 3 and 4, which reported increase in SERT-IR fibre and SERT-Te density at same age in 3xTg-AD mice compared to non-Tg controls fed with standard rodent chow (Noristani et al. 2010; Noristani et al. 2011). Increased SERT-IR fibre density was associated with an increase in the area density ($S_v$, #/mm$^2$) of the fibres and increased numerical density ($N_v$, #/mm$^3$) of SERT-positive terminals, suggesting heterotrophic sprouting of hippocampal serotonergic fibres in 3xTg-AD mice as an intrinsic defensive mechanism against intraneuronal accumulation of Aβ (Noristani et al. 2010; Noristani et al. 2011).
Aβ-induced neurotoxicity involves glutamatergic excitotoxicity that is mediated by intracellular calcium (Ca\(^{2+}\)) influx (Brorson et al. 1995; Miguel-Hidalgo et al. 2002; Stutzmann 2007; Supnet & Bezprozvanny 2010). Increased 5-HT input counteracts the excitotoxic effect of glutamate by blocking Ca\(^{2+}\) channels and preventing membrane hyperpolarisation (Bayliss et al. 1997; Muramatsu et al. 1998; Williams et al. 1998). Previous result in chapter 4 also reported decreased asymmetric perforated synapses that further support altered hippocampal glutamatergic neurotransmission in 3xTg-AD mice at 3 months of age (Noristani et al. 2011). Increased hippocampal serotonergic input in the 3xTg-AD mice may indicate an intrinsic neuro-protective response to intraneuronal Aβ-induced damage by maintaining hippocampal functionality and connectivity.

Although HT\(\text{P}\) diet reduced intraneuronal Aβ density (by 17%) in 3xTg-AD mice, increased SERT-IR fibre density was still observed in this group (Fig. 5.3 and 5.4). This may be due to rather limited decrease in intraneuronal Aβ in the hippocampus. Further decrease in intraneuronal Aβ aggregates may be required to prevent the observed increase in SERT-IR fibre density. This can be achieved by (i) starting the administration of HT\(\text{P}\) diet at an earlier time-point (1 month of age or before), (ii) prolonging the HT\(\text{P}\) diet to an older age that is associated with Aβ plaque formation (9 – 12 months) and (iii) further increasing even more the TrP content in the HT\(\text{P}\) diet (see also chapter 6 for further discussion and consideration of intraneuronal Aβ in inducing SERT-IR fibre sprouting).

5.5.4. Altered dietary TrP content has no affect on 5-HT neurones in the dorsal and the median raphe nuclei

Consistent with previous results, no changes in the total 5-HT neurones were observed in the DR and MR nuclei in 3xTg-AD mice at 3 months of age compared to age-matched non-Tg controls (Noristani et al. 2010). Altered dietary TrP content also had no effect on the total 5-HT-positive neurones in the DR and MR nuclei, suggesting heterotypic sprouting of serotonergic projections in the hippocampus (Fig. 5.5). Contrarily, Orozco-Suárez and colleagues (2003) reported a decrease in 5-HT neurones of rats subjected to chronic decrease in dietary TrP content (Orozco-Suárez et al. 2003). However, this discrepancy is probably due to different
experimental paradigm compared to the current study. Specifically, Orozco-Suarez and colleagues (2003) placed rats in LTrP diet before/during pregnancy and observed a decrease in 5-HT neurones in their offspring at 30 and 60 days of age (Orozco-Suarez et al. 2003), whereas in the current study LTrP diet was given to mice at 2 months of age, once the development of serotonergic system is completed. 5-HT plays an important role in the normal development of serotonergic neurones and projections in the brain (Ahmad & Zamenhof 1978; Haydon et al. 1984; Shemer et al. 1991). The reported decrease in 5-HT neurones by (Orozco-Suarez et al. 2003) followed prenatal administration of LTrP diet may be due to its effect on 5-HT neurones early during brain development.

In conclusion, these results support a possible neuroprotective role of increased 5-HT neurotransmission in AD pathology. Increasing 5-HT level by TrP supplement not only improves behavioural abnormalities, but it may also reduce the underlying neuropathology associated with AD, as shown by a clear diminution of intraneuronal Aβ accumulation in the hippocampus. Direct increase in 5-HT neurotransmission may provide a promising therapeutic approach to the halt or better treatment of AD.
Supplementary Figure 1. Bar graphs showing the effect of altered dietary TrP intake on overall food (A) and water (B) intake over 1 month period in non-Tg control and 3xTg-AD mice. Bars represent mean ± S.E.M., (n = 5 – 7). *, ** and *** = p<0.05, p<0.01 and p<0.001 compared to genotype-matched animals fed with normal TrP diet, un-paired t-test. Key: LTrP: low TrP diet, NTrP: normal TrP diet, HTrP: high TrP diet.
**Supplementary Figure 2.** Bar graphs showing the effect of altered dietary TrP intake on intraneuronal Aβ density in the CA3 subfield of the hippocampus (A) and the amygdala in 3xTg-AD mice. Bars represent mean ± S.E.M., (n = 5 – 7). Key: **IOD**: inverse optical density, **LTrP**: low TrP diet, **NTrP**: normal TrP diet, **HTrP**: high TrP diet.
Chapter 6
General Discussion
6. General discussion

6.1. General overview
AD is an age-related, irreversible and progressive neurodegenerative disease that causes a severe and irreparable decline in various cognitive faculties, including learning and memory. There are more than 24 million people with AD worldwide and this number is set to quadruple by the year 2050 (Ferri et al. 2005). AD affects specific brain regions involved in cognitive function including the neocortex and the hippocampus. Although AD was described over 100 years ago, currently there are no effective treatments to halt the progression of the disease (Alzheimer 1907; Patel & Grossberg 2011). The work of this thesis was dedicated to studying the alteration of serotonergic (5-HT) neurotransmission and the impact that this has on the progression of AD in an animal model of the disease. The majority of 5-HT neurones are located in the dorsal (DR) and median raphe (MR) nuclei giving rise to 5-HT projections that innervate multiple brain regions including the cortex, the thalamus and the hippocampus (Vertes 1991; Vertes et al. 1999; Schmitt et al. 2000). 5-HT neurotransmission mediates numerous physiological functions such as food intake, aggression, sleep-wake cycle, learning and memory (as described in Chapter 1). In this thesis, the majority of the experimental work was centred on investigating the alteration of the 5-HT system (Chapters 3 and 4). Initially, changes in 5-HT projections to the hippocampus and the total number of 5-HT neurones in the DR and MR nuclei were studied using immunohistochemistry combined with light and confocal microscopy (Chapter 3). Secondly, immunoperoxidase immunohistochemistry and electron microscopy were used to investigate the ultrastructural alteration of 5-HT terminals and their relation to altered synaptic connectivity within the hippocampus (Chapter 4). Finally, in the last experimental Chapter (Chapter 5), 5-HT neurotransmission was modified via dietary intake of its precursor, tryptophan, (TrP) to assess the effects of this manipulation on 5-HT projections in the hippocampus, the total number of 5-HT neurones in the DR and MR nuclei as well as intraneuronal Aβ deposition in the hippocampus.

The experimental work of this thesis was carried out using a triple transgenic (3xTg-AD) mouse model of AD. The latter is one of the most advanced and
relevant models of AD due to its temporal- and region-specific expression and progression of Aβ and tau neuropathology as well as age-related progressive cognitive decline that closely resembles that is seen in humans (Oddo et al. 2003a; Oddo et al. 2003b; Billings et al. 2005) (see also Chapter 1, section 1.2.3.). In addition, the work of this thesis focused principally on the hippocampus since this is one of the earliest and most profoundly affected brain regions in AD and plays an essential role in the cognitive decline that is a hallmark of AD (Braak & Braak 1991; Du et al. 2001). Furthermore, various 5-HT components are affected in the AD hippocampus, including 5-HT receptors and 5-HT transporter (SERT) (Cross et al. 1984; Jansen et al. 1990; Kepe et al. 2006; Truchot et al. 2007; Truchot et al. 2008; Ouchi et al. 2009; Lai et al. 2011; Mizukami et al. 2011).

In this general discussion section, the overall findings are discussed in relation to previously reported studies in post-mortem AD brains and other animal models of the disease. Furthermore, based on the results described in this thesis, a possible neuro-protective effect of increased 5-HT neurotransmission in reducing AD-related neuropathology (namely, Aβ) is suggested. Finally, some additional suggestions for further research will be proposed to advance these findings, which are hoped to broaden and enlighten the understanding of how altered 5-HT neurotransmission may affect the underlying neuropathology associated with AD.

6.2. Serotonergic system in the 3xTg-AD mouse model of AD
Contrary to the orthodox view that identifies deficits in the cholinergic (ACh) neurotransmitter system as the main neurochemical alteration in AD, research over the last 40 years has confirmed that multiple neurotransmitter systems are affected in AD, including the glutamatergic (Neary et al. 1986; Kowall & Beal 1991), noradrenergic (Lyness et al. 2003; Zarow et al. 2003), dopaminergic (Reinikainen et al. 1990) and 5-HT systems (Noristani et al. 2010; Noristani et al. 2011) (see also Chapter 1, section 1.1.1.6.). As described in detail in the Introduction Chapter (Chapter 1, section 1.5), the development of AD neuropathology is particularly associated with severe alterations of the 5-HT system, including reduced 5-HT neurotransmitter levels, loss of 5-HT neurones, reduced 5-HT receptors and SERT binding sites in AD brains.
Post-mortem studies have relied upon a decrease in SERT binding sites as a measure to support the reduction of 5-HT projections in AD brains (Bowen et al. 1983; Chen et al. 1996; Tsang et al. 2003; Thomas et al. 2006; Bowen et al. 2008; Lai et al. 2011). However, several questions remain unanswered regarding the AD-associated alterations in 5-HT projections using such binding studies in post-mortem AD brains because:

- Post-mortem AD studies only allow a one-time ('snap-shot') assessment that is usually at the end-stage of the disease. These studies provide no information related to changes in 5-HT content and/or degree of 5-HT projections throughout the progression of the disease.

- Binding studies depend on the affinity of the radioligand to their target sites as well as the density of target protein in the brain (Meltzer et al. 1999). Reduced radioligand affinity may be mistakenly interpreted as a decrease in the density of target protein.

- End-stage binding studies do not provide information regarding AD-associated alterations in axonal morphology that are evident prior to degeneration (Hardy et al. 1986; Marcyniuk et al. 1986; Burke et al. 1988).

As described previously, immunohistochemical studies using 5-HT- and SERT-specific antibodies have been extensively used to visualise and quantify 5-HT projections in rodent and primate brains (see Chapter 1, section 1.3.4.6.). Unfortunately, to our knowledge, no dedicated post-mortem analysis of human brains using immunohistochemical staining has been performed to visualise and quantify AD-related changes in 5-HT projections. The current understanding of AD-associated alterations in 5-HT projections (with respect to axonal morphology and axonal ramification) arises primarily from studies using a range of animal models of AD, which exhibit only some of the neuropathology associated with AD.

Contradictory findings have been reported regarding 5-HT projections in different animal models of AD (Aucoin et al. 2005; Liu et al. 2008; Verdurand et al. 2011). Transgenic mouse models expressing single mutations, such as the APPswe and PS1ΔE9 mice, display no changes in 5-HT fibre density in the cortex and the hippocampus up to 18 months of age (Liu et al. 2008). On the other hand, the
APP_{sw}/PS_{1AE9} double transgenic mouse model showed degeneration of 5-HT fibres in the cortex, the amygdala and the hippocampus between 12 – 18 months of age (Liu et al., 2008). However, a more recent study in APP_{sw}/PS_{1AE9} mice (up to 11 months of age) revealed no alterations in SERT binding sites (measured using [^3]Hescitalopram radioligand), despite the evident increase in Aβ plaque deposition within the cortex and the hippocampus (Holm et al., 2010). Similarly, other double transgenic mouse model of AD carrying the APP_{Sw,Ind} mutations displayed a stable 5-HT fibre density in the parietal cortex and the hippocampus up to 18 months of age, despite pronounced Aβ deposition in these brain regions (Aucoin et al. 2005).

In Chapter 3, a SERT-specific antibody was used for the visualisation and quantification of serotonergic fibres in the 3xTg-AD mouse model of AD. The results showed a generalised increase in the SERT-immunoreactive (SERT-IR) fibre density throughout the hippocampus at 3 and 18 months of age compared to age- and sex-match non-transgenic (non-Tg) controls (Chapter 3, Fig 3.1 and 3.2). Increased SERT-IR fibre densities were particularly localised in the CA1 subfield and more specifically, in the CA1 stratum lacunosum moleculare (S.Mol), the region that displays the highest density of 5-HT fibres in the hippocampus (Vertes 1991; Vertes et al. 1999). In the CA1 S.Mol, the initial increase in SERT fibre density was observed at an early age (3 months, by 61%). No changes were observed in SERT-IR fibres densities between 6, 9 and 12 months of age, while increased SERT-IR fibre density reappeared at a more advanced age (18 months, by 74%).

To uncover whether the observed increase in hippocampal SERT-IR fibre density was due to increased innervations from the DR and/or the MR nuclei, SERT-IR fibres in the hippocampus were quantified based on their axonal morphology (Chapter 3, Fig. 3.3). Previous immunohistochemical studies have classified 5-HT fibres originating from the DR nucleus as fine fibres (FF), due to their thin appearance with small fusiform or granular varicosities found regularly spaced along the fibre (Kosofsky & Molliver 1987; Mulligan & Tork 1988; Bjarkam et al. 2005). 5-HT fibres arising from the MR nucleus are described as beaded fibres (BF), due to their thick fibre structure with large and spherical varicosities that are irregularly spaced (Kosofsky & Molliver 1987; Mulligan & Tork 1988; Bjarkam et
In addition, the MR nucleus also gives rise to thick, straight, non-varicose 5-HT axons that are referred as stem axons (SA) (Kosofsky & Molliver 1987; Mulligan & Tork 1988; Bjarkam et al. 2005). Quantitative analysis of SERT-IR fibre morphology revealed that the increase in SERT fibre density at 3 months of age was specifically due to an increase in BF (by 18%), while at 18 months of age pronounced increases in both BF (by 43%) and FF (by 155%) were observed in the hippocampus. No changes were observed in SA at any of the examined ages between controls and 3xTg-AD mice (Chapter 3, Fig. 3.3). These findings strongly suggest that the increase in hippocampal SERT-IR fibre density is due to fibres that are originate from both the DR and MR nuclei in the 3xTg-AD mouse model of AD. This is the first description of increased BF and FF density in an animal model of AD.

A detailed morphological analysis of the hippocampus revealed no degenerative profiles of SERT-IR fibres, such as swollen and tortuous varicosities or abnormal thickened and ballooned spherical axon terminals (van Luijtelaar et al. 1988; van Luijtelaar et al. 1989; Steinbusch et al. 1990). These results suggest that the observed increase in SERT-IR fibre density in the 3xTg-AD mouse model of AD is due to a specific outgrowth from undamaged axons (Harkany et al. 2001). These findings contradict a previous report in the APP<sub>Sw</sub>,<sub>Ind</sub> double transgenic mouse model of AD that displayed distorted 5-HT-immunostained fibres, particularly in close proximity to Aβ neuritic plaques (Aucoin et al. 2005). This discrepancy may be due to differences in transgenic lines (APP<sub>Sw</sub>,<sub>Ind</sub> vs. 3xTg-AD) and/or differences in the expression of AD-related neuropathology between different mouse models of the disease (Aucoin et al. 2005; Noristani et al. 2010). More specifically, the APP<sub>Sw</sub>,<sub>Ind</sub> transgenic mouse model of AD displays extracellular Aβ deposition starting from 6 months of age (Aucoin et al. 2005), a much earlier time-point when compared to the 3xTg-AD mouse model of AD. The latter first displays Aβ plaques between 9–12 months of age and these become more evident at 18 months of age in the hippocampus (Mastrangelo & Bowers 2008; Noristani et al. 2010; Olabarria et al. 2010; Olabarria et al. 2011). In fact, early Aβ plaque deposition may account for the distortion of 5-HT-immunostained fibres in APP<sub>Sw</sub>,<sub>Ind</sub> mice (Aucoin et al. 2005). However, and despite accelerated Aβ plaque deposition, APP<sub>Sw</sub>,<sub>Ind</sub> mice do not express the neurofibrillary tangles (NFTs) seen
in the 3xTg-AD mouse model of AD, which express both AD-related neuropathologies (Aβ plaques and NFTs). Such difference in the expression of NFTs may also further contribute to the contradictory findings on 5-HT fibre alterations between the APP_{Sw, Ind} and the 3xTg-AD mouse models of AD (Aucoin et al. 2005; Noristani et al. 2010).

As mentioned earlier, hippocampal 5-HT fibres originate from the DR and MR nuclei of the brain stem (Azmitia & Segal 1978; Vertes 1991; Vertes et al. 1999). To investigate whether the increased SERT-IR fibre density was due to alteration in 5-HT neurone complement, the total number of 5-HT-immunoreactive neurones was determined in the DR and the MR nuclei. No changes were found in the total 5-HT neurone numbers in either the DR or the MR nuclei (Chapter 3, Fig. 3.4), suggesting that the number of 5-HT neurones in the DR and MR nuclei is not affected in the 3xTg-AD mouse model of AD.

These findings are in agreement with a previous study in the APP_{swe} and the PS1_{ΔE9} single transgenic mouse models, which also displayed no changes in the numbers of 5-HT neurones in the DR nucleus, although the authors did not investigate 5-HT-positive neuronal changes in the MR nucleus (Liu et al. 2008). In contrast, the same study using the APP_{swe}/PS1_{ΔE9} double transgenic mouse reported a severe (~50%) loss of 5-HT neurones in the DR nucleus (Liu et al. 2008). Likewise, ~33% loss of 5-HT neurones was found in the DR and MR nuclei in a canine model of AD, which displays severe cortical Aβ deposits, neurodegeneration and memory impairments (Bernedo et al. 2009). Such contradictory findings may again be due to different in species used (dog/mouse) or different trasgenic models carrying either single (PS1_{ΔE9} and PS1_{ΔE9}), double (APP_{swe}/PS1_{ΔE9}) or triple (3xTg-AD) mutations (Liu et al. 2008; Noristani et al. 2010).

Unlike animal models of AD, studies in postmortem AD brains have constantly reported reduced numbers of 5-HT neurones in the DR and MR nuclei (Chen et al. 2000; Kovacs et al. 2003; Hendricksen et al. 2004) (see also Chapter 1, Table 1.8). AD-associated loss of 5-HT neurones has been linked with increased Aβ neuritic plaques and NFTs deposition in the raphe nuclei of AD brains (Curcio & Kemper 1984; Ebinger et al. 1987; German et al. 1987; Halliday et al. 1992; Hendricksen et
al. 2004). Unlike post-mortem AD brains, no evident Aβ neuritic plaques and NFTs were found within the raphe nuclei in the 3xTg-AD mouse model of AD (Overk et al. 2009). The absence of AD-related neuropathology in the raphe nuclei may account for stable numbers of 5-HT-positive neurones in the DR and MR nuclei in the 3xTg-AD model (Chapter 3, Fig. 3.4). However, these 5-HT neurones appear to grow new fibres in brain area with increased Aβ deposition such as the hippocampus.

Previous immunohistochemical studies examining SERT-specific antibody distribution under the electron microscope have reported labelling of serotonergic terminals (in hippocampus) as well as axons arriving from the raphe nuclei (Zhou et al. 1998; Pickel & Chan 1999). To establish whether SERT-IR fibre sprouting was due to increase in either terminals or axons, peroxidase immunohistochemistry and electron microscopy were used to examine the ultrastructural changes in hippocampal SERT axons (SERT-Ax) and terminals (SERT-Te) in the 3xTg-AD mouse model of AD compared to age-matched controls (Chapter 4, Fig. 4.4). The results showed a specific increase in the numerical density (Nv, number of terminals per given volume, #/mm³, Chapter 4, Fig. 4.4) of SERT-Te in the 3xTg-AD mouse at 3 and 18 months of age, whilst no changes were observed in the Nv of SERT-Ax in any of the examined ages.

Based on findings from Chapter 3 and Chapter 4, it was suggested that the observed increase in SERT-IR fibre density is due to sprouting of SERT fibres due to (i) the lack of increase in the total number of DR and the MR 5-HT neurones that project to the hippocampus; (ii) an increase in both SERT-IR fibre density and the area density (Sv, number of fibres per given area, #/mm²) of both BF and FFs in the hippocampus; and (iii) an increase in the Nv of hippocampal SERT-Te with no changes in SERT-Ax that arise from the DR and the MR nuclei.

To uncover the relationship between altered 5-HT neurotransmitter level and SERT-IR fibre sprouting, both non-Tg control and 3xTg-AD mice were fed with a modified diet containing altered TrP contents for a period of 1 month (between 2 and 3 months of age Chapter 5, Fig. 5.1). Similar to results described in Chapter 3, increased SERT-IR fibre density was observed within the hippocampus of the
3xTg-AD mouse model compared to controls in low, normal and high TrP diets (Chapter 5, Fig. 5.4). In addition, the total number of 5-HT neurones remained stable in the 3xTg-AD mouse model of AD and non-Tg controls irrespective of dietary TrP contents (Chapter 5, Fig. 5.5). Interestingly, high TrP diet reduced intraneuronal A\(\beta\) density in the 3xTg-AD mouse model of AD (see section 6.3.1 for detailed discussion and consideration of increased TrP intake on intraneuronal A\(\beta\) deposition).

To summarize the above, the results from Chapters 3, 4 and 5 suggest that: (a) increased SERT fibre sprouting is specifically observed in the 3xTg-AD mouse; (b) this change is associated with an increase in the N\(_v\) of hippocampal SERT-Te; and (c) that increased SERT-IR fibre sprouting occurs irrespective of differences in dietary TrP intake. These findings are, to our knowledge, the first account of serotonergic fibre sprouting in a transgenic mouse model of AD.

The observed sprouting of hippocampal SERT-IR fibres in the 3xTg-AD mouse is in broad agreement with previous studies that show 5-HT fibre sprouting in different regions of the rodent brains (including the hippocampus) following experimental-induced brain damage (Gasser & Dravid 1987; Zhou et al. 1995; Harkany et al. 2000; Harkany et al. 2001). Serotonergic fibres sprout in response to damage of neighbouring 5-HT fibres (homotypic sprouting) (Azmitia et al. 1978; Zhou & Azmitia 1984; Zhou & Azmitia 1986). Homotypic sprouting of 5-HT fibres was observed following the microinjection of 5-HT-specific neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the cingulum bundle-induseum griseum - a substance that causes selective and long-lasting degeneration of hippocampal 5-HT fibres (Azmitia et al. 1978; Zhou & Azmitia 1984; Zhou & Azmitia 1986). 5-HT fibres also sprout in response to damage in non-5-HT fibres (heterotypic sprouting) (Zhou et al. 1991). Heterotypic sprouting of 5-HT fibres was reported following microinjection of dopaminergic (DA)-specific neurotoxin, 6-hydroxydopamine (6-OHDA) directly into the substantia nigra, which causes selective and long-lasting degeneration of DA neurones and fibres (Snyder et al. 1986; Zhou et al. 1991; Yamazoe et al. 2001).
Injection of ibotenic acid within the striatum and the hippocampus triggered vigorous 5-HT fibre sprouting in rats, particularly around the lesion sites with the most neurodegeneration (Zhou et al. 1995). In addition, injection of glutamate analogue N-methyl-d-aspartate (NMDA) into the nucleus basalis magnocellularis (NBM) caused neuronal loss that was also associated with increased 5-HT fibre sprouting (Harkany et al. 2000). Similarly, Aβ1-42 infusion into the NBM triggered prominent neurodegeneration accompanied by increased 5-HT fibre sprouting, again particularly surrounding the most pronounced lesion sites (Harkany et al. 2001). These findings are in line with the obtained results from Chapter 3, which showed SERT-IR fibre sprouting to be most pronounced nearest Aβ plaques (<20µm) (Chapter 3, Fig. 3.5). Interestingly, in the Tg2576 APP transgenic mouse model of AD severe degenerative profiles within the 20µm distance from Aβ plaques edges include disrupted neurite processes and reduced dendritic spine density (Spires et al. 2005). These findings point out that increased SERT-IR fibre sprouting is particularly evident in brain regions with severe neuronal damage.

Besides experimentally-induced lesions, 5-HT fibre sprouting was also reported in animal models of other neurodegenerative disorders including Menkes’ disease (a severe neurodegenerative disease with widespread cortical neuronal loss) (Martin et al. 1994) and amyotrophic lateral sclerosis (ALS) (Bose & Vacca-Galloway 1999). Immunohistochemical analysis revealed increased 5-HT fibre sprouting in the cortex of brindled mottled mice (a mouse model of Menkes’ disease) that was supported by neurochemical studies showing an increase in 5-HT and 5-HIAA concentrations (Martin et al. 1994). Similar to AD, brindled mottled mice display a severe deficit in noradrenergic (NA) neurotransmission and this may trigger 5-HT fibre sprouting (Martin et al. 1994; Lyness et al. 2003; Zarow et al. 2003; Gulyas et al. 2010) (Chapter 1, section 1.1.1.6.5.).

The Wobbler mouse is used as an animal model of motoneurone degeneration (Duchen & Strich 1968; Sillevis Smitt & de Jong 1989). Immunohistochemical staining (using a 5-HT-specific antibody) revealed pronounced 5-HT fibre sprouting in the cervical ventral horn of the spinal cord in the Wobbler mice (Papapetropoulos & Bradley 1972; Bose & Vacca-Galloway 1999). Similar to studies in experimentally-induced lesion models of AD, 5-HT fibre sprouting was
particularly observed in regions with severe degeneration of motoneurones, suggesting that 5-HT sprouting is closely linked to neurodegenerative processes associated with ALS (Bose & Vacca-Galloway 1999). Taken together, these data suggest that 5-HT fibre sprouting occurs not only as a consequence of experimental lesion, but it is also evident in other rodent models of neurodegenerative diseases.

In addition, Gaspar and colleagues (1993) reported vigorous 5-HT fibre sprouting in monkeys that developed permanent hemi parkinsonism following intracarotid injection of a DA neurotoxin (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) (Gaspar et al. 1993). Intracarotid injection of the MPTP caused selective degeneration of DA neurones in the substantia nigra, the putamen and the caudate nucleus ipsilaterally to the injected side that was associated with increased density of 5-HT fibres in these regions (Gaspar et al. 1993). No changes were observed in the number of 5-HT neurones in the raphe nuclei, suggesting a heterotypic 5-HT fibres sprouting in response to DA denervation in adult primates (Gaspar et al. 1993). 5-HT fibre sprouting in monkeys was supported by an earlier neurochemical study showing increased 5-HT neurotransmitter level in the striatum following chronic administration of MPTP (Schneider 1990). These finding suggest that the 5-HT fibre sprouting phenomenon is not specific to rodents, but is also evident in primate models of neurodegenerative disease.

Furthermore, early post-mortem study in AD brains reported reduced [³H]paroxetine binding sites in the temporal cortex; however, no changes in SERT binding sites were observed in the frontal cortex, which the authors suggest may be due to sprouting of remaining 5-HT fibres in the latter region (Chen et al. 1996). However, to date, no direct evidence showing 5-HT fibre sprouting in post-mortem AD brains has been reported. Also, in frontotemporal dementia (FTD) analyses using the same radioligand SERT binding sites in the frontal, the temporal and the parietal cortex were stable (Bowen et al. 2008). In agreement with the earlier report in AD, sprouting of the remaining 5-HT fibres in the cortex was suggested as a possible mechanism for preserved SERT binding sites in FTD (Bowen et al. 2008), although no supportive data were provided for such 5-HT fibre sprouting in either AD or FTD post-mortem brains.
The lack of a demonstration for 5-HT fibre sprouting in AD brains is perhaps not surprising given that there are no post-mortem reports on AD-related alterations in 5-HT innervations using immunohistochemical staining. Interestingly, earlier post-mortem AD studies reported sprouting of ACh fibres in the hippocampus (measured using staining against acetylcholinesterase, AChE) (Geddes et al. 1985; Hyman et al. 1987), which was also more evident adjacent to Aβ plaques (Geddes et al. 1986). Furthermore, AChE fibre sprouting was also reported in the APP/London transgenic mouse model of AD within the CA1 subfield of the hippocampus and the dentate gurus (DG) (Bronfman et al. 2000), therefore, supporting our assumption of 5-HT fibre sprouting in AD brains. However, and to expand these ideas and concepts, the verification of 5-HT fibre sprouting in post-mortem AD brains should be carried out.

Nevertheless, the novel finding from the experiments carried out in this thesis was the confirmation of serotonergic fibre sprouting in the 3xTg-AD mouse model of AD that express both pathological hallmarks (Aβ plaques and NFTs) associated with AD.

Because SERT fibres sprouting were observed at two time-points (3 and 18 months of age, but not between these points), in the 3xTg-AD model, the following sections describe possible mechanisms that might account for the independent early (3 months) and the late (18 months) SERT fibre sprouting.

### 6.2.1. Possible mechanism responsible for the early serotonergic fibre sprouting in the 3xTg-AD mouse model of AD

Previous studies, including our own, have shown intraneuronal Aβ aggregates at an early age (3 months) in the 3xTg-AD mouse model of AD (Mastrangelo & Bowers 2008; Rodríguez et al. 2008). In fact, accumulating evidence suggests that intraneuronal Aβ deposition initiates the neurodegenerative processes underlying AD (Rodríguez et al. 2008; Wirths et al. 2009; Umeda et al. 2011), for review see (Wirths et al. 2004; Gimenez-Llort et al. 2007; Gouras et al. 2010). More specifically, in the 3xTg-AD mouse model of AD, intraneuronal Aβ accumulation correlates with cognitive deficits including impaired long-term synaptic plasticity and memory retention (Oddo et al. 2003b; Billings et al. 2005).
Given the lack of changes in 5-HT neuronal number, the triggering signal for SERT-IR fibre sprouting appears to originate from the hippocampus where the neurotrophic factors are released. One such neurotrophic factor is the S-100β protein that is produced, stored and released by astrocytes and can stimulate the outgrowth of 5-HT fibres (Azmitia et al., 1990; Whitaker-Azmitia et al., 1990; Liu and Lauder, 1992; Azmitia, 2001). Inhibiting S-100β synthesis (via application of S-100β antisense gene) blocked 5-HT fibre sprouting in the C6 glioma cells (Ueda et al. 1995). The release of S-100β is mediated by activation of astrocytic 5-HT1A receptors (Whitaker-Azmitia et al. 1990). Intrahippocampal injection of Aβ1-40 peptide in the rat hippocampus caused a transient increase in the expression of 5-HT1A receptors in the astrocytes (Verdurand et al. 2011), similar to that seen in patients with pre-AD mild cognitive impairment (Truchot et al. 2007; Truchot et al. 2008). Given the appearance of intraneuronal Aβ deposition at 3 months of age in the 3xTg-AD mouse model of AD, one cannot exclude the possibility that such intraneuronal Aβ aggregation may also induce a transient increase in the expression of 5-HT1A receptors in the S-100β-expressing astrocytes and trigger an increase in the release of S-100β protein (Fig. 6.1).

Recent studies suggest a direct interaction between S-100β and Aβ during the progression of AD (Mori et al. 2010; Roltsch et al. 2010). Over-expression of S-100β in the Tg2576 AD model increased Aβ plaque deposition and glia activation in the cortex (Mori et al. 2010). On the other hand, reduced S-100β protein (by administration of S-100β synthesis inhibitor arundic acid) decreased Aβ neuropathology in the Tg2576 transgenic mouse model of AD (Mori et al. 2006). Similarly, the PSAPP transgenic mouse model of AD with reduced S-100β expression (PSAPP/S-100β0.5) displayed reduced Aβ plaque load in the cortex, suggesting an interaction between S-100β and Aβ during the development of AD-related neuropathology (Roltsch et al. 2010).

These data suggest that intraneuronal Aβ accumulation plays a central role in SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD at 3 months of age. Interestingly, increased SERT-IR fibre sprouting was still evident in 3xTg-AD mice fed with high TrP diet despite a decrease in intraneuronal Aβ aggregates.
5, Fig. 5.3 and 5.4). However, it has to be considered that chronic administration of high TrP diet reduced intraneuronal Aβ density only by 17% compared to 3xTg-AD mice fed with normal TrP diet. It is likely that further reduction in intraneuronal Aβ density may be required to stop SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD.

One possibility to further decrease intraneuronal Aβ aggregation is early administration of high TrP diet from a young age (1 month or younger) in the 3xTg-AD mouse. In Chapter 5, 3xTg-AD and control animals were placed in high TrP diet for a period of 1 month (between 2 – 3 months of age) in order to avoid the effect of altered dietary TrP intake on the development of 5-HT system in the brain (Chapter 5, Fig. 5.1). Given that at 3 months of age a high TrP diet reduced intraneuronal Aβ density equivalent to that at the start of the diet exposure (2 months of age), this suggests that increased TrP diet prevents accumulation of intraneuronal Aβ rather than reversing intracellular deposition. Therefore, early administration of a high TrP diet will provide a longer treatment window that may have a greater effect on intraneuronal Aβ deposition as well as SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD.

Similarly, administration of a high TrP diet can also be prolonged to an age that is associated with extracellular Aβ deposition (9 – 12 months of age in the 3xTg-AD). In Chapter 5, 3xTg-AD and control animals were perfused at 3 months of age (as this point represents the specific age for the increase in SERT-IR fibres and terminals; Chapters 3 and 4). However, given the late recurrence of SERT-IR fibre sprouting at 18 months of age, prolonged treatment with high TrP diet would likely have a greater effect on Aβ neuropathology in the 3xTg-AD mouse model of AD.

Alternatively, diets with higher TrP content may be needed to further increase 5-HT neurotransmission and induce a greater effect on intraneuronal Aβ deposition. The high TrP diet used in Chapter 5 contained 0.40g TrP/100g, which is double the TrP level compared to normal TrP diet. SERT-IR fibre sprouting was significantly increased in non-Tg control animals fed with this high TrP diet compared to low TrP diet groups, which had 10 times more TrP content between the two diets (Chapter 5, Fig. 5.4).
Figure 6.1. Schematic diagram representing the different stages of AD progression from healthy brain to severe AD pathology. Note that 5-HT fibres are represented in green, pyramidal neurones in orange and S-100β-expressing astrocytes in blue.

Healthy stage. 5-HT fibres innervating the hippocampus and their relation with hippocampal pyramidal neurones and S-100β-expressing astrocytes.

Early AD-stage. Intraneuronal Aβ accumulation becomes evident in the pyramidal neurones that in turn trigger a transient increase in astrocytic 5-HT₁A receptors that mediate the release of S-100β from astrocytes. There is also a transient increase in S-100β-expressing astrocytes and a possible increase in S-100β neurotrophic factor released by these S-100β-expressing astrocytes that in turn may trigger the early SERT-IR fibre sprouting in the hippocampus.

From personal library.
Mid AD-stage. 5-HT fibres remain stable during the mid-stage of AD with low densities of extracellular Aβ deposits. In addition, the density of 5-HT$_{1A}$ receptors and S-100β-expressing astrocytes return to control levels.

Severe AD-stage. Late SERT-IR fibres sprouting occurs concomitantly with the extracellular Aβ neuritic plaque deposition and severe glial reactivity. Aβ plaque formation induces a local inflammatory response that is associated with increased microglia activation and the release of interleukin-1 (IL-1). The liberated IL-1 activates astrocytes and triggers the release of neurotrophic factors such as S-100β that promote the late SERT-IR fibre sprouting. In addition, Aβ neuritic plaque deposition is also associated with neurodegeneration and a possible increase in the release of brain derive neurotrophic factor (BDNF) from degenerative neurone may also act as neurotrophic factor and can stimulate the late SERT-IR fibre sprouting in the hippocampus. Atrophic astroglia populate the Aβ-free brain parenchyma.

From personal library.
Recent findings from our laboratory suggest that the early increase in SERT-IR fibre sprouting at 3 months of age in the 3xTg-AD mouse model of AD may be due to a transient increase in S-100β-positive astrocytes in the hippocampus (Chapter 3, supplementary Fig. 3) that has a neurotrophic effect on 5-HT fibre sprouting (Azmitia et al., 1990; Whitaker-Azmitia et al., 1990; Liu and Lauder, 1992; Azmitia, 2001). Interestingly, this increase in S-100β-positive astrocytes was particularly evident in hippocampal regions that had previously showed increased SERT-IR fibre sprouting; namely the total CA1 subfield and specifically the CA1 S.Mol (Chapter 3, supplementary Fig. 3).

These results are in agreement with previous findings in S-100β over-expressing mice, which also display an early increase in SERT-IR fibres associated with concomitant increase in S-100β-positive astrocytes in the hippocampus at 10 weeks of age (Shapiro et al. 2010). This conclusion is supported by evidence that: (a) transgenic mice with reduced S-100β expression display fewer S-100β-expressing astrocytes and reduced 5-HT fibre density in the hippocampus (Ueda et al. 1994); and (b) age-associated decrease in hippocampal 5-HT fibre density is associated with reduced S-100β-positive astrocytes in rats (Nishimura et al. 1995).

The gene coding for S-100β protein is located on chromosome 21, within the obligate region for Down's syndrome, which displays comparable neuropathological hallmarks (Aβ plaques and tangles) as AD (Gullesserian et al., 2000; Azmitia, 2001). Indeed, increased S-100β protein expression has been reported in the temporal cortex in Down’s syndrome and in AD (Griffin et al., 1989). This increased S-100β expression is more pronounced in young Down's syndrome patients (Griffin et al. 1989). In addition, Western blot analysis of the frontal cortex also showed increased expression of SERT protein in Down's syndrome brains (Gullesserian et al. 2000).

Taken together, these data suggest that intraneuronal Aβ deposition may cause a transient increase in 5-HT1A receptor expression in S-100β-expressing astrocytes, which, together with the observed increased in the Ns of these astrocytes, may be responsible for observed SERT-IR fibre sprouting at 3 months of age (Fig. 6.1). However, there are no data on either the expression of 5-HT1A receptors,
extracellular level of S-100β protein or the underlying mechanisms responsible for the transient increase in the Nv of S-100β-positive astrocytes in 3xTg-AD mice at 3 months of age. No changes were observed in the Nv of S-100β-positive astrocytes at older age (Chapter 3, supplementary Fig. 3), suggesting that an alternative mechanism(s) may be responsible for the late (18 months) SERT fibre sprouting in the 3xTg-AD mouse model of AD.

6.2.2. Possible mechanism responsible for the late serotonergic fibre sprouting in the 3xTg-AD mouse model of AD

Extracellular deposition of Aβ plaques in the 3x-Tg-AD mice appear between 9 and 12 months and consolidates by 18 months of age (Mastrangelo & Bowers 2008; Rodríguez et al. 2008). Extracellular Aβ plaque deposition progressively increases with advanced age and mainly targets the CA1 subfield of the hippocampus (Oddo et al. 2003b; Rodríguez et al. 2008; Rodríguez et al. 2009a; Rodríguez et al. 2009b). 18 months old 3xTg-AD mice exhibit pronounced extracellular Aβ plaques that may induce neurotoxic damage associated with local inflammatory response and subsequent release of glia derived neurotrophic factors (such as inflammatory cytokines and S-100β) that may trigger the observed late sprouting of hippocampal SERT-IR fibres in the 3xTg-AD mouse model of AD (Fig. 6.1). Lack of SERT-IR fibre sprouting at 9 and 12 months in 3xTg-AD mice may be due to lower level of extracellular Aβ plaque deposition compared to that at 18 months of age. In support of this phenomenon, our preliminary results emphasise that, indeed, SERT fibre sprouting is further increased within the hippocampus at 24 months of age in 3xTg-AD mice, concomitant with the increased extracellular Aβ neuritic plaque deposition (H.N.N & J.J.R.A. unpublished observation).

The presence of neurotrophic factors is critical for stimulating 5-HT fibre sprouting following neuronal damage and more specifically in neurodegenerative disease such as AD. Extracellular deposition of Aβ plaques triggers glial reactivity in different regions of the AD brains including the entorhinal cortex and the hippocampus (Rodríguez et al. 2009b; Olabarria et al. 2010; Verdurand et al. 2011). A previous study in our laboratory had shown that in 3xTg-AD mice astrocytes located within the close proximity of Aβ plaques undergo severe hypertrophy at 18 months of age in the hippocampus (Olabarria et al. 2010). The
late (18 months) sprouting of hippocampal SERT-IR fibres in 3xTg-AD mice may be triggered by these reactive astrocytes present adjacent to Aβ plaques, as previously suggested in Chapter 3 (Rodríguez et al. 2009b; Olabarria et al. 2010; Verdurand et al. 2011) (see also Chapter 3, Fig. 3.6). Astrocytic hypertrophy and subsequent release of neurotrophic factors such as S-100β may be associated with the increased SERT-IR fibre sprouting in the vicinity of Aβ plaques (Noristani et al. 2010) (see also Fig. 6.1). As mentioned earlier, astrocytes can produce, accumulate and liberate S-100β protein following activation of astrocytic 5-HT1A receptors, which acts as a neurotrophic factor and stimulate 5-HT fibre sprouting (Azmitia et al., 1990; Whitaker-Azmitia et al., 1990; Liu and Lauder, 1992; Azmitia, 2001).

A previous study in rats had shown vigorous 5-HT fibre sprouting particularly in the striatum and the hippocampus where the reactive astrocytes also displayed increased expression of S-100β protein (Zhou et al. 1995). Although there are no changes in Nv of S-100β-positive astrocytes at 18 months of age (Chapter 3, supplementary Fig. 3), S-100β-positive astrocytes located adjacent to Aβ plaques may undergo severe astrogliosis and hypertrophy leading to increased release of S-100β protein (Fig. 6.1). Hypertrophic astrocytes with increased S-100β expression have been reported in the vicinity of Aβ neuritic plaques both in post-mortem AD brains (Sheng et al. 1994) and in different transgenic mouse models of AD including the APPV717F (Sheng et al. 2000) and the Tg2576 transgenic mouse models of AD (Mori et al. 2010). Indeed, preliminary results from our laboratory suggest that S-100β-positive astrocytes located in close vicinity of Aβ plaques undergo severe hypertrophy compared to S-100β astrocytes located (> 50µm) away from Aβ plaques (Rodríguez et al. in preparation).

Taken together, these data highlight that Aβ plaque deposition triggers an increase in the activation of S-100β-expressing astrocytes and subsequent release of S-100β protein that may be responsible for the late (18 months) SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD (Fig. 6.1).

In addition, increased Aβ-induced local inflammation and microglia activation may
also contribute to the late (18 months) SERT-IR fibre sprouting in 3xTg-AD mice. A previous study in rats had shown that heterotypic sprouting of 5-HT fibres (following microinjection of 6-OHDA) (Zhou et al. 1991), is also associated with increased number of activated (or ameboïd) microglia in the substantia nigra and the striatum (Rodrígues et al. 2001). Interestingly, the increased density of activated microglia was particularly evident in the brain regions with increased 5-HT fibre sprouting (Revuelta et al. 1999), suggesting a close relation between microglia activation and 5-HT fibre sprouting.

AD-associated increase in microglia activation and proliferation was discovered 2 decades ago, when McGree and colleagues (1987) reported increased number of activated microglia in the hippocampus of AD brains concomitant with increased deposition of Aβ plaques (McGeer et al. 1987). Increased number of activated microglia has been regularly reported in the vicinity of Aβ plaques in multiple transgenic mouse models of the disease including the APPswe (Frautschy et al. 1998), the PS1xAPP, (Jimenez et al. 2008) and APPswe/PS1d9xYFP mice (Meyer-Luehmann et al. 2008). More specifically, a previous study in our laboratory has shown that, indeed, 3xTg-AD mice display increased number of not only activated microglia, but also resting (ramified) in the CA1 subfield of the hippocampus (Rodríguez et al. 2010), where SERT fibre sprouting was previously observed (Chapters 3 and 4).

In AD brains, reactive microglia and astrocytes co-exist in the close vicinity of Aβ plaques, suggesting a possible interaction between two glia cells during the development of AD neuropathology. It has been suggested that Aβ deposition activate interleukin-1 (IL-1)-secreting microglia that in turn trigger the activation of astrocytes and S-100β release (Sheng et al. 1996; Mrak & Griffinbc 2001; Mori et al. 2010) (Fig. 6.1). Interestingly, in 3xTg-AD mice, increased microglia proliferation occurs (12 months) before astrocyte activation (18 months), suggesting that activation of microglia and the possible release of inflammatory cytokines may contribute to astrocyte activation (Olabarria et al. 2010; Rodríguez et al. 2010). Increased IL-1 expression has been reported in post-mortem AD brains concomitant with increased S-100β protein (Griffin et al. 1989). Over-expression of S-100β in Tg2576 transgenic mice increased microglia proliferation (Mori et al. 2010).
2010), whilst the PSAPP/S-100β− transgenic mouse model of AD displayed reduced microgliosis (Roltsch et al. 2010), further supporting the interaction between astrocytes and microglia.

In addition, to their role as a sensor for pathological events in the brain, activated microglia also secretes a broad range of cytokines and neurotrophic factors that may support axonal growth and tissue re-modelling (Kreutzberg 1996). However, the precise identity of the neurotrophic factors released by activated microglia, which may contribute to SERT-IR fibre sprouting, awaits further research. Further in vitro and in vivo experiments are necessary to uncover the possible neurotrophic effect of IL-1 on 5-HT fibre sprouting in AD. Although the glial cell line-derived neurotrophic factor (GDNF) have been show to promote survival of DA neurones (Bohn 1999), to date, there are no published data demonstrating their role in 5-HT fibre sprouting.

All in all, these data suggest that accumulation of Aβ plaques trigger a local inflammatory response that activates microglia and increases the release of IL-1. The released IL-1 stimulate activation of astrocytes that in turn liberate neurotrophic factors such as S-100β that promotes the late SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD (Fig. 6.1).

Besides glial derived neurotrophic factors, the density of 5-HT projections can also be regulated by neuronal origin neurotrophic factor such as the brain derived neurotrophic factor (BDNF) that is highly present in the hippocampus (Mamounas et al. 2000; Azmitia 2001; Luellen et al. 2007). Transgenic mice with reduced BDNF expression (BDNF−/−) exhibit an accelerated loss of 5-HT fibres and reduced extracellular level of 5-HT in the CA1 subfield of the hippocampus, suggesting that the presence of BDNF is critical for 5-HT fibre outgrowth (Luellen et al. 2007). Others reported BDNF-induced sprouting of 5-HT fibres in the neocortex, the spinal cord and the hippocampus (Bregman et al. 1997; Mamounas et al. 2000; Grider et al. 2005). Subcutaneous injection of 5-HT neurotoxin (p-chloroamphetamine, PCA) caused severe degeneration of 5-HT fibres in the rat neocortex (Mamounas et al. 1995; Mamounas et al. 2000; Grider et al. 2005). Intracortical BDNF infusion triggered sprouting of both intact and PCA-lesioned 5-
HT fibres resulting in increased 5-HT innervations of the rat neocortex (Mamounas et al. 1995; Mamounas et al. 2000). *In situ* expression of BDNF (achieved by injection of adenoviral vectors containing cDNAs for BDNF) also triggered cortical 5-HT fibre sprouting following PCA-induced lesion (Mamounas et al. 1995; Grider et al. 2005). Similarly, application of exogenous BDNF increased 5-HT fibre sprouting following spinal cord injury in adult rats (Bregman et al. 1997).

In AD, post-mortem studies have consistently reported reduced BDNF expression in multiple brain regions including the cortex and the hippocampus (Ferrer et al. 1999; Murer et al. 2001). However, it is important to note that these post-mortem studies had investigated the BDNF protein levels at the end stages of the disease, where extensive neuronal loss has already taken place (Braak & Braak 1991). Such severe neurodegeneration indiscriminately affects both BDNF-expressing and non-BDNF-expressing neurones resulting in an overall loss of BDNF level in AD brains (Murer et al. 2001). Increased BDNF expression in AD brains may be an early event prior to widespread neurodegeneration, whilst reduced BDNF may be specifically associated with the end stage of the disease. In agreement with this phenomenon, a previous study in the APP-23 transgenic mouse model of AD with only moderate neurodegeneration reported increased BDNF expression, suggesting that increased BDNF expression occurs before rigorous widespread neurodegeneration in AD brains (Burbach et al. 2004). Furthermore, a recent study in 3xTg-AD mice has also shown increased expression of BDNF in the hippocampus at 13 months of age that may contribute to SERT-IR fibre sprouting (Rothman et al. 2012).

Taken together, these data suggest that increased BDNF level in the close vicinity of Aβ plaques exert a neurotrophic effect that may contribute to the observed late (18 months) SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD. However, currently to the best of our knowledge, there are not data regarding the expression levels of BDNF in the close vicinity of Aβ plaques in 3xTg-AD mice.

In summary, the mechanism responsible for triggering the observed late (18 months) SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD can be narrowed down to the following sequence of events:
Increased Aβ plaque deposition triggers a local inflammatory response that induces an increase in microglia proliferation and activation of IL-expressing microglia.

Activated microglia cells secrete IL-1 that, in turn, promotes astrocyte activation.

Activated astrocytes liberate neurotrophic factors such as S-100β that promote SERT-IR fibre sprouting.

Increased BDNF released by moderate number of degenerated neurones in the close proximity of Aβ plaques may also contribute to the late (18 months) SERT-IR fibre sprouting in 3xTg-AD mice (see Fig. 6.1).

Discovering the precise mechanism and functional consequences of SERT-IR fibre sprouting is of fundamental significance in understanding how the AD brains respond to Aβ plaque deposition.

6.2.3. Functional significance of late serotonergic fibre heterotypic sprouting

Increased 5-HT input counteracts glutamate-induced excitotoxicity by inhibiting voltage-dependent calcium (Ca²⁺) currents (Muramatsu et al. 1998; Williams et al. 1998). Electrophysiological studies have shown that application of 5-HT inhibited voltage dependent Ca²⁺ channels including the N- and the P/Q-type channels (Bayliss et al. 1997). 5-HT-mediated Ca²⁺ current inhibition have been reported in slice preparations from multiple brain regions including the cortex (Foehring 1996), the caudal raphe nucleus (Bayliss et al. 1997), the hypoglossal motoneurones (Bayliss et al. 1995) and the ventromedial hypothalamus (Koike et al. 1994).

5-HT-induced inhibition of glutamatergic neurotransmission is mediated by activation of 5-HT₁A and 5-HT₁B receptors (Williams et al. 1998; Guo & Rainnie 2010). Activation of 5-HT₁A receptors inhibited Ca²⁺ current in ACh neurones of the NBM, suggesting that increased activation of 5-HT₁A receptors may be neuroprotective in preventing Ca²⁺ influx-induced excitotoxicity (Williams et al. 1998). Others also reported 5-HT₁A-mediated inhibition of Ca²⁺ current in the amygdala (Lin et al. 2001), the prefrontal cortex (Cai et al. 2002; Yuen et al. 2005) and the spinal cord (Hill et al. 2003). Intrahippocampal injection of the glutamate analogue
(kainic acid) caused seizure in freely-moving rats that was inhibited by pre-treatment with 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)-tertralin (8-OH-DPAT), suggesting that activation of 5-HT_{1A} receptors has an inhibitory role on glutamatergic neurotransmission (Gariboldi et al. 1996). Activation of 5-HT_{1B} receptors suppresses glutamatergic neurotransmission by reducing glutamate release from pre-synaptic terminals (Guo & Rainnie 2010). 5-HT_{1B}-mediated pre-synaptic inhibition of glutamatergic neurotransmission has been demonstrated in multiple brain regions including the suprachiasmatic nucleus (Pickard et al. 1999), the nucleus accumbens (Muramatsu et al. 1998) and the entorhinal cortex (Schmitz et al. 1998).

The majority of the released glutamate is taken up by astrocytes through the Na^+-dependent glutamate transporters (Danbolt 2001; Kirischuk et al. 2007). Astrocytes convert glutamate to glutamine by an astrocytes-specific enzyme called glutamine synthetase (GS) (Danbolt 2001). GS expression is critical for glutamatergic neurotransmission (Walton & Dodd 2007) and the prevention of glutamate-mediated neurotoxicity (Danbolt 2001). A recent study in our laboratory has reported impaired astrocytic GS expression in the CA1 subfield of the hippocampus in 3xTg-AD mice at 18 month of age, suggesting impaired glutamatergic homeostasis (Olabarria et al. 2011). Interestingly, reduced GS expression was more evident in astrocytes located adjacent to Aβ plaques, emphasising that the brain parenchyma surrounding Aβ plaques are particularly vulnerable to glutamatergic-induced excitotoxicity (Olabarria et al. 2011). In addition, our recent findings of reduced N_0 of hippocampal perforated synapses in Chapter 4 further support impaired glutamatergic neurotransmission in the 3xTg-AD mouse model of AD at both 3 and 18 months of age (Noristani et al. 2011).

In AD, Aβ plaque deposition triggers multiple neurodegenerative processes including synaptic and neuronal loss that are intimately linked with cognitive decline associated with the disease (Supnet & Bezprozvanny 2010). In vitro studies have shown that neuronal exposure to Aβ or its fragment increased glutamate-mediated excitotoxicity and elevated intracellular Ca^{2+} [Ca^{2+}]_i influx (Mattson et al. 1992; Brorson et al. 1995; Stutzmann 2007; Supnet & Bezprozvanny 2010). Application of Aβ_{1-42} peptide or its fragment (Aβ_{25-35}) into human neuronal cultures
triggered irreversible opening of a Ca\textsuperscript{2+} channel, increased extracellular Ca\textsuperscript{2+} entry and elevated [Ca\textsuperscript{2+}]\textsubscript{i} (Mattson \textit{et al.} 1992; Blanchard \textit{et al.} 1997). Whole cell current recording from individual neurones revealed that the Aβ\textsubscript{25-35} fragment caused increased bursts of excitatory action potential firing in the rat hippocampal neuronal culture (Brorson \textit{et al.} 1995). An \textit{ex vivo} study using dissociated neurones from adult mice has shown that the Aβ\textsubscript{25-35} fragment also amplified K\textsuperscript{+}-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}, suggesting Aβ-induced destabilisation of Ca\textsuperscript{2+} homeostasis (Hartmann \textit{et al.} 1993).

These data highlight that Aβ accumulation increases Ca\textsuperscript{2+} entry into the neurones via irreversible opening of Ca\textsuperscript{2+} channels that destabilises neuronal Ca\textsuperscript{2+} homeostasis and renders neurones more vulnerable to excitotoxic damage.

Given the 5-HT-mediated inhibition of Ca\textsuperscript{2+} current, SERT-IR fibre sprouting may be beneficial in reducing neurotoxic damage associated with AD neuropathology (see \textit{Chapter 1}, Fig. 1.30). Interestingly, morphometric analysis of SERT-Te surface area showed an increase in terminal size, suggesting a greater “potential to release and uptake” of 5-HT neurotransmitter level in 3xTg-AD mice compared to non-Tg controls (\textit{Chapter 4}, Fig. 4.5). Therefore, it is proposed that the observed increase in hippocampal SERT-IR fibre sprouting in 3xTg-AD mice at the later age (18 month) may indicate an intrinsic defence mechanism in response to the extracellular Aβ plaque deposition, increased glutamatergic neurotransmission and neurotoxic damage. Increased 5-HT input may substantially contribute to minimise the glutamate-induced neurotoxicity in the surrounding neuropil.

Several issues need to be addressed in order to confirm the above possible neuroprotective role of 5-HT in AD including: (i) the presence of 5-HT receptors in the hippocampus, (ii) the existence of signalling mechanisms responsible for 5-HT-mediated neuro-protection and (iii) the extend of neurodegeneration in the 3xTg-AD mouse model of AD.

Firstly, the functional effect of 5-HT depends on the presence of post-synaptic 5-HT receptors and their associated signal transduction mechanisms (Barnes & Sharp 1999; Hoyer \textit{et al.} 2002). Previous studies have shown high densities of 5-HT
receptors in multiple brain regions including the hippocampus in rodents, primates and humans (Chapter 1, section 1.3.4.7.). AD-associated alterations in 5-HT receptors have been reported in post-mortem binding studies of AD brains as well as in vivo imaging studies in AD patients (Chapter 1, Table 1.11 and Table 1.12). Unlike 5-HT$_{2A}$ receptors (which is consistently reduced in AD brains), numerous post-mortem studies have reported stable 5-HT$_{1A}$ receptors in multiple regions of AD brains including the temporal cortex, the frontal cortex and the hippocampus (Cross et al. 1986; Cross et al. 1988; Jansen et al. 1990; Lai et al. 2003b; Tsang et al. 2010). Interestingly, Lai and colleagues (2002) reported an increase in 5-HT$_{1A}$ receptor binding sites in the frontal cortex of AD brains, which is in agreement with more recent in vivo imaging studies that also reported increased 5-HT$_{1A}$ expression during the early stages of the disease progression (Lai et al. 2002; Truchot et al. 2007; Truchot et al. 2008). Similarly, intrahippocampal injection of Aβ$_{1-42}$ peptide reduced 5-HT$_{2A}$ receptor expression, whilst injection of Aβ$_{1-40}$ peptide triggered a transient increase in 5-HT$_{1A}$ receptor expression in the rat hippocampus (Christensen et al. 2008; Verdurand et al. 2011). Given the preserved (or even increased) expression of 5-HT$_{1A}$ receptors in AD brains and animal models of the disease, it is likely that these receptors are still present in 3xTg-AD mice at 18 months of age. However, currently there are no reported studies on 5-HT$_{1A}$ receptor expression in any transgenic mouse models of AD including the 3xTg-AD mouse model.

Secondly and as mentioned earlier, previous electrophysiological studies have reported 5-HT-mediated inhibition of Ca$^{2+}$ current via blocking of Ca$^{2+}$ channels, which may provide neuro-protections against Aβ-induced neurotoxicity. However, currently there are no data on the existence of specific signalling mechanisms responsible for 5-HT-mediated neuro-protection in 3xTg-AD mice.

Thirdly, 3xTg-AD mice express both pathological hallmarks of AD namely: Aβ plaques and NFTs that are associated with impaired cognitive function (Chapter 1, section 1.2.3.). However, thus far, no degeneration of hippocampal or cortical neurones have been reported in 3xTg-AD mice (Wirths & Bayer 2010). This suggests that in 3xTg-AD mice, indeed, there are enough neurones to be rescued from excitotoxicity by the putative increase in 5-HT input. The cognitive
impairment observed in the 3xTg-AD mouse model of AD may be associated with neuronal dysfunction such as loss of synapses rather than severe neurodegeneration.

6.3. Altered synaptic density in the 3xTg-AD mouse model of AD

Ultrastructural analysis of biopsied cortical tissue from AD patients, at mild to moderate stage of the disease, revealed severe loss of synapses (DeKosky & Scheff 1990), which further declined in post-mortem AD brains and showed a good correlation with cognitive impairment associated with the disease (DeKosky & Scheff 1990; Scheff et al. 2006).

Pre-clinical studies on different transgenic mouse models of AD usually employ qualitative or semi-quantitative light immunohistochemistry and assess overall changes in the synaptic associated proteins such as synaptophysin, as a measure of altered synaptic density (Irizarry et al. 1997a; Hu et al. 2003; Zhong et al. 2008). Contradictory findings have been reported in different transgenic mouse models of AD including increase, decrease or no overall alterations in synaptophysin immunoreactivity (see Chapter 4, Supplementary Table 1). Previous studies in 3xTg-AD mice found stable synaptophysin immunoreactivity (Yao et al. 2005; Julien et al. 2010), although a single electron microscopic study on the morphological analysis of different types of synapses have shown reduced perforated synapses in the pyramidal cell layer of the hippocampal CA1 subfiled at 13 months of age (Bertoni-Freddari et al. 2008).

The second major finding from the results in Chapter 4 was a decrease in the $N_v$ of hippocampal perforated axospinous synapses in 3xTg-AD mice compared to age-matched non-Tg controls (Chapter 4, Fig. 4.6C and 4.7C) (see also Fig. 6.2). These results further extend the previous findings by Bertoni-Freddari and colleagues (2008) by analysing all hippocampal CA1 strata in 3xTg-AD mice up to 18 months of age. Although the overall synaptic $N_v$ remained stable, a significant decrease in the $N_v$ of perforated axospinous synapses was found in the CA1 subfield of the hippocampus and more specifically in the CA1 S.Mol that was evident at an early age (3 months, 56%) and continued into advanced age (18 months, 52%) (Chapter 4, Fig. 4.6C and 4.7C).
Figure 6.2. Schematic diagram illustrating the early (3 months) and the late (18 months) SERT-IR fibre sprouting that occurs not only in response to intraneuronal Aβ accumulation and extracellular Aβ plaque deposition, but also concomitant with a decrease in perforated synapse in the hippocampus.

From personal library.
Perforated synapses are considered as indicative of synaptic remodelling and turnover in the CNS, for review see (Calverley & Jones 1990). In the hippocampus, perforated axospinous synapses increase synaptic efficacy and play a pivotal role in spatial and working memory (Geinisman et al. 1986b; Calverley & Jones 1990; Nicholson et al. 2004). Induction of long-term potentiation (LTP) and improved memory performance in rodents have been linked with an increase in the number of perforated synapses (Stewart et al. 2005). On the other hand, deterioration of cognitive functions, including learning and memory correlate well with reduced perforated synapses in the hippocampus (Geinisman et al. 1986; Nicholson et al. 2004).

Given the importance of perforated axospinous synapses in cognitive functions, the reduced $N_v$ of perforated synapses reported in Chapter 4 may reflect an impaired synaptic plasticity and synaptic efficacy in the 3xTg-AD mouse model of AD, which may contribute to the deterioration of learning and memory performance in these mice (Oddo et al. 2003a; Oddo et al. 2003b; Berton-Freddari et al. 2008).
6.4. Increased serotonergic Neurotransmission as a potential therapeutic approach in AD

Data from Chapter 5 reported that increased 5-HT neurotransmission, via high dietary TrP intake, reduced intraneuronal Aβ accumulation in the hippocampus. These findings are in general agreement with a previous study in 3xTg-AD mice, where increasing 5-HT neurotransmission (via chronic treatment with SSRI, paroxetine) reduced intraneuronal Aβ in the hippocampus (Nelson et al. 2007b). In addition, chronic treatment with another commonly prescribed SSRI (citalopram) reduced Aβ plaque burden in the cortex and the hippocampus in the PS1APP transgenic mouse model of AD (Cirrito et al. 2011). Furthermore, in vivo imaging had reported that chronic exposure to SSRI antidepressants reduced Aβ plaque load in cognitively normal individuals (Cirrito et al. 2011).

Previous in vitro studies, uncovering the molecular mechanism involved in 5-HT-mediated decrease in Aβ deposition, have shown that application of 5-HT stimulate the non-amyloidogenic pathway in amyloid precursor protein (APP) metabolism (Nitsch et al. 1996; Robert et al. 2001). Application of SSRI (paroxetine) reduced APP translation and diminished pathogenic Aβ peptide secretion in vitro (Payton et al., 2003; Morse et al., 2004). Both SSRI (citalopram) and tricyclic anti-depressant (imipramine) have been shown to promote the secretion of soluble form of APP (SAPPα) in vitro that may potentially prevent Aβ accumulation in AD (Pakaski et al., 2005) (Chapter 5, Fig. 5.6). These reports are in agreement with an in vivo study where chronic administration of 5-HT2A/2C agonist dextrofenfluramine (DEXNOR) increased SAPPα in the CSF and reduced Aβ secretion in primary basal forebrain neuronal culture obtained from guinea pigs (Arjona et al., 2002).

Stimulation of the non-amyloidogenic pathway may be responsible for alleviation of intraneuronal Aβ aggregates following high dietary TrP intake and increased 5-HT neurotransmission, where the secreted protein is no longer available for the amyloidogenic accumulation mediated by β- and γ- secretases cleavages (Fig. 6.3), as previously suggested for treatment with SSRI (Arjona et al. 2002; Pakaski et al. 2005). In addition, inhibition of β- secretase activity, as a result of increased central 5-HT neurotransmission, is another possible mechanism that may mediate the high
TrP diet-induced decrease in intraneuronal Aβ aggregates (Fig. 6.3). In support of the latter possible mechanism, a recent study by Takahashi and Miyazawa (2011) have reported that 5-HT derivatives inhibit β-secretase hence possibly reducing the amyloidogenic accumulation of Aβ in AD (Takahashi & Miyazawa 2011).

In Chapter 1, clinical studies that administered serotonergic drugs alone or in combination with acetylcholinesterase (AChE) inhibitors were comprehensively reviewed (Chapter 1, section 1.6.). It was shown that although several studies reported improved memory and reduced cognitive disturbances (Schneider et al. 1991; Roth et al. 1996; Mowla et al. 2007; Mossello et al. 2008), all clinical studies have reported improved depressive behaviour following treatment with drugs acting on 5-HT neurotransmission (serotonominetic) in AD (Chapter 1, Table 1.14).
Figure 6.3. Photomicrographs summarising the effect of increased dietary TrP intake on intraneuronal Aβ accumulation (A, B) and a potential explanation of the relationship between extracellular Aβ plaques (D) and hyperphosphorylation of intraneuronal tau protein (F) as well as their possible effect on the underlying cognitive deficit associated with AD. From personal library.
Given that AD-associated neuropathology triggers severe degeneration of ACh and 5-HT systems, stimulation of both neurotransmitter systems have been shown to improve clinical outcomes in AD patients (Dringenberg 2000; Abe et al. 2003; Toda et al. 2003; Smith et al. 2009). Pre-clinical studies using a dual inhibitor of AChE and SERT has been shown to elevate extracellular levels of 5-HT and ACh in the hippocampus that was associated with improved memory performance in rodents (Abe et al. 2003; Toda et al. 2010). Similarly, increased cerebral metabolism and memory performance was reported in AD patients following combined treatment with AChE and SERT inhibitors (Finkel et al. 2004; Mossello et al. 2008; Smith et al. 2009; Rozzini et al. 2010). In addition, combined treatment with AChE and SERT inhibitors also improved the activity of daily living in AD patients, suggesting that concomitant increase in ACh and 5-HT neurotransmissions provide a better therapeutic approach in AD treatment (Nyth & Gottfries 1990; Lyketsos et al. 2003; Mowla et al. 2007).

It is important to note that AChE inhibitors are prescribed to patients with mild to moderate AD, whilst AD patients at moderate to severe stage of the disease are treated with memantine (see Chapter 1, section 1.1.1.6.4.). To the best of our knowledge there are no reported study on combined treatment with SSRI and memantine in AD patients at moderate to severe stage of the disease. However, a recent pharmacokinetic study had shown that combined memantine and SSRI (fluoxetine) is safe with respect to drug-drug interaction in rats (Zendulka et al. 2011). Furthermore, combined memantine and fluoxetine treatment induced a synergetic effect in improved behaviour in an animal model of obsessive-compulsive disorder (Wald et al. 2009). Altogether these pre-clinical studies suggest that combined treatment with memantine and SSRIs may provide better therapeutic outcomes in management of AD. Given that increased 5-HT neurotransmission not only improved cognitive performance and reduced behavioural abnormalities in patients with AD, but it also diminished the underlying neuropathology in animal models of the disease (Nelson et al. 2007b; Cirrito et al. 2011; Noristani et al. 2012), future studies on combined treatment with SSRI and memantine may be more efficacious in AD management.
6.5. Future directions
Currently alterations in hippocampal astrocytic populations specifically expressing the S-100β protein are being explored in our laboratory throughout the progression of the disease in 3xTg-AD mice as a possible neurotrophic factor responsible for triggering the SERT-IR fibre sprouting. Changes in morphology (volume and surface) and the \( N_v \) of S-100β-expressing astrocytes in 3xTg-AD mice compared to age-matched non-Tg controls are being examined, using methods that had previously been described (Olabarria et al. 2010). In addition, the study also investigates the alteration in S-100β-expressing astrocytes in relation to Aβ plaque deposition in 3xTg-AD mice at 18 and 24 months of age. As mentioned earlier, our preliminary data suggest that S-100β-expressing astrocytes located in the close vicinity of Aβ plaques undergo severe hypertrophy compared to S-100β astrocytes located > 50µm away from Aβ plaques. These finding are in line with results in Chapter 3 suggesting that increased SERT fibre sprouting in the close vicinity of Aβ plaques may be associated with increase astrocytic S-100β expression in this region. This may help us to uncover not only the link between glial cells and neurones but also the role of S-100β as a possible mechanism for observed SERT-IR fibre sprouting in the 3xTg-AD mouse model of AD.

The experimental work of this thesis primarily focused on the hippocampus due to its critical role in memory function and it being one of the first brain regions affected in AD brains. Although the results in Chapter 3 showed increased SERT-IR fibre sprouting adjacent to Aβ plaques, the analysis mainly focused on S.Mol of the CA1 subfield due to high abundance of Aβ plaques in this region. High density of Aβ plaque deposition also occurs in the cortex and the amygdala in 3xTg-AD mice. To establish that SERT-IR fibre sprouting is driven by Aβ plaques, irrespective of different brain region, further studies measuring SERT-IR fibre density in the cortex and the amygdala are required.

In addition, as another plausible follow-up study it is important to investigate the expression of hippocampal 5-HT receptors in 3xTg-AD mice throughout the progression of the disease. Post-mortem and clinical studies have shown that 5-HT receptors are deeply affected in AD neuropathology (see Chapter 1) and other
animal models of AD. In particular, to decipher the effect of AD-related neuropathology, it is important to investigate the alteration in 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor expression in relation to the development of extracellular Aβ plaques and intraneuronal hyperphosphorelated tau protein accumulation in 3xTg-AD mice. These studies may help to uncover the role of specific 5-HT receptors responsible in mediating 5-HT response during the progression of AD. However, currently there are no preliminary data on altered 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor expressions in the 3xTg-AD mouse model of AD.

Results from Chapter 5 reported that increased dietary TrP intake reduced intraneuronal Aβ accumulation that may prevent extracellular Aβ plaque deposition in the hippocampus (Fig. 6.3). A potential future experiment may include extending the increased dietary TrP intake and assessing its effect not only on AD-related neuropathology but also on cognitive function in the 3xTg-AD mouse model of AD.

All together, these studies could be of great relevance, value and importance to understand AD pathology and its progression and may further lead to potential development of improved serotonomimetic drugs in targeting AD.
6.6. Concluding remarks
Serotonergic neurotransmission plays a fundamental role in multiple physiological functions including food intake, aggression, sleep, learning and memory processes. Impaired serotonergic system contributes to the pathophysiology of various mental disorders including depression, anxiety, stress and schizophrenia. Accumulating evidence emphasises the involvement of 5-HT system in AD. Our research has shown that there is a biphasic increase in hippocampal serotonergic fibre sprouting, which corresponds with intraneuronal/extracellular Aβ deposition and reduced perforated synapses in the 3xTg-AD mouse model of AD. Increased serotonergic input may act as an intrinsic neuro-protective mechanism to counteract Aβ-induced neurotoxicity, suggesting that over-activation of 5-HT neurotransmission may be beneficial in reducing AD-related neuropil damage. Indeed, increased dietary TrP intake reduced intraneuronal Aβ accumulation in the hippocampus, possibly via stimulating the non-amyloidogenic processing of APP. Given the protective role of increased 5-HT neurotransmission, treatment with serotonomimetic drugs (MAOIs and SSRIs) may not only improve behavioural and cognitive abnormalities but they also may be beneficial in reducing the underlying neuropathology associated with AD. Direct increase in 5-HT neurotransmission may provide a promising therapeutic approach to the halt or better treatment of AD.
Chapter 7

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Serotonin fibre sprouting and increase in serotonin transporter immunoreactivity in the CA1 area of hippocampus in a triple transgenic mouse model of Alzheimer’s disease

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative disease that deteriorates cognitive functions and associated brain regions such as the hippocampus, being the primary cause of dementia. Serotonin (5-HT) is widely present in the hippocampus, being an important neurotransmitter involved in learning and memory. Although recent evidence suggests alterations in 5-HT neurotransmission in AD, it is not clear how hippocampal 5-HT innervation is modified. Here, we studied hippocampal 5-HT innervation by analysing: (i) the expression, density and distribution of 5-HT transporter (SERT)-immunoreactive fibres; (ii) the specific morphological characteristics of serotonergic fibres and their relation to amyloid plaques; and (iii) the total number of 5-HT neurons within the raphe nuclei in triple transgenic animals of different ages (3, 6, 9, 12 and 18 months). The transgenic animals showed a significant increase in SERT fibres in the hippocampus in a subfield-, strata- and age-specific manner. The increase in SERT fibres was specific to the CA1 stratum lacunosum-moleculare. An increase in SERT fibres in transgenic animals was observed at 3 months (by 61%) and at 18 months (by 74%). No changes, however, were found in the total number of raphe 5-HT neurons at any age. Our results indicate that triple transgenic mice display changes in the expression of SERT and increased SERT fibres sprouting, which may account for imbalanced serotonergic neurotransmission associated with (or linked to) AD cognitive impairment.

Introduction

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative disease impairing cognition and memory (Braak et al., 1999; Garcia-Alloza et al., 2005). AD neuropathological hallmarks are plaques (amyloid beta; Ab), neurofibrillary tangles (NFT), neuronal death, synaptic loss and synaptic plasticity impairment (Braak et al., 1999; Scheff et al., 2006). Classically, the loss of cholinergic neurons and a decrease in choline acetyltransferase activity were linked with AD (Birks et al., 2008). However, drugs enhancing cholinergic function have modest efficacy, suggesting the involvement of other neurotransmitters (Birks et al., 2008).

Serotonin (5-HT) plays a critical role in cognition and memory (Schmitt et al., 2006; Evers et al., 2007). The dorsal (DR) and medial (MR) raphe nuclei, which contain the majority of 5-HT neurons, widely project throughout the CNS including the hippocampus (Vertes et al., 1999), showing different axon morphology (Bjarkam et al., 2005; Keuker et al., 2005; Hensler, 2006). DR axons are fine fibres (FF) with small and regularly spaced varicosities, whilst MR axons are either non-varicose (SA; stem axons) or thick with large irregular varicosities (beaded fibres – BF; Bjarkam et al., 2005; Keuker et al., 2005; Hensler, 2006).

Increasing evidence emphasizes the involvement of 5-HT in AD (Toghi et al., 1995; Meltzer et al., 1998; Lai et al., 2002; Mowla et al., 2007; Truchot et al., 2007). Patients with AD have a 5-HT deficit, and treatments with selective 5-HT re-uptake inhibitors or specific receptor ligands enhance cognitive functions (Porter et al., 2000; Mowla et al., 2007; Mossello et al., 2008; Terry et al., 2008).

Patients with AD have altered levels of 5-HT, its metabolite and receptors within the cortex and hippocampus (Meltzer et al., 1998; Lai et al., 2002; Garcia-Alloza et al., 2005; Kepe et al., 2006; Lorke et al., 2006; Bowen et al., 2008; Hasselbalch et al., 2008), and a decrease in 5-HT neurons and projections (Chen et al., 2000; Thomas et al., 2006), which are correlated with cognitive and behavioural alterations (Lai et al., 2002; Garcia-Alloza et al., 2005). However, these studies are prone to misinterpretation due to contradictory results. Sprouting of 5-HT fibres and increased activity is observed...
following neurotoxic lesions and Aβ accumulation in rats and amyloid precursor mutant mice (APPsw) transgenic mice within the hippocampus and cortex (Gasser & Dravid, 1987; Zhou et al., 1995; Phinney et al., 1999; Harkany et al., 2000; Verdurand et al., 2009). In contrast, APPsw/PS1M146V mice show 5-HT fibres degeneration (Liu et al., 2008). Hence, the exact changes and alterations in 5-HT transmission associated with AD require further study.

Therefore, the use of animal models reproducing a complete AD pathology is fundamental for understanding 5-HT involvement in AD and the development of potential therapies (Rodriguez et al., 2008, 2009a,b). Thus, by studying changes in hippocampal serotonergic innervations and raphe 5-HT neurons we aim to determine the exact 5-HT alterations throughout AD progression using the triple transgenic (3 × Tg-AD) mouse model – expressing both Aβ and NFT with an spatiotemporal distribution, which resembles AD and exhibits age-related cognition deficits (Oddo et al., 2003a,b; Clinton et al., 2007; Frazer et al., 2008).

Materials and methods

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

Animals

The procedure for generating 3 × Tg-AD mice has been described previously (Oddo et al., 2003a,b; Frazer et al., 2008). Briefly, human cDNA harbouring the Swedish APP mutation (KM670/671NL) and human P301L four repeats mutated Tau were co-injected into single-cell embryos of homozygous PS1M146V knockin mice. The background of the PS1 knockin mice is a hybrid 129/C57BL6. The non-Tg control mice were used from the same strain and genetic background as the PS1 knockin mice, but they express the endogenous wild-type mouse PS1 gene. All 3 × Tg-AD and non-Tg mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12 h light–dark cycles with free access to food and water.

Fixation and tissue processing

Male 3 × Tg-AD mice and their respective non-transgenic controls were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg) at different ages (3, 12, 18 and 12 months; n = 4–11). Mice were perfused through the aortic arch with 3.75% acrolein (25 mL; TAAB, UK) in a solution of 2% paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde (75 mL). Brains were then removed and cut into 4–5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously (Rodriguez et al., 2008, 2009b). The brain sections were postfixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40–50 μm-thick slices using a vibrating microtome (VT1000S; Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4.

Immunohistochemistry

The sections were incubated for 30 min in 30% methanol in 0.1 M PB and 30% hydrogen peroxide (H2O2; Sigma, UK). Sections were then rinsed with 0.1 M PB for 5 min and placed in 1% sodium borohydride (Aldrich, UK) for 30 min. The sections were then washed with PB profusely before rinsing in 0.1 M Trizma base saline (TS) for 10 min. Brain sections were then incubated in 0.5% bovine serum albumin (Sigma, UK) in 0.1 M TS and 0.25% Triton X-100 (Sigma, UK) for 30 min. Sections were incubated for 48 h at room temperature in primary antibody (rabbit anti-SERT, 1 : 2500 and rabbit anti-5-HT, 1 : 5000; Immunostar, Hudson, WI, USA). The sections were rinsed in 0.1 M TS for 30 min and incubated in 1 : 400 dilutions of biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Stratford Scientific, Soham, UK) for 1 h at room temperature. Sections were rinsed with 0.1 M TS for 30 min followed by incubation for 30 min in avidin-biotin peroxidase complex (Vector Laboratories, Peterborough, UK). The peroxidase reaction product was visualized by incubation in a solution containing 0.022% 3,3′-diaminobenzidine (DAB; Aldrich, Gillingham, UK) and 0.003% H2O2 for 6 min, as described previously (Rodriguez et al., 2008, 2009b). The reaction was stopped by rinsing the sections in 0.1 M TS for 6 min followed by 0.1 M PB for 15 min. Brain sections were permanently mounted onto gelatinized slides and allowed to dry overnight. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and, finally, xylene. Coverslips were applied using Entellan (Merck KGaA, Germany) and slides were left to dry overnight.

For detection and determination of SERT-IRF and its relationship with Aβ senile plaques, we used dual indirect immunofluorescence labelling. The sections were incubated for 48 h at room temperature in primary antibody cocktail containing: (i) mouse anti-Aβ monoclonal antibody (1 : 2000; Covance, USA) and (ii) rabbit anti-SERT polyclonal antibody (1 : 2500; Immunostar, Hudson, WI, USA) simultaneously. Subsequently, Aβ and SERT-IRF were detected in a sequential manner on the same sections by incubation with rhodamine (TRITC)-conjugated goat anti-mouse and FITC conjugated goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1 M TS for 30 min and permanently mounted in an aqueous medium (Vectashield; Vector Laboratories, Peterborough, UK).

Optical density (OD) measurement

Using computer-assisted imaging analysis (ImageJ 1.32j; NIH, USA) we analysed the expression and density of SERT-IRF by
measuring their OD, as described previously (Cordero et al., 2005). Brieﬂy, to exclude any experimental errors and/or bias, all images were taken at constant light intensity. Optical ﬁlters were used to ensure the speciﬁcity of the signal recorded by the camera. The staining was observed throughout the thickness of the section (40 μm) using confocal scanning microscopy (Leica SP5 upright) recording optical sections at every 0.2 μm. No differences were observed in SERT antibody penetration (Pickel et al., 1992) and immunoactivity throughout the thickness of the section between 3 × Tg-AD and non-Tg control animals, hence the changes in OD were used as a measure of increased transporter and ﬁbre sprouting. The OD was calculated from a relative scale of intensity ranging from 0 to 250, with a measurement of 250 corresponding to the area with very low SERT-IRF and 0 corresponding to the densest area of labelling. The calibration density was kept constant for measuring all sections to avoid experimental variances. Non-speciﬁc OD in sections was measured from the corpus callosum. SERT-IRF density of the complete CA1 subﬁeld of the hippocampus and its different layers [stratum pyramidale (PCL), stratum oriens (SO), stratum radiatum (S.Rad) and stratum lacunosum-moleculare (S.Mol)], except CA3 where we also studied stratum lucidum, were measured independently. Similarly, SERT-IRF density of the dentate gyrus (DG) and its different layers (granule cell layer, molecular layer and hilus) were measured individually. To analyse the change in SERT-IRF density against constant control, 250 was divided by control region (corpus callosum) and the obtained factor was multiplied by the region of interest in every given section. Inverse OD was obtained by subtracting from the obtained background level (250). Measurements of mean density were taken and averaged, after background subtraction, from each hippocampal layer in both the left and right hemisphere of each slice. The results are shown as inverse SERT-IRF density (IOD/pixel). To determine SERT-IRF density in relation to Aβ plaques, we used confocal scanning microscopy (Leica SP5 upright), recording layers at every 0.2 μm throughout the thickness of the section. Parallel confocal planes from the entire thickness of the section (40 μm) were superimposed and SERT-IRF density was measured using computer-assisted imaging analysis (ImageJ 1.32j; NIH, USA). We considered all SERT-IRF within 20 μm from plaque borders as plaque associated (around), and SERT-IRF at more than 20 μm as not plaque associated (away), as described previously (Spires et al., 2005).

**Morphological analysis of hippocampal SERT ﬁbres**

We carried out a detailed morphological analysis in order to determine the different types of serotonergic ﬁbres present within the CA1 subﬁeld of the hippocampus in relation to their nuclei of origin. For each condition, at least three sections from 12 animals were examined for quantiﬁcation of SERT-IRF. Images were taken using Nikon Eclipse 80i microscope with a 100 × oil immersion objective. All visible SERT-IRF ﬁbres were analysed in 15 random images within the S.Mol of CA1, each representing an area of 10 800 μm², with a total surface area of 162 000 μm². Therefore, our results are expressed as area density S₀ (Rodriguez et al., 2008). SERT-IRF’s were classiﬁed as BF, FF and SA according to their morphological features, as already described (Bjarkam et al., 2005; Keuker et al., 2005; Hensler, 2006) – BF, thick axons with irregularly spaced varicosities; FF, thin axons with typically small and evenly distributed varicosities; SA, large thick straight axons with no varicosities (Bjarkam et al., 2005; Keuker et al., 2005; Hensler, 2006; see Fig. 3).

**Cell count of 5-HT neurons in the raphe nuclei**

To determine whether the changes in hippocampal SERT-IRF density might be due to the loss of 5-HT neurons, we estimated the total number of 5-HT-immunoreactive (5-HT-IR) neurons in DR and MR nuclei of 3 × Tg-AD and non-Tg control mice. The areas analysed for 5-HT cell count included the DR dorsal, DR ventral, DR interfacicular part, MR and paramedian raphe nuclei. The boundaries of areas in which cells were to be counted were clearly delineated; thus, counts were reproducible, and counting 5-HT-stained cell proﬁles in every third section constituted a true random sample (Vertes & Crane, 1997). All 5-HT-IR neurons were intensively labelled against a light background, which made them easy to identify with an equal chance of being counted (Vertes & Crane, 1997). The main source of error in using this calculation is the potential multiple counting of the same proﬁle in more than one section, as suggested previously (Vertes & Crane, 1997). However, in this case, one has to consider that the maximum cell diameter of the neurons counted was approximately 25–30 μm, and every third 40-μm section was 120 μm distant from the adjacent one, making multiple counting of the same cell proﬁle in adjacent sections unlikely, as described previously (Vertes & Crane, 1997). To obtain a systemic random sampling of 5-HT-IR neurons, a sampling grid, consisting of a counting frame, was positioned over DR and MR nuclei on each section. The number of 5-HT-IR neurons was determined blindly by a single observer using 10 × 10 mm graticule. All visible 5-HT-IR neurons were counted in every third 40-μm-thick coronal section throughout the rostrocaudal extent of the different subdivisions of DR nucleus corresponding to bregma −4.36/−4.96 mm (Paxinos & Franklin, 2004). To deﬁne DR and MR as well as their subnuclei boundaries, adjacent sections were counterstained with toluidine blue. The estimated total 5-HT-IR neurons within different raphe nuclei were calculated according to the Konigsmark equation (Konigsmark, 1970), as described previously (Vertes & Crane, 1997):

\[
N_i / N_s = V_i / V_s
\]

where \(N_i\) is total count, \(N_s\) is sample count, \(V_i\) is total volume (range from 0.55 to 0.65 mm³) and \(V_s\) is sample volume.

**Statistical analysis**

Two-way analysis of variance (ANOVA) was used to determine changes in SERT-IRF density and 5-HT neurons at different ages, followed by Tukey post hoc test. Unpaired t-test was used to determine changes in SERT-IRF associated with Aβ plaques. Signiﬁcance was accepted at \(P \leq 0.05\). The data were analysed using Minitab (Minitab 15 Software). Results are expressed as mean ± SEM.

**Results**

**Distribution of hippocampal SERT ﬁbres**

In both 3 × Tg-AD and non-Tg control animals the SERT-IRFs were heterogeneously distributed throughout the hippocampal formation (Figs 1 and 2). SERT-IRF appeared mainly as ﬁne and thick processes with numerous varicosities, which are characteristic of axonal proﬁles (Fig. 1E and F). The highest density of SERT-IRF was observed within the S.Mol of CA1. Thick axons with large circular and irregular spaced varicosity that are classiﬁed as BFs accounted for 94% of total SERT-IRF in S.Mol (Fig. 3B). FFs accounted for less than 5% of total SERT-IRF, whereas large thick straight SERT-IRF (SAs) without varicosities were rare and
accounted for the remaining percentage (Fig. 3B, D and F). This pattern of distribution of BF, FF and SA was consistent within all layers of the hippocampus. S.Rad and SO exhibited moderate expression, whilst the lowest density of SERT-IRF was determined in the PCL (Fig. 1). In the DG, it was possible to observe a gradient in the density of SERT-IRF that was increasing from the inner to the outer molecular layer. However, this expression was not as marked as in the different CA subfields (Figs 1A and B, and 2). The granule cell layer of the DG and the hilus showed a low to moderate SERT-IRF density, with the majority of fibres exhibiting morphology of BFs (Fig. 1A and B).

**Temporal changes of SERT-IRF density in 3 Tg-AD mice**

The global analysis showed no general changes in SERT-IRF density in the DG and CA subfields (Fig. 2A–C), except for CA1 where a clear trend towards significant increase was detected at 3 and 18 months ($F_{4,77} = 4.451$, $P = 0.083$ and $P = 0.081$; Fig. 2C). A detailed quantitative analysis of individual CA1 layers revealed that this increase was due to a specific and significant increase in SERT-IRFs density with the CA1 S.Mol (Fig. 1B, D and F, and 2D), which also exhibited the highest density of SERT-IRFs in control animals (Fig. 1A, C and E). Such an increase in SERT-IRFs density was also restricted to 3 and 18 months old in 3 Tg-AD mice compared with age-matched controls (61%; $F_{1,180} = 20.65$, $P = 0.0394$ and 74%; $P = 0.0347$, respectively; Fig. 2D). Despite the increase in CA1 S.Mol, no significant changes were observed in either the CA1 S.Rad or SO (data not shown). Furthermore, neither in the DG nor in the CA3 subfield were significant changes observed in SERT-IRF densities observed ($F_{4,77} = 0.63$, $P = 0.997$, $P = 1.0$, $F_{4,77} = 3.13$, $P = 0.945$, $P = 1.0$; Fig. 2A and B).

**BFs and FFs increase in 3 Tg-AD mice**

At both 3 and 18 months old we found a significant increase in the density of BFs in 3 Tg-AD compared with the age-matched non-Tg (18%; $F_{1,180} = 50.85$, $P = 0.0309$ and 43%; $P < 0.001$, respectively; Fig. 3A and B). Conversely, FFs density increased only at 18 months old (155%; $F_{1,180} = 5.70$, $P = 0.0440$, respectively; Fig. 3C and D) in 3 Tg-AD. No significant changes were observed in the density of SAs in either age group (Fig. 3E and 3F). These changes were not accompanied by the appearance of any age-related degenerative profiles, such as swollen and tortuous varicosities, abnormal thickened axons, ballooned or spherical axon terminals (Phinney et al., 1999; Spires et al., 2005; Fig. 3B, D and F).
The number of 5-HT neurons remains constant throughout aging and in 3×Tg-AD mice

5-HT-IR neurons were distributed throughout the different subdivisions of both the DR (Fig. 4A–E) and MR (Fig. 4F). 5-HT-IR somatodendritic profiles were characterized by small rounded cell bodies with sparse dendritic arborizations (Fig. 4C and D). The highest number of 5-HT-IR neurons was observed in the DR (Fig. 4A–F). The 5-HT-IR neuronal population was also detected within both the MR and paramedian raphe nuclei (Fig. 4F). The number and distribution of 5-HT-IR neurons showed no significant difference in either the DR and MR nuclei between 3×Tg-AD and non-Tg mice in every age group (F4,32 = 0.091, P = 0.767; F4,32 = 2.92, P = 0.097, respectively; Fig. 4E and F).

Increased SERT-IRF density associated with Aβ plaques

Extracellular accumulation of Aβ plaques in the hippocampus of the 3×Tg-AD animals starts between 9 and 12 months, and increases with age, being maximal at 18 months old. It is primarily localized in the CA1 subfield, and more specifically within the S.Mol (Fig. 5A). The overall density of SERT-IRF in the S.Mol of 18-month-old 3×Tg-AD mice is increased significantly independent of the presence of plaques (27.25%; P = 0.005; Fig. 5C). This is further confirmed by the fact that in 3×Tg-AD mice compared with non-Tg control the SERT-IRF density away from amyloid plaques shows an equivalent significant increase (20.79%; P = 0.015; Fig. 5C). However, the sprouting of SERT-IRF was much more marked in the vicinity of the amyloid plaques compared with non-Tg control (33.67%; P = 0.003; Fig. 5B and C). Sprouting of SERT-IRF adjacent to amyloid plaques was also significantly higher compared with fibres away from the plaques (10.7%; P = 0.029; Fig. 5B and C). No distorted SERT-IRF has been observed either around or away from amyloid plaques (Fig. 5B).

Discussion

Our main finding is that 3×Tg-AD mice display age-dependent changes in 5-HT innervation. Increased sprouting of SERT-IRF in 3×Tg-AD mice starts at 3 months; and does not appear again when compared with controls till 18 months old. This sprouting is region-specific, being restricted to the S.Mol of CA1, which in normal conditions is also known to host the highest density of SERT within the hippocampal formation (Vertes, 1991; Vertes et al., 1999; Keuker et al., 2005). A detailed morphological analysis of SERT-IRF revealed no degenerative profiles of serotonergic axons (swollen and tortuous varicosities or abnormal thickened and ballooned and spherical axon terminals; Phinney et al., 1999; Spires et al., 2005) in both 3×Tg-AD and non-Tg mice of every age. These results suggest that the observed increase in SERT-IRF density in 3×Tg-AD is due to a specific outgrowth from undamaged axons, as described previously (Harkany et al., 2001), from both the DR and MR nuclei because of sprouting of BF (from MR) and FF (from DR; Bjarkam et al., 2005; Hensler, 2006). Increased sprouting is not associated with changes in the total number of 5-HT neurons—which stayed constant and was equivalent to that found in other AD transgenic models (Liu et al., 2008).
Our results are in broad agreement with previous studies, reporting that brain damage induces sprouting of serotonergic fibres in different regions of the rodent brain, including the hippocampus (Phinney et al., 1999; Harkany et al., 2000, 2001; Liu et al., 2008). This sprouting can be triggered during several pathological events. First, direct damage to either 5-HT or non-5-HT fibres induced by neurotoxins such as ibotenic acid and N-methyl-D-aspartate (NMDA) can stimulate homotypic and heterotypic sprouting in the forebrain, striatum and hippocampus mainly of BFs (Gasser & Dravid, 1987; Harkany et al., 2000, 2001; Liu et al., 2008). Second, it could be triggered by intracerebral injection of Aβ1–42 (Harkany et al., 2001), which is the primary neurotoxin in AD pathology (Moreno et al., 2007). In fact, Aβ intracellular accumulation in 3 × Tg-AD mice initiates intracellularly at an early age (3 months) that may induce damage to the affected neurons (Moreno et al., 2007; Rodriguez et al., 2008). Deposition of extracellular Aβ1–42 in the 3 × Tg-AD mice appears between 9 and 12 months, and then progressively increases with age, being mainly targeted to the CA1 hippocampal subfield (Oddo et al., 2003b; Rodriguez et al., 2008, 2009a,b). Such a build-up of plaques at a later age (18 months) may induce neurotoxic effects, resulting in neuronal damage that in turn may also stimulate the observed sprouting of serotonergic fibres in 3 × Tg-AD (Fig. 6A and B), which is in agreement with recent findings showing rapid serotonergic fibres sprouting within the basal forebrain, cerebral cortex and occasionally the hippocampus as a trophic response to various agents, such as S-100β protein (Zhou et al., 1995; Whitaker-Azmitia et al., 1997; Harkany et al., 2000, 2001). Furthermore, the pronounced increase in the density of BFs that we observed may suggest that increased sprouting of specific SERT-IRF may be followed by later fibre degeneration due to Aβ accumulation and/or related to Aβ-induced damage to their postsynaptic targets, as suggested previously (Harkany et al., 2000, 2001).

Lack of SERT-IRF sprouting at 9 and 12 months in 3 × Tg-AD may be due to a lower level of extracellular amyloid accumulation compared with at 18 months (in CA1 subfield of the hippocampus). In agreement with this phenomenon, preliminary results in our laboratory suggest that SERT-IRF sprouting is further increased within the hippocampus at 24 months in 3 × Tg-AD parallel to enhanced formation of large extracellular amyloid plaques (H.N. Noristani & J.J. Rodriguez, unpublished observation). In addition, our finding of early sprouting (3 months) may indicate that this process could even start with the accumulation of intracellular Aβ1–42, as we have recently shown (Rodriguez et al., 2008), which in turn might account for an initial protective response to restore hippocampal functionality, but later diminishes with the formation of extracellular plaques. Furthermore, transgenic mice overexpressing S-100β protein exhibit an early increase in maturation of hippocampal dendrite (1 month; Whitaker-Azmitia et al., 1997) and impairment in hippocampus-dependent spatial memory at 3 months old (Gerlai & Roder, 1996), as happens also in the 3 × Tg-AD (Oddo et al., 2003a; Clinton et al., 2007; Frazer et al., 2008).

Alternatively the early increase in SERT-IRF in 3 × Tg-AD could be due to genetic manipulation and overexpression of APP and Tau (Oddo et al., 2003a; Clinton et al., 2007; Frazer et al., 2008), which could have an effect on the normal 5-HT system development. The 5-HT neurons are one of the earliest to emerge during development of the brain and spinal cord (Azmitia, 2001). 5-HT neurons are detected at embryonic day 11 and 12 (E11–12) in mice and rats (Azmitia, 2001). The expression of SERT protein is initially detected at E12–14 and is then increased during migration of 5-HT axons from raphe nuclei between E20 and postnatal day 21 (P21; Zhou et al., 2000). Such a gradual increase in SERT expression is followed by a significant decrease at P28 that is then maintained at adult level (Azmitia, 2001). We may assume that 3 × Tg-AD could have an extended increase of SERT expression and/or a delay in its postnatal downregulation. However, further studies during early development are required to address this phenomenon.

The sprouting of serotonergic fibres can also be induced by trophic factors such as brain-derived neurotrophic factor (BDNF), which is highly present in the hippocampus (Luellen et al., 2007). BDNF-induced serotonergic axonal sprouting has been demonstrated in the neocortex, spinal cord and hippocampus (Bregman et al., 1997; Mamounas et al., 2000; Grider et al., 2005). The level of BDNF has been shown to increase after neurotoxic insults (p-chloroamphetamine lesion), being directly associated with 5-HT sprouting (Bregman et al., 1997; Grider et al., 2005). This fact is further corroborated by the
Evidence that accelerated loss of serotonergic fibres in the CA1 of transgenic mice with constitutive loss of BDNF (Luellen et al., 2007) is associated with a loss of serotonergic fibres and decrease of extracellular levels of 5-HT in the hippocampus (Luellen et al., 2007). Furthermore, BDNF is increased in AD brain, particularly in the vicinity of amyloid plaques (Murer et al., 2001; Burbach et al., 2004). Thus, a possible mechanism for the observed late serotonergic fibres sprouting can be associated with abnormal levels of BDNF (Fig. 6B).

Extracellular build-up of plaques also stimulates glial reactivity in different regions of the AD brain, including the entorhinal cortex and hippocampus (Rodríguez et al., 2009a; Verdurand et al., 2009; Fig. 6B). Increased release of neurotrophic factors such as S-100β by reactive astrocytes is therefore another candidate mechanism for the phasic induction of SERT-IRF sprouting in the hippocampal CA1 S.Mol (Fig. 6B). Other studies in our laboratory have shown astrocytes closely associated to neuritic plaques exhibit prominent and hence astrogliosis (Olabarria et al., 2010). As shown by excitotoxic lesions, the astrogliosis, which results in the enhanced level of S-100β, is associated with large serotonergic fibres sprouting (Zhou et al., 1995). In addition, enhanced expression of S-100β has been observed in the temporal lobe and frontal cortex of patients with AD, as well as in Down’s syndrome (DS; Griffin et al., 1989). Furthermore, the gene for S-100β is located on chromosome 21, within the so-called obligate region for DS, which also can exhibit AD-type neuropathology (Griffin et al., 1989; Azmitia, 2001).

The Aβ-induced neurotoxicity involves increased activation of glutamatergic system (Brorson et al., 1995; Miguel-Hidalgo et al., 2002) and impairment of calcium homeostasis (Brorson et al., 1995). Increased serotonergic input may help to counteract NMDA-induced neurotoxicity by inhibition of calcium influx and membrane hyperpolarization, as suggested previously (Harkany et al., 2000, 2001). Such an effect is mediated by activation of 5-HT1A and 5-HT1B receptors that are highly expressed in hippocampal formation (Kepe et al., 2006; Peddie et al., 2008). In support of the above phenomenon, a recent study by Verdurand et al. (2009) reported enhanced 5-HT neurotransmission and 5-HT1A receptors following intrahippocampal infusion of Aβ (Verdurand et al., 2009). Therefore, increased sprouting of serotonergic fibres may be an intrinsic protective mechanism in response to Aβ-induced excitotoxic damage.

On the other hand, the expression of APP and Aβ1-42 has been shown to promote neurite outgrowth. This potential neurotrophic...
effect on transmitter presence and homeostasis, hence contributing to enhanced synthesis of the neurotransmitter, which might have an be directly associated with either an increased re-uptake of or system by counteracting A

these results suggest that the sprouting of serotonergic fibres in AD

heterotypic sprouting of serotonergic neurons within the hippocampus

an abnormal sprouting of serotonergic fibres within the S.Mol of CA1

alterations. However, deep and specific behavioural tests are required AD in relation to their AD pathology.

Furthermore, altered 5-HT neurotransmission is intimately involved in the disturbance of mood disorder, including clinical depression (Cannon et al., 2007). PET studies have consistently shown increased SERT density in drug-free depressed patients (Cannon et al., 2007). Depression is also considered a risk factor for AD development and, in fact, 20–50% of patients with AD exhibit depressive symptoms (Garcia-Alloza et al., 2005; Messorello et al., 2008). Therefore, increased SERT-IRF sprouting obviously results in abnormal 5-HT neurotransmission that might also induce behavioural alterations in our 3 x Tg-AD model resulting in depression-like and mood-like alterations. However, deep and specific behavioural tests are required to confirm the onset and development of these alterations in 3 x Tg-AD in relation to their AD pathology.

In conclusion, this study demonstrates that 3 x Tg-AD mice exhibit an abnormal sprouting of serotonergic fibres within the S.Mol of CA1 at 3 and 18 months. Increased sprouting occurs in different types of serotonergic fibres irrespective of their nuclei of origin. Lack of changes in 5-HT-IR neurons within raphe nuclei support the heterotypic sprouting of serotonergic neurons within the hippocampus that is induced at the target region by non-5-HT neurons. Altogether these results suggest that the sprouting of serotonergic fibres in AD might act as a neuroprotective mechanism that defends the nervous system by counteracting Aβ-induced neurotoxicity. This in turn could be directly associated with either an increased re-uptake of or enhanced synthesis of the neurotransmitter, which might have an effect on transmitter presence and homeostasis, hence contributing to cognitive deficit associated with AD.

Acknowledgement

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Abbreviations

3 x Tg-AD, triple transgenic mouse model of Alzheimer’s disease; 5-HT, serotonin; Aβ, amyloid beta; AD, Alzheimer’s disease; APPβ, amyloid precursor protein mutant mice; BDNF, brain-derived neurotrophic factor; BF, beaded fibre; CA, hippocampus (Ammon’s horn); DG, dentate gyrus; DR, dorsal raphe; DS, Down’s syndrome; FF, fine fibre; IB, immunoreactive; MR, medial raphe; NFT, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; OD, optical density; PB, phosphate buffer; PCL, stratum pyramidale; SA, stem axon; SERT, 5-HT transporter; S.Mol, stratum lacunosum-moleculare; SO, stratum oriens; S.Rad, stratum radiatum; TS, Trizma base saline.

References


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preceptor protein expression and serotonergic sprouting following excito-


t receptors in the prefrontal cortex of Alzheimer and normal aging patients. BMC Neurosci., 7, 36.


Alzheimer’s disease (AD) is a neurodegenerative pathology that deteriorates mnesic functions and associated brain regions including the hippocampus. Serotonin (5-HT) has an important role in cognition. We recently demonstrated an increase in 5-HT transporter (SERT) fibre density in the hippocampal CA1 in an AD triple transgenic mouse model (3xTg-AD). Here, we analyse the ultrastructural localisation, distribution and numerical density (Nv) of hippocampal SERT axons (SERT-Ax) and terminals (SERT-Te) and their relationship with SERT fibre sprouting and altered synaptic Nv in 3xTg-AD compared with non-transgenic control mice. 3xTg-AD animals showed a significant increase in SERT-Te Nv in CA1 at both, 3 (95%) and 18 months of age (144%), being restricted to the CA1 stratum molecular (S. Mol; 227% at 3 and 180% at 18 months). 3xTg-AD animals also exhibit reduced Nv of perforated axospinous synapses (PS) in CA1 S. Mol (56% at 3 and 52% at 18 months). No changes were observed in the Nv of symmetric and asymmetrical synapses or SERT-Ax. Our results suggest that concomitant SERT-Te Nv increase and PS reduction in 3xTg-AD may act as a compensatory mechanism maintaining synaptic efficacy as a response to the AD cognitive impairment.

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Subject Category: Neuroscience

Alzheimer’s disease (AD) is an age-related, irreversible and progressive neurodegenerative disease that deteriorates memory and cognition.1 Neuropathological hallmarks of AD include neuritic plaques (amyloid beta (Aβ)), neuro-fibrillary tangles, neuronal death and synaptic loss, which occurs early in AD, and is linked to impaired synaptic connectivity and plasticity.2,3

Although, AD affects primarily the cholinergic system,4 other neurotransmitter systems are also implicated such as glutamate5 and serotonin (5-HT).6,7 Majority of 5-HT neurones are located within the dorsal and median raphe nuclei projecting to numerous brain regions including the thalamus, amygdala, cortex and the hippocampus, where they have important role in mnesic and cognitive functions.8,9 AD patients exhibit reduced 5-HT neurotransmission, which correlates with disease severity.10 Treatment with selective serotonin re-uptake inhibitors (SSRIs) and specific 5-HT receptor ligands improves cognition in AD patients.8

The degree and importance of changes in the 5-HT system in AD remains elusive in human studies, however, experiments on animal models indicate its pathological relevance. APPswe/PS1E9 double transgenic mouse display degeneration of 5-HT fibres, while APPswe,Ind transgenic mice overexpressing APP Swiss/Indian mutations show stable 5-HT fibre density in parietal cortex and the hippocampus.11,12 However, subsequent study in APPswe/PS1E9 transgenic mice revealed no alterations in 5-HT transporter binding sites (measured using [3H]-escitalopram radioligand) up to 11 months of age despite Aβ accumulation in the cortex and the hippocampus.13 Similarly, increased 5-HT fibre sprouting was reported following neurotoxin lesion and Aβ accumulation in striatum and hippocampus14–17 (see also Table 1). In addition, other transgenic mice (APP-23) show aberrant non-5-HT hippocampal axonal sprouting, which is directly related to Aβ accumulation.18 Serotonin transporter (SERT) is expressed in serotonergic axons and axonal terminals being critical for 5-HT re-uptake.19 Recently, we reported increased hippocampal SERT-immunoreactive (SERT-IR) fibres density in the triple transgenic (3xTg-AD) mouse model of AD,7 which mimics the spatiotemporal pathology and mnesic alterations of AD.20 Interestingly, the increase in SERT-IR fibre density is more evident in proximity to amyloid plaques suggesting that aberrant SERT-IR axonal sprouting and amyloid deposition are closely linked in AD neuropathology.7

Studies of synaptic density in AD patients and transgenic mouse models of AD also reveal inconsistent results. Whereas post-mortem studies show a steady decrease in synapses,21,22 different transgenic mouse models exhibit
either increase, decrease or no overall alterations\textsuperscript{23,24} (see also Supplementary Table 1). Indeed, 3xTg-AD mice show no changes in total synapse density\textsuperscript{25} although the numerical density (N,) of hippocampal perforated axosominal synapses seems to be reduced.\textsuperscript{26}

We recently reported increased SERT-IR fibre density in the CA1 stratum lacunosum moleculare (S. Mol) of the hippocampus in 3xTg-AD mice.\textsuperscript{7} This increase in SERT-IR fibre density initially appears at 3 months, which is in parallel with the evident intraneuronal accumulation of A\(\beta\).\textsuperscript{7,20} However, SERT-IR fibre density between 6, 9 and 12 months of age, is comparable to non-transgenic (non-Tg), while there is a late sprouting recurrence at 18 months, concomitant with the build-up of large extracellular neuritic plaques in 3xTg-AD animals.\textsuperscript{7,20} In this study, we extend our previous findings by analysing the ultrastructural changes in hippocampal SERT axons (SERT-Ax), terminals (SERT-Te) and their association with synaptic N,, and connectivity in the hippocampus in 3xTg-AD compared with non-Tg control, by using a quantitative three-dimensional immunoperoxidase method, at these two ostensibly key points in either the start-up of anomalous intraneuronal A\(\beta\) accumulation (3 months) and the consolidation of A\(\beta\) neuropil plaque formation and aggregation (18 months).

### Results
At light microscopic level, in both 3xTg-AD and non-Tg control animals SERT-immuoreactive (SERT-IR) fibres were heterogeneously distributed throughout the hippocampal formation (Figures 1, 2a and b). SERT-IR fibres appeared mainly as fine and thick processes with numerous varicosities, which are characteristic of axonal profiles (Figures 1c, d, 2a and b). The highest densities of SERT-IR fibres are evident in the S. Mol of CA1 area (Figures 1c, d, 2a and b). Strata radiatum and oriens exhibited moderate expression while the lowest densities of SERT-IR fibres are present in the stratum pyramidale (S. Py).

The 3xTg-AD group showed increased density of SERT-IR fibres in the S. Mol of CA1 exclusively at 3 and 18 months compared with age-matched controls (Figures 1c, d, 2a and b), as shown by augmented SERT-IR fibre optical and N,, which corresponded to our previous and recently described findings during the progression of AD.\textsuperscript{7} Increased SERT-IR fibre density, in 3xTg-AD, was even more evident in the proximity of A\(\beta\) plaques (Figures 2d–f).

### Ultrastructural distribution of hippocampal SERT profiles.
SERT-EM analysis confirmed that the majority of SERT-IR profiles were small axons and axon terminals and/or varicosities with different size and morphological features according to their raphe nuclei origin (Figures 3a, b, d–f and 4c–f).\textsuperscript{7} SERT-IR profiles were identified by the presence of morphous electron-dense DAB reaction product that was mainly associated with the plasma membrane of axons and terminals (Figures 3d–f and 4c–f) with or without association with the synaptic specialisation (Figure 3a and d–f). From the 17 993 axonal profiles analysed, 592 (3.3\%) contained peroxidase labelling for SERT. The majority of SERT-IR profiles consisted of morphologically heterogeneous population of axonal terminals and/or varicosities (SERT-Te) that made up 81\% of the SERT contained profiles (n = 479 out of 592; Figures 3a, b, d–f, 4c–f, 5b–e). Occasionally, the labelling was directly associated with cytoplasmic organelles, including dense core vesicles, which are characteristic of serotonergic terminals (Figure 3f).\textsuperscript{19} Dense labelling for SERT was also observed in small unmyelinated axons (SERT-Ax), which were predominantly apposed to other small axons and/or terminals (Figure 3c). SERT-Ax comprised 17\% of the SERT-IR profiles (n = 104 out of 592) and was seldomly in contact with dendritic profiles. SERT labelling was absent in postsynaptic sites and was rarely observed in glial profiles. From the 479 immuno-labelled SERT-Te identified in this study, only 64 (13\%) showed synaptic specialisation mostly represented by asymmetrical synapses with dendritic spines and dendrites (Figures 3a and b). SERT-Te area varied in size from 0.12 to 1.01 \(\mu\text{m}^2\) (mean 0.2 \(\pm\) 0.01 \(\mu\text{m}^2\)). The main postsynaptic target of SERT-Te terminals were unlabelled dendrites onto which these terminals establish asymmetrical

### Table 1 Summary of studies on 5-HT alterations in animal models of Alzheimer’s disease

<table>
<thead>
<tr>
<th>AD model</th>
<th>Neuropathology</th>
<th>Brain area investigated</th>
<th>5-HT alteration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP(<em>{\text{swe}})/PS1(</em>{\text{A}E9})</td>
<td>Plaques</td>
<td>C, Am, H</td>
<td>5-HT fibre degeneration (12 and 18 months)</td>
<td>Liu et al.\textsuperscript{11}</td>
</tr>
<tr>
<td>APP(_{\text{swe}})</td>
<td>Plaques</td>
<td>H</td>
<td>Aberrant non-5-HT axonal sprouting (18–20 months)</td>
<td>Phinney et al.\textsuperscript{18}</td>
</tr>
<tr>
<td>Intra hippocampal A(_{\beta}(1–40)) injection</td>
<td>Aggregated amyloid material</td>
<td>H</td>
<td>Increased 5-HT activity within the vicinity of injection site</td>
<td>Verdurand et al.\textsuperscript{16}</td>
</tr>
<tr>
<td>Intrastriatal ibotenic acid injection</td>
<td>Neurodegeneration</td>
<td>S</td>
<td>Vigorous sprouting of 5-HT fibres</td>
<td>Zhou et al.\textsuperscript{17}</td>
</tr>
<tr>
<td>MBN NMDA injection</td>
<td>Increased APP expression+cholinergic lesion</td>
<td>MBN</td>
<td>Abundant sprouting of 5-HT fibres within damaged area</td>
<td>Harkany et al.\textsuperscript{14}</td>
</tr>
<tr>
<td>MBN A(_{\beta}(1–42)) injection</td>
<td>Cholinergic lesion</td>
<td>MBN, FC, PreC, H</td>
<td>5-HT fibre sprouting</td>
<td>Harkany et al.\textsuperscript{15}</td>
</tr>
<tr>
<td>APP(<em>{\text{swe}})/PS1(</em>{\text{A}E9}) hAPP(_{\text{swe,Ind}})</td>
<td>Plaques and tangles</td>
<td>H</td>
<td>No change</td>
<td>Holm et al.\textsuperscript{13}</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td></td>
<td></td>
<td>Biphasic &gt;65% increased SERT-Fibre density (3 and 18 months)</td>
<td>Noristani et al.\textsuperscript{7}</td>
</tr>
</tbody>
</table>

Abbreviations: Am, Amygdala; APP, amyloid precursor protein; C, cortex; FC, frontal cortex; H, hippocampus; MBN, magnocellular nucleus basalis; NMDA, \(N\)-methyl-d-aspartate, PreC, prefrontal cortex; S, stratum
synapses, which sometimes localised in a close vicinity to glutamatergic excitatory synapses (Figures 3a, 4c–f, 5b–e).

**Temporal changes in SERT-Te Nv and size in 3xTg-AD mice.** We observed an increase in SERT-Te Nv in the CA1 subfield of the hippocampus in a strata-specific manner (Figures 4a and b). The global analysis showed a significant increase in SERT-Te Nv at 3 and 18 months in 3xTg-AD compared with control group (95%, $P = 0.0436$ and 148%, $P = 0.0064$, respectively; Figure 4a). A more detailed quantitative analysis of CA1 individual strata revealed that the increased SERT-Te Nv was restricted to the CA1 S. Mol, which also exhibited the highest density of SERT-IR fibres within the hippocampal formation in basal conditions (Figures 1c, d, 2a and b). This increase also appeared at the same ages doubling or almost doubling the values from age-matched control mice (227%, $P = 0.0148$ at 3 months, and 180%, $P = 0.0329$ at 18 months; Figure 4b). SERT-Te Nv showed no alterations in either stratum oriens (S. Or) or stratum radiatum (S. Rad) at any examined age ($P = 0.5659$, $P = 0.1948$ for S. Or and $P = 0.0571$, $P = 0.2607$ for S. Rad at 3 and 18 months, respectively; data not shown). The increase in SERT-Te Nv is associated with a generalised increase in their size as measured by determining their area, which was age specific (by 108%, $P = 0.0264$ at 3 months and by 89%, $P = 0.0474$, at 18 months; Figures 5a–e) but not strata specific. No age-associated changes were observed in either SERT-Te Nv or their size in 3xTg-AD animals between 3 and 18 months of age. However, non-Tg animals showed an age-associated decrease in SERT-Te Nv in the CA1 of the hippocampus (39% decrease, $P = 0.0413$) although their size remain stable (Figures 4a and 5a). In addition, SERT-Ax Nv and size remained stable in 3x-Tg-AD and non-Tg control in all strata and examined ages.

**Reduced Nv of perforated synapses in the hippocampus of 3xTg-AD mice.** Synaptic elements and postsynaptic densities were heterogeneously distributed throughout the different hippocampal layers (Figures 3b, 4c–f, 5b–e) as revealed by the use of serial EM sections, which allowed us to distinguish and quantify different synapse populations, according to their postsynaptic densities and synaptic vesicles composition (see Supplementary Figure S1). Symmetric synapses have thin pre- and postsynaptic densities with axons containing pleomorphic (round and elongated) synaptic vesicles (Supplementary Figure S1A), while asymmetric synapses displayed round synaptic vesicles in the axons and were characterised by the presence of a thin presynaptic density and with a thick and more prominent postsynaptic membrane specialisation (Supplementary Figure S1B). Some of these asymmetric synapses presented a notable discontinuity ($> 50$ nm) in the electron density of the postsynaptic junction as previously described being considered as perforated (Supplementary Figure S1C). The majority of the total unlabelled (SERT−) identified synapses were asymmetric being characterised by a thin pre- and a thick postsynaptic membrane specialisation (Figures 3e and e). In total, 11 108 SERT− synapses were identified of which 10 889 (98.03%) were asymmetrical type between axons and dendritic spines; the remaining 219 (2%) were symmetrical synapses mainly established onto dendrites and dendritic shafts (Figure 3a). Only 7.9% (863) of the asymmetric synapses were identified as perforated.

Although, global analysis of hippocampal CA1 area showed no difference in Nv of total, symmetric and asymmetric SERT−
synapses; there was a significant decrease in perforated axospinous \( \text{N}_v \) in 3xTg-AD compared with non-Tg control (Figure 6c). Analysis of the whole hippocampus revealed significant decrease in perforated axospinous \( \text{N}_v \) in 3xTg-AD animals at 3 but not at 18 months of age (64% loss, \( P = 0.0056 \) and 39% loss, \( P = 0.1453 \)). The layer-specific analysis also revealed that the S. Mol exhibits significant deficit in \( \text{N}_v \) of perforated axospinous synapses (Figure 7c) at 3 and 18 months compared with age-matched control (56% loss, \( P = 0.0260 \) and 50% loss, \( P = 0.0394 \)). Furthermore, the non-Tg control group also showed an age-associated decrease in perforated axospinous \( \text{N}_v \) both in CA1 subfield of the hippocampus and S. Mol of the CA1 (38% loss, \( P = 0.0260 \) and 34% loss, \( P = 0.0070 \), Figures 6c and 7c), this was not the case for \( \text{N}_v \) of other types of SERT-\(^+\) synapses. However, we did not find any changes in the synaptic \( \text{N}_v \) specifically established by SERT-labelled (SERT\(^+\)) profiles when comparing 3xTg-AD with non-Tg animals either in the whole CA1 subfield or S. Mol (\( P = 0.4796 \), \( P = 0.3739 \) at 3 months and \( P = 0.1529 \), \( P = 0.5185 \) at 18 months, respectively (Figures 6d and 7d). SERT\(^+\) synapses were mainly asymmetric synapses with dendritic spines and dendrites.
Ultrastructural analysis of SERT synaptical specialisation also showed no difference in SERT axo-spine and axo-dendritic synapses between 3xTg-AD and non-Tg control animals.

Discussion

Age-dependent and CA1 strata-specific increase in SERT terminals. Our main finding is that 3xTg-AD mice display an age-dependent increase in Nv of SERT-IR terminals. Global quantitative analysis of hippocampal CA1 revealed that SERT-Te Nv is increased at 3 and 18 months when compared with age-matched non-Tg controls. The increase in SERT-Te Nv is more evident within the S. Mol of CA1 area, which in normal conditions is also known to host the highest density of serotonergic projections in the hippocampal formation.8 In addition, to the increased Nv, the size of SERT-Te was also increased in the CA1 subfield of the hippocampus. No changes were observed in SERT-Ax Nv. These data suggest that there is an age-dependent increase in hippocampal serotonergic input that is closely associated with development of AD pathology in 3xTg-AD. Furthermore, our finding of increased SERT-Te Nv confirm our previous hypothesis of SERT fibres sprouting in the CA1 S. Mol of the hippocampus (as shown by increased optical and Nv of SERT-IR fibres) at 3 and 18 months 3xTg-AD mice compared with age-matched non-Tg control animals.7 Serotonergic fibre sprouting is generally defined as fibre outgrowth reflected by increase in size and diameter of axons, appearance of new spindle varicosities with no sign of damaged SERT axons.7,14,17 Serotonergic fibre sprouting may act as a compensatory mechanism by maintaining an overall stable hippocampal circuitry and synaptic connectivity. Thus, our results provide the first ultrastructural evidence for increased serotonergic input within the hippocampus of 3xTg-AD mouse model. Previously, increased 5-HT fibre sprouting was found only after acute brain damage such as Aβ, ibotenic acid and NMDA injections in rats (Table 1).14–17 Similar changes in non-5-HT fibre sprouting were observed in the entorhinal cortex and thalamic nucleus in the APP23 transgenic mice.18 In 3xTg-AD mice, intracellular Aβ accumulation starts at an early age (3 months) that may also cause early damage to the affected neurons.28 Extracellular deposition of Aβ contributes to plaque formation, which in 3xTg-AD begins between 9 and 12 months of age and consolidates at 18 months, primarily targeting the CA1 hippocampal subfield and in particular stratum molecular.7,20 The 3xTg-AD mice exhibit age-related increase in extracellular build-up of plaques that continues into advanced age.20 Indeed, previous studies including our recent studies in the same cohort of animals confirmed that the 3xTg-AD animals exhibit extensive Aβ and tau pathology at 18 months of age7,20 (see also Figure 2e). Such massive build-up and consolidation of

![Diagram](https://example.com/diagram.png)
plaques at a later age (18 months) may induce neurotoxic effects resulting in neuronal damage that in turn may also stimulate an increase in SERT-Te in 3xTg-AD. Lack of SERT fibre sprouting at earlier ages (6 to 12 months) in 3xTg-AD animals may be due to lower level of extracellular amyloid accumulation in CA1 of the hippocampus, as well as to a less compromised synaptic connectivity compared with 18 months. In agreement with this phenomenon, a recent study in the Aβ/APPsw/PS1ΔE9 double transgenic model with associated severe amyloid pathology showed no alteration in cortical and hippocampal SERT density up to 11 months of age. In addition, our preliminary results suggest that SERT-IR fibre sprouting is further increased in the hippocampus at 24 months of age in 3xTg-AD animals, concomitant with formation of large extracellular Aβ plaques (Noristani et al., unpublished observation). Furthermore, our finding of early increase in SERT-Te (3 months) may indicate that this process could even start with the accumulation of intracellular Aβ/Δε20, which in turn might account for an initial protective response to restore hippocampal functionality.

The neurotoxic effect of Aβ involves increased activation of glutamatergic neurotransmission and impairment of calcium homeostasis. Increased 5-HT input may help to counteract neurotoxicity by inhibiting calcium influx and inducing membrane hyperpolarisation. This effect is mediated by activation of 5-HT1A and 5-HT1B receptors that are highly expressed in the hippocampal formation. In support of the above hypothesis, a recent study by Verdurand et al. reported enhanced 5-HT neurotransmission and 5-HT1A receptors expression following intra-hippocampal infusion of

**Figure 4** Bar graphs showing the age effect on SERT-Te Nv within overall CA1 subfield of the hippocampus (a) and stratum moleculare of the CA1 (b) at 3 and 18 months in non-Tg control and 3xTg-AD. Bars represent mean ± S.E.M. *P < 0.05, **P < 0.01 compared with age-matched non-Tg control, #P < 0.05 compared with 3 months non-Tg control. (c-f) Representative electron micrographs illustrating SERT-Te density in the CA1 stratum moleculare subfield of the hippocampus at 3 and 18 months in non-Tg control (c and e) and 3xTg-AD mice (d and f). Scale bar 500 nm (c-f). M, mitochondria.
Aβ. Increased expression of hippocampal 5-HT₁₅ receptors was also reported in patients with mild cognitive impairment who exhibit greater risk of developing AD. Therefore, an increased 5-HT input may represent an intrinsic protective mechanism in response to Aβ-induced excitotoxic damage. In fact, increased SERT-Te area in 3xTg-AD suggests that these terminals might release greater quantities of neurotransmitter, thus enhancing the synaptic strength. We have also observed a pronounced increase in SERT-Te Nv in the S. Mol. Such layer-specific effect may be due to the highest density of 5-HT projections compared with other hippocampal layers. In addition, and as mentioned earlier, in 3xTg-AD animals excess extracellular Aβ plaques are evident in the S. Mol. This combined phenomenon would account not only for the SERT-IR fibres sprouting but also directly affect the synaptic density and connectivity in the hippocampus.

AD-associated changes in synaptic density. Contrary to the accepted phenomenon linking AD to synaptic loss, we found no overall deficit in synaptic Nv in the hippocampal CA1 subfield. Our results are in agreement with previous studies, which, by employing qualitative or semi quantitative light immunohistochemistry, reported no overall changes in synaptic-associated proteins such as synaptophysin in the hippocampus and in the cortex of APP23, PDAPP, PSAPP, APPSw and recently in 3xTg-AD animals (see Supplementary Table 1). Similarly, no changes in total Nv of synapses were observed in the pyramidal layer of the hippocampal CA1 in 3xTg-AD animals at 13 months of age. We further extend this finding by analysing all the strata in 3xTg-AD animals up to 18 months of age. Although we found no changes in the overall synaptic Nv, there was a decrease in the Nv of perforated axospinous synapses in the CA1 area of the hippocampus and more specifically in the S. Mol that was evident at early age (3 months, 56%) and continued into advanced age (18 months, 52%). Our analysis has also shown that this synaptic population is affected during aging. The age-related decrease is consistent with previous results in aged rats in which hippocampal perforated synapses showed decrease in Nv and a reduction in the size of postsynaptic density, although others have reported no changes in total number of all perforated and non-perforated axospinous synapses. One factor that may account for this discrepancy includes variations in hippocampal volume.
Figure 6  Bar graphs showing the age effect on unlabelled (SERT−) total (a), asymmetrical (b), and perforated synapses (c) as well as the specific synaptic Nv of SERT-labelled (SERT+) profiles (d) in the CA1 of the hippocampus at 3 and 18 months in non-Tg control and 3xTg-AD group. Bars represent mean ± S.E.M. **P < 0.01, *P < 0.05.

Figure 7  Bar graphs showing the age effect on unlabelled (SERT−) total (a), asymmetrical (b), and perforated synapses (c) as well as the specific synaptic Nv of SERT-labelled (SERT+) profiles (d) at 3 and 18 months in non-Tg control and 3xTg-AD in the CA1 S. Mol of the hippocampus. Bars represent mean ± S.E.M. (n = 3). *P < 0.05, **P < 0.01
Conflicting results have been reported in relation to hippocampal volume alterations in different transgenic models of AD. Previous studies in single and double transgenic mouse models of AD including (PS1, APP/PS1-1 and PSAPP mice) showed no alterations in hippocampal volume using longitudinal in vivo MRI scanning or Cavalieri’s principle for estimation of total hippocampal volume. Contrary, Oberg et al. reported decreased hippocampal volume in APP/PS1 double transgenic mice using in vivo longitudinal MRI scanning. On the other hand, Maheswaran et al. have demonstrated increased hippocampal volume in the TASTPM mice showing severe amyloidosis, which could be associated with an accumulation of amyloid plaques and reactive glial cells. In this study, we analysed an equal hippocampal volume between 3xTg-AD and non-Tg control animals, which reliably eradicates the effect of possible volume alterations on synaptic Nv. In addition, ultrathin section series were cut at a constant thickness (see Materials and methods section) between the two groups to avoid the bearing effect of section thickness on synaptic density. Furthermore, the criteria we used for synapse determination includes not only the presence of postsynaptic density but also the occurrence of the adjacent presynaptic element containing the synaptic vesicles, which provides a reliable and un-biased estimation of synaptic Nv compared with that of just profile count. Finally, and in parallel with the report in the majority of AD transgenic models, our findings are consistent with a recent electron microscopic study that also reported reduced Nv of perforated synapses in 3xTg-AD, when quantifying synapses per unit volume.

Perforated axospinous synapses in the hippocampus have an important role in spatial and working memory. Increased perforated synapses have been linked with the induction of long-term potentiation and enhanced memory performance in rodents. Reduced perforated synapses show good correlation with deterioration of cognitive function including learning and memory. Given the pivotal role of perforated axosynaptic synapses in cognitive function, the reduced Nv of perforated synapses that we have found may reflect the impaired synaptic plasticity and subsequent deterioration of learning and memory in 3xTg-AD animals. In this direction, post-mortem studies on human tissue have consistently reported a decrease in synaptic density in the cortex and in the hippocampus. In transgenic models, which exhibit plaque pathology, both an increase and a decrease in synaptic density was found in the neocortex and in the hippocampus. Transgenic models with tangle pathology have also showed reduced hippocampal synaptic density (see also Supplementary Table 1). Different factors may account for these controversies including differences in (i) transgenic models (ii) strains, (iii) age, (iv) brain area studied and (v) methods used to quantify synaptic density. In conclusion, this study suggest that sprouting of serotonergic axons is directly related with an increased Nv, and area of SERT-Te that may act as a compensatory mechanism in maintaining overall synaptic efficacy. In addition, this increased serotonergic input may be an intrinsic neuroprotective response to counteract Aβ-induced neurotoxicity and altered hippocampal glutamatergic circuitry, as revealed by the decrease in asymmetric perforated Nv, accounting for the early behavioural alterations and later establishment of severe and permanent cognitive and mnesic impairments in AD. In fact, enhanced 5-HT neurotransmission via chronic treatment with SSRI (paroxetine) improves memory performance and retards the development of amyloid and tau pathologies in 3xTgAD mice. Thus, increasing 5-HT neurotransmission in AD may provide a better therapeutic approach not only to improve behavioural abnormalities, but also to interfere with underlying neuropathology associated with AD.

Materials and Methods
All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

Animals. The procedures for generating 3xTg-AD mice have been described previously. Briefly, human APP cDNA harbouring the Swedish mutation (KM670/671NL) and human four repeat Tau, harbouring the P301L mutations were co-microinjected into single-cell embryos of homozygous PS1M146V knockin mice. The background of the PS1 knockin mice is a hybrid 129/C57BL6. The non-Tg control mice used were also from the same strain and genetic background as the PS1 knockin mice, but they harbour the endogenous wild-type mouse PS1 gene. All 3xTg-AD and non-Tg mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12-h light–dark cycles with free access to food and water.

Fixation and tissue processing. Male 3xTg-AD and their respective non-Tg controls were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 3 and 18 months of age (n = 3). Mice were perfused through the aortic arch with 3.75% acrolein (TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, Gillingham, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde. Brains were then removed and cut into 4–5 mm coronal slabs of tissue consisting of the entire rostrocaudal extent of the hippocampus, as described previously. The brain sections were post-fixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40–50 µm thickness using a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4 were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal vibratome hemissections at level –2.06 mm posterior to Bregma (dorsal hippocampus), were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin. To optimise the penetration of immunoreagents, the tissue sections were freeze-thawed as described previously. For this procedure, the sections were (i) incubated in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB, pH 7.4, (ii) rapidly immersed in chloroformfluoromethane, followed by liquid nitrogen and (iii) placed in 0.1 M PB at room temperature to thaw the tissue. Then the sections were rinsed in 0.1 M PB, followed by 0.1 M Trizma base saline (TS), pH 7.6.

Antibodies. A polyclonal rabbit antibody raised against a synthetic peptide sequence corresponding to amino acids 602–622 of rat 5HT transporter (Immunostar, Hudson, WI, USA) was used for determination of SERT-positive axons and terminals in the hippocampus. For the identification of Aβ plaques, we used a monoclonal mouse antisera monoclonal antibody against amino acid resides 1–16 of beta amyloid (Covance, Emeryville, CA, USA). The specificity of the antibody has been reported previously using immunohistochemistry and western blots. To further determine the specificity of the antibody, adsorption controls were done using SERT peptide, which resulted in total absence of target labelling. Furthermore, omission of primary and/or secondary antibody also showed no immunoreactivity (data not shown).

Immunohistochemistry. The sections were incubated for 30 min in 30% methanol in 0.1 M PB and 3% hydrogen peroxide (H2O2, Sigma). Sections were then rinsed with 0.1 M PB for 5 min and placed in 1% sodium borohydride (Aldrich, Gilligam, UK) for 30 min. The sections were then washed with PB profusely before
Cavalieri principle as described elsewhere.49 Ax and synapses was determined, on serial ultrathin sections, according to the profiles/0.022% of 3,3′-diaminobenzidine (DAB, Aldrich) and 0.003% H2O2 for 6 min as described previously.7 The reaction was stopped by rinsing the sections in 0.1 M TS for 6 min followed by 0.1 M PB for 15 min.

For detection and determination of SERT-IR fibre and its relationship with Aβ senile plaques, we used dual indirect immunofluorescence labelling. The sections were incubated for 48 h at room temperature in primary antibody (rabbit anti-SERT, 1:2500, Immunostar) simultaneously. Subsequently, Aβ plaques and SERT-IR fibres were detected in a sequential manner on the same sections by incubation with Alexa 595 goat anti-mouse and Alexa 488 goat anti-rabbit (Invitrogen, Paisley, UK) IgG, respectively. Finally, sections were rinsed with 0.1 M PB for 30 min and permanently mounted in an aqueous medium ( VECTORshield; Vector Laboratories Ltd, Peterborough, UK).

Electron microscopy. Following the DAB treatment (immunohistochemistry), brain sections were rinsed and flattened at 0.1 M PB and post-fixed in 2% osmium tetroxide in 0.1 M PB for 1 h as described previously.27,30 The sections were then washed in 0.1 M PB for 10 min followed by sequential dehydroxylation through replacement with graded ethanol series (30, 50, 70 and 95%, 5 min each). The sections were further dehydrated using 100% ethanol (10 min), propylene oxide (2 × 15 min) and propylene oxide/EPOX (1:1, Agar Scientific Ltd, Stanstead, UK) overnight at room temperature, rotating. The propylene oxide/EPOX was replaced with 100% EPOX and rotated for further 2 h. The sections were then flat embedded between sheets of Acar fluo halocarbon film (Agar Scientific Ltd).37,30 Polymerisation was carried out by incubating at 80°C overnight. Following polymerisation, the region of interest (CA1 subfield of the hippocampus) was selected and micro-dissected from the flat-embedded tissue and mounted on the tip of EPO blocks27 (Figure 1), keeping the same levels and coordinates between the different animals. Diamond knife was used to cut series of ultrathin sections of these regions of a thickness of approximately 70 nm. Series of sections were collected on degreased copper mesh grids (200 mesh) and counterstained using uranyl acetate and lead citrate before examining under Philips FEI Tecnai 12 BioTwin electron microscope (FEI, Eindhoven, The Netherlands). Random images of the areas of interest (blindly taken) were collected and the negatives digitalised on an Imacon Flextight 848 scanner (Imacon Inc, Hasselblad A/S, Copenhagen, Denmark), each representing a volume of 12.39 μm3, for a total analysed CA1 volume of 396.48 μm3. Using computer-assisted imaging analysis (ImageJ 1.32j, NIH, Bethesda, MD, USA), we also analysed the area of SERT-Te in all hippocampal layers, as well as their synaptic specialisations found in labelled (SERT +) terminals in both 3xTg-AD and non-Tg control animals. Composite figures, adjusted for brightness, contrast and sharpness, were generated using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).

Labelling profiles and nomenclature. The labelled profiles were classified as somata, dendrites, dendritic spines, unmyelinated axons, axon terminals and glia, according to their morphological features as described previously.27,30 and defined by Peters et al.46 Synapses were defined as either symmetric when having thin pre- and postsynaptic densities or asymmetric when having a thin pre- and a thick postsynaptic membrane specialisation.27 Perforated synapses were defined as those asymmetric synapses with a notable discontinuity (>50%) in the electron density of the postsynaptic junction as previously described.27

Numerical density. To determine the Nv of labelled profiles ( labelled profiles/μm3), ultrastructural analysis was entirely carried out on the most superficial portions of the tissue in contact with the embedding plastic; from both vibratome section sides, to minimise artificial differences in labelling attributed to potential differences in the penetration of reagents as described previously.27 Regions used for this analysis were chosen randomly by a single investigator without any information about the genotype or the age of the animal. The Nv of SERT-Te, SERT-Ax and synapses was determined, on serial ultrathin sections, according to the Cavalieri principle as described elsewhere.49

\[ N_v = \sum P/[t - x(p)] \]

Where \( N_v \) is the numerical density, \( \lambda P \) is the number of SERT-Tg, SERT-Ax or synapses counted; \( t \) is the average ultrathin section thickness and \( x(p) \) is the corresponding surface area. All sections used for quantitative analysis were of identical anatomical level corresponding to −2.06 mm posterior to Bregma (dorsal hippocampus) in both 3xTg-AD and non-Tg control animals.

Three vibratome sections from each of the 12 animals (n = 3 per group) were examined for ultrastructural quantification of SERT immunolabelled profiles. The Nv of labelled profiles were determined and identified by the presence of amorphous electron-dense DAB reaction product (Figures 3–5). We selected this method of immunod gold labelling, because the latter is less sensitive compared with peroxidase labelling.50 Although gold labelling allows a more selective subcellular localisation, it has reduced tissue penetration and limited diffusion that may result in an underestimation of the relative abundance of immunoreactive profiles.27,50 Furthermore, immunogold labelling provides less reliable quantitative 3D estimation of labelled profiles because it is primarily regarded as 2D (surface) method and is not reliably considered as quantitative for 2D labelling.52

The total labelled profiles numbered 592 over a corresponding total volume of 4,556.16 μm3. Using computer-assisted imaging analysis (ImageJ 1.33), NIH, Bethesda, MD, USA), we also analysed the area of SERT-Te in all hippocampal layers, as well as their synaptic specialisations found in labelled (SERT +) terminals in both 3xTg-AD and non-Tg control animals. Composite figures, adjusted for brightness, contrast and sharpness, were generated using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).

Statistical analysis. Results are expressed as mean ± S.E.M. Unpaired Student’s t-test was applied to determine differences in Nv of SERT-Te, SERT-Ax and synapses between 3xTg-AD and non-Tg control animals at 3 and 18 months of age. Significance was accepted at P < 0.05. The data were analysed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

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