CORTICAL ASTROGLIAL ATROPHY IN AGEING AND ALZHEIMER'S DISEASE

A thesis submitted to the University of Manchester for the degree of PhD in Neuroscience in the Faculty of Life Science

2013

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# List of Content

**Chapter 1 - General Introduction**

- **Abstract**

  p. 21

- **1. Function and Dysfunction of the Entorhinal cortex**

  p. 23

  1.1 Anatomy and Organisation of the EC
      - **1.1.1 Subdivisions of the EC**
      - **1.1.2 Lamination and Cytoarchitecture**
      - **1.1.3 Differences and Similarities in Lamination between the LEC and MEC**
      - **1.1.4 Entorhinal-Hippocampal Connections**
      - **1.1.5 Entorhinal-Cortical and Entorhinal-Subcortical Connections**
      - **1.1.5.1 Entorhinal-Cortical Connectivity**
      - **1.1.5.2 EC Connections with Subcortical Structures**
      - **1.1.6 Intraentorhinal Connections**

  p. 23

  1.2 Functional Roles of the EC
      - **1.2.1 EC as Information Transfer and Integration Hub**
      - **1.2.2 Entorhinal Activation and Neuronal Activity in Cognition**
      - **1.2.2.1 Working memory**
      - **1.2.2.2 Long-term Memory**
      - **1.2.2.3 Spatial Representation and Memory**

  p. 35

  1.3 Entorhinal Dysfunction in Neuropathology
      - **1.3.1 EC in temporal lobe epilepsy and schizophrenia**
      - **1.3.2 EC in non-AD dementia and Ageing**

  p. 44

  1.4 **Summary**

  p. 50
2. Alzheimer's Disease Pathology in the EC

2.1 Overview of Alzheimer's Disease

2.2 Pathological Hallmarks

2.3 Neurochemical Changes

2.3.1 Cholinergic System

2.3.2 Glutamatergic System

2.3.3 Serotonergic and Cannabinoid Systems

2.4 Affected Brain Regions

2.4.1 Nucleus basalis of Meynert

2.4.2 Hippocampus

2.4.3 Prefrontal Cortex

2.4.4 Entorhinal Cortex

2.4.4.1 Atrophy and Neuronal Alteration in the EC

2.4.4.2 Tangles and Plaques in EC

2.4.4.3 Neurochemical Alterations in the EC

3. Astrocytic Involvement in AD within Cortical Regions

3.1 Astroglial cytoskeleton

3.2 Physiological role of astrocytes

3.2.1 Regulation of Synaptogenesis and the Number of Synapses

3.2.2 Potassium Buffering

3.2.3 Induction and Maintenance of Blood-Brain Barrier
3.2.4 Metabolic Support

3.2.5 Glutamate Homeostasis—Glutamate-Glutamine Cycle

3.2.6 Bidirectional Communication between Neurone and Astrocyte

3.3 Astroglia in Neuropathology

3.4 Astroglial Alteration in the cortex during ageing and AD

3.4.1 Effects of Ageing on Cortical Astroglia

3.4.2 Astroglial Alteration in the Cortex during AD Progression

4. Animal Models of AD

4.1 Lesion Model

4.2 Single and Double Transgenic Animal Model

4.3 Triple Transgenic Animal Model

Scope and Outline

Chapter 2 – Material and Methods

1. Animal

2. Fixation and Tissue Processing

3. Immunohistochemistry

3.1 Antibodies

3.2 Immunoperoxidase Labelling

3.3 Immunofluorescence Labelling

4. Morphological Analysis of GFAP-IR and/or GS-IR Astrocytes

5. Cell Count in the EC

6. Optical Density (OD) Measurement
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Identifying the region of interest (ROI)</td>
<td>p. 101</td>
</tr>
<tr>
<td>6.2 Acquiring and saving images for OD measurement</td>
<td>p. 102</td>
</tr>
<tr>
<td>6.3 Calibrating the optical density</td>
<td>p. 103</td>
</tr>
<tr>
<td>6.4 Measuring OD</td>
<td>p. 103</td>
</tr>
<tr>
<td>6.5 Performing the subtraction of background staining</td>
<td>p. 103</td>
</tr>
<tr>
<td>7. Cell Surface Measurement</td>
<td>p. 105</td>
</tr>
<tr>
<td>8. Statistical Analysis</td>
<td>p. 106</td>
</tr>
<tr>
<td>Chapter 2S – Supplement Material</td>
<td>p. 107</td>
</tr>
<tr>
<td>Chapter 3 – Early Astrocytic Atrophy in the Entorhinal Cortex of a Triple Transgenic Animal Model of Alzheimer’s Disease</td>
<td>p. 111</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>p. 113</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>p. 115</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>p. 118</td>
</tr>
<tr>
<td>RESULTS</td>
<td>p. 122</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>p. 129</td>
</tr>
<tr>
<td>Chapter 4 – Astrocytes in the Entorhinal cortex of the Triple Transgenic Animal Model of Alzheimer’s Disease Show No Alterations in Glutamate-Glutamine Shuttle Components</td>
<td>p. 141</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>p. 143</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>p. 145</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>p. 147</td>
</tr>
<tr>
<td>RESULTS</td>
<td>p. 151</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>p. 157</td>
</tr>
</tbody>
</table>
Chapter 5 – Region specific ageing of astrocytes: hippocampal hypertrophy and entorhinal cortex atrophy.

Rodriguez, J.J, Yeh, C.Y., Olabarria, M., Kulijewicz-Nawrot, M., Verkhratsky A.
Neurobiol Aging (submitted)

ABSTRACT

INTRODUCTION

MATERIAL AND METHODS

RESULTS

DISCUSSION

Chapter 6 – General Discussion

1. General Overview

2. Astrocytic changes in AD

   2.1 Changes in the Astroglial Cytoskeleton in the EC of 3xTg-AD Mice
   2.2 Glutamate Homeostasis in the 3xTg-AD Animals
   2.3 Heterogeneous Alterations in Astroglial Morphology during Ageing

3. General Conclusion

4. Future work

References

Appendix

Final Word Count: 65523
# List of Figures

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Figure</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>Figure 1.1</td>
<td>Schematic illustration of the rodent EC sub-divisions</td>
<td>p. 24</td>
</tr>
<tr>
<td></td>
<td>Figure 1.2</td>
<td>Brightfield micrograph of a toluidine blue stained section demonstrating the lamination of the rodent EC</td>
<td>p. 25</td>
</tr>
<tr>
<td></td>
<td>Figure 1.3</td>
<td>Schematic illustration summarising the entorhinal projections to the hippocampus.</td>
<td>p. 29</td>
</tr>
<tr>
<td></td>
<td>Figure 1.4</td>
<td>Schematic representation of the entorhinal-cortical connectivity</td>
<td>p. 32</td>
</tr>
<tr>
<td></td>
<td>Figure 1.5</td>
<td>Schematic illustration of intraentorhinal circuit.</td>
<td>p. 35</td>
</tr>
<tr>
<td></td>
<td>Figure 1.6</td>
<td>Schematic diagram showing the non-spatial and spatial information streams through the EC and the hippocampus.</td>
<td>p. 37</td>
</tr>
<tr>
<td></td>
<td>Figure 1.7</td>
<td>Diagram showing the process of APP cleavage</td>
<td>p. 49</td>
</tr>
<tr>
<td></td>
<td>Figure 1.8</td>
<td>Fluorescent microphotograph showing astrocytic morphology of GFAP-IR and Alexa 488-filling astrocyte.</td>
<td>p. 63</td>
</tr>
<tr>
<td></td>
<td>Figure 1.9</td>
<td>Schematic Illustration of Astroglial functions in the brain</td>
<td>p. 69</td>
</tr>
<tr>
<td></td>
<td>Figure 1.10</td>
<td>Schematic Illustration of tripartite synapse.</td>
<td>p. 71</td>
</tr>
</tbody>
</table>

| Chapter 2 | Figure 2.1 | Diagram illustration and brightfield micrograph showing a representative level of LEC | p. 99 |
|          | Figure 2.2 | Brightfield micrograph showing OD measurement of GS-labelling | p. 101 |
|          | Figure 2.3 | Standard step-tablet used for calibration | p. 102 |
|          | Figure 2.4 | Calibration curve used for all the OD measurement | p. 103 |
|          | Figure 2.5 | Diagram illustration and brightfield micrographs showing a representative level of dorsal hippocampus and GS-IR cell | p. 105 |

| Chapter 2-Supplementary Material | Figure 2.S.1 | Standard step-tablet for calibrating the system before OD measurement | p. 105 |
|                                  | Figure 2.S.2 | Standard OD values obtained from calibration | p. 106 |
|                                  | Figure 2.S.3 | Standard OD calibration curve | p. 106 |

| Chapter 3 | Figure 3.1 | Illustration scheme of entorhinal inputs to hippocampus | p. 116 |
|           | Figure 3.2 | Brightfield micrographs and bar graph showing the distribution of GFAP-IR astrocytes in the EC of 3xTg animals compared to non-Tg group. | p. 124 |
|           | Figure 3.3 | Bar graphs and confocal micrographs showing decreased GFAP-IR surface, volume, and body volume in the EC of 3xTg-AD mice when compared with control group. | p. 125 |
|           | Figure 3.4 | Bar graphs showing decreased GFAP surface and volume within specific layers of the EC of 3xTg-AD animals. | p. 127 |
|           | Figure 3.5 | Confocal micrographs of dual labelling of GFAP and β-amyloid | p. 128 |

| Supplementary | Figure 3.1 | Brightfield micrographs showing the amyloid and PHF immunoreactivity in the EC. | p. 132 |

<p>| Chapter 4 | Figure 4.1 | Brightfield micrographs of distribution of GS-IR astrocytes in the EC of non-Tg control and 3xTg-AD groups | p. 151 |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.2</td>
<td>Bar graphs illustrating comparison of GS or GFAP surface and volume</td>
<td>p. 153</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Confocal micrographs and bar graphs showing three subsets of astrocytes</td>
<td>p. 154</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Bar graphs illustrating comparison of GS or GFAP surface and volume</td>
<td>p. 156</td>
</tr>
<tr>
<td><strong>Chapter 5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Confocal micrographs showing the GFAP-IR astrocytes in the DG, CA1 and EC during ageing process</td>
<td>p. 175</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Brightfield micrographs showing GS-IR cells in the DG, CA1 and EC</td>
<td>p. 177</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Bar graphs showing the comparisons of GFAP surface, volume, and soma volume in the DG, CA1 and EC</td>
<td>p. 179</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Bar graph showing regional comparison of GS-positive cell area in the DG, CA1 and EC</td>
<td>p. 181</td>
</tr>
<tr>
<td><strong>Chapter 6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>Schematic Illustration of astroglial changes in the EC during ageing and AD</td>
<td>p. 197</td>
</tr>
</tbody>
</table>
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 1.1</td>
<td>Transgenic mouse models of AD</td>
<td>p. 83</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Summary of primary antibodies used and their respective sources</td>
<td>p. 95</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Summary of secondary antibodies used for immunofluorescence and their respective sources</td>
<td>p. 97</td>
</tr>
</tbody>
</table>
Abstract

Ageing is a process correlated with cellular stress and increased risks of neurodegenerative diseases, in particular Alzheimer’s disease (AD), which is accompanied with severe cognitive and memory impairments. Both ageing and AD affect many brain regions and thus induce brain malfunctions. Among the brain regions, the entorhinal cortex (EC) has drawn more and more attentions due to its pivotal role in cognition and memory functions as well as its vulnerability to ageing process and AD neuropathology. Synaptic and neuronal degenerations, which are also manifest features of AD, occur in the EC during the ageing process and at the early stage of AD. In addition, both pathological hallmarks of AD, namely abnormal accumulation of β-amyloid (Aβ) and hyperphosphorlation of tau proteins, initially appear in the EC and then progress to other brain regions such as the hippocampus and the neocortex. Glial alterations in AD and ageing process have been considered as secondary event to neuronal changes. Nevertheless, accumulating evidence indicates the relevant and primary involvement of astroglia, which is responsible for brain homeostasis, in AD and ageing. In this thesis, we have focused on the astroglial alterations in the EC during the progression of AD in an animal model of the disease as well as in ageing process in non-transgenic control mice. We have used the triple transgenic mouse model of AD (3xTg-AD), which is the most relevant animal model of AD and resembles the spatiotemporal progression of human AD pathology.

Our results revealed cytoskeletal atrophy of astrocytes in the EC of 3xTg-AD animals (Chapter 3), shown by significant decrease in GFAP surface and volume. This astroglial alteration began at very early age (1 month) and sustained till more advanced age (12 month). Moreover, Aβ plaques did not trigger astrogliosis, and there was rare presence of GFAP labelled astrocytes in the vicinity of Aβ deposition. This may reflect the relative indifference of astroglia in the EC and thus explain the susceptibility of the EC at the early stage of AD. To study whether astroglial atrophy in cytoskeleton compromise astrocytic function in glutamate homeostasis, we investigated the expression of glutamine synthetase (GS), which is specifically expressed in astrocytes and is critical for glutamate balance (Chapter 4). Our results showed constant GS expression and the density of GS positive astrocytes in the EC. However, dual labelling of GS and GFAP revealed 3 different subsets of astrocytes, being GS-, GFAP-, GS/GFAP- positive astrocytes. The morphology of GS-IR cells, measured by surface and volume, did not change in spite of the evident GFAP atrophy. Therefore, GFAP atrophy does not disturb glutamate homeostasis in the EC, suggesting diverse functional populations of astrocytes, which may show distinct responses during AD progression. In addition we also analysed astroglial changes during the ageing process in the EC and its major projection area, the hippocampus (Chapter 5). Astrocytes in the hippocampus exhibited prominent hypertrophy, shown by increased GFAP whereas entorhinal astrocytes in the EC had profound reduction in GFAP expression. This may implicate heterogeneous astrocytic responses to ageing in different brain regions. The general atrophy of astrocytes in the EC of 3xTg-AD mice and aged controls, suggests astroglial atrophy may results in reduced astrocytic coverage and modulation of synapses, accounting for the synaptic dysfunction in ageing and AD.
Declaration

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Research work of
Chia-Yu Yeh

Supervised by:
Prof. Alexei Verkhratsky
Prof. José Julio Rodríguez Arellano (co-supervisor)

2013

For the degree of PhD in Neuroscience
in the
Faculty of Life Science
The University of Manchester
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### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3xTg-AD</td>
<td>Triple transgenic mouse model of AD</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
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<tr>
<td>AChT</td>
<td>Choline acetyltransferase</td>
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<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<td>ANLS</td>
<td>Astrocyte-neurone lactate shuttle</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>APPswe</td>
<td>APP transgenic mice with Swedish mutation</td>
</tr>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Aβ</td>
<td>Amyloid beta (beta amyloid)</td>
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<td>BACE-1</td>
<td>Beta secretase 1</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DLPFC</td>
<td>Dorsolateral prefrontal cortex</td>
</tr>
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<td>DMPFC</td>
<td>Dorsomedial prefrontal cortex</td>
</tr>
<tr>
<td>DMS</td>
<td>Delayed match-to-sample</td>
</tr>
<tr>
<td>DNMS</td>
<td>Delayed non-match-to-sample</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
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<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, Radixin, Moesin</td>
</tr>
<tr>
<td>Ext Cap</td>
<td>External capsule</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial form of AD</td>
</tr>
<tr>
<td>FP</td>
<td>Frontopolar region of prefrontal cortex</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>FTD-P-17</td>
<td>FTD with Parkinsonism linked to chromosome 17</td>
</tr>
<tr>
<td>GFAP</td>
<td>Gial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFAP-IR</td>
<td>Gial fibrillary acidic protein-immunoreactive</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate-aspartate transporter</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate transport 1</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GLUT 1</td>
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</tr>
<tr>
<td>GrcL</td>
<td>Granular cell layer</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>GS-IR</td>
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</tr>
<tr>
<td>InsP3</td>
<td>Inostitol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IOD</td>
<td>Inverse Optical density</td>
</tr>
<tr>
<td>K&lt;sub&gt;ir&lt;/sub&gt;</td>
<td>Rectifying K&lt;sup&gt;+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>Lac Mol</td>
<td>Lacunosum moldeculare</td>
</tr>
<tr>
<td>LEC</td>
<td>Lateral entorhinal cortex</td>
</tr>
</tbody>
</table>
PUBLICATIONS IN PEER REVIEWED JOURNALS


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

General Introduction
Abstract

The entorhinal cortex (EC) has been regarded as an information transfer and integration hub between the hippocampus and neocortex, contributing to mnesic and cognitive functions. Therefore, alterations of the EC structure and/or changes in it have been found to have a determinant role in multiple neurological and neurodegenerative diseases, such as Alzheimer’s disease (AD) by mainly compromising mnesic functions; which also happens during physiological aging. AD is an irreversible and progressive disease, which is the most common type of elderly dementia and is characterised by β-amyloid and tau pathology as well as loss of neurones and synapses. AD pathology initiates in the EC, progressing to the hippocampus and eventually advancing to other brain regions. The EC, being the first and most affected brain region in AD, accounts for the cognitive and memory impairments observed at early stages of AD.

So far, most of research has focused on the neuronal and/or synaptic abnormalities since neurone is regarded as the main “active” element in the brain. Nonetheless, more and more studies investigate the role of glia in neurodegenerative diseases including AD. Glial changes were first observed and described by Alzheimer and Cajal in early 20th century despite the fact that glial pathology has been regarded as a secondary event during the progression of the disease. Among the various types of glia, astroglia are the most abundant and ubiquitous in the CNS, maintaining brain homeostasis and functions. Moreover, a growing body of evidence has suggested that astroglia can actively respond to neuronal activities and modulate synaptic connectivity. Therefore, astrogial alterations could not only compromise its housekeeping functions in brain physiology but also have a remarkable influence on the neuropathology. As a result, astroglia play a key role in neuropathogenesis and the development of the disease as well as in the progression of pathology. However, the specific astrogial changes in AD and ageing remain not fully understood.

In chapter 1, we present a general introduction of EC functions in cognition and memory as well as EC malfunctions in neuropathology, especially focusing on ageing and AD. Furthermore, we extensively review the literature on neuroglia involvement in AD pathology. In particular, we centred it in astrogial (patho) physiology as well as their role in both physiological ageing and AD.
Chapter 1
Introduction

1. Function and Dysfunction of the Entorhinal cortex

The entorhinal cortex (EC) is located at the rostral end of the temporal lobe in human brains and is mainly referred to as Brodmann’s area 28 (Brodmann, 1909). The name arose from the region proximity to the rhinal sulcus with several cortices surrounding it. The EC borders the periamygdaloid cortex and piriform cortex anteriorly, faces medially the parasubiculum, whilst laterally and posteriorly it is adjacent to the perirhinal and postrhinal cortices, respectively (Ramon y Cajal, 1902). Due to anatomical location, the EC plays a pivotal role of relay station between the hippocampus and the neocortices, which all are involved in cognitive and memory processes. The EC not only conveys but also actively selects and integrates bi-directional information flow between the hippocampus and the cortex (Ramon y Cajal, 1902); reviewed by (Kerr et al., 2007; Canto et al., 2008). Therefore, the EC is perceived the key element in cognitive and mnesic processes.

1.1 Anatomy and Organisation of the EC

1.1.1 Subdivisions of the EC

The EC is divided into sub-regions based on its connectivity and/or cytoarchitectural organisation (Ramon y Cajal, 1902; Brodmann, 1909; Insastui et al., 1997; Canto et al., 2008). As was initially proposed by Cajal and afterwards corroborated by later studies, the EC is divided into two regions, the lateral entorhinal cortex (LEC) and the medial entorhinal cortex (MEC) based on the terminal distribution of EC input to the dentate gyrus (DG) of the hippocampus (Insastui et al., 1997; Dolorfo and Amaral, 1998a); Fig 1.1). Fibres arising from the LEC terminate in the outer one-third of the molecular layer of the DG, whilst fibres from the MEC target the middle one-third part (Hjorth-Simonsen, 1972; Hjorth-Simonsen and Jeune, 1972). According to Brodmann the EC is divided into two subfields numbered 28a and 28b on the basis of their cytoarchitecture (Brodmann, 1909); these two subfields roughly correspond to the LEC and MEC respectively (Brodmann, 1909; van Groen et al., 2003; Witter, 2007). Noteworthy, the
terms LEC and MEC are not only related to the projections to the DG of the hippocampus but also linked to its topographical position. In general, LEC occupies a more rostrolateral position while MEC has a more caudomedial position (Dolorfo and Amaral, 1998a; Canto et al., 2008).

Figure 1.1 Schematic illustration of the rodent EC sub-divisions: The EC is subdivided into the LEC and MEC, based on their terminal projections to the hippocampal DG. Whilst the LEC projects to the outer part of molecular layer (also known as outer molecular layer), the MEC projects to the middle portion (also known as middle molecular layer) of the molecular layer of DG. (Modified from (Paxinos and Franklin, 2004).

In addition to these classical divisions, the EC can be further sub-divided into more subfields based on the anatomical position and cytoarchitectonic characteristics, with differences between species. In rodents, the EC is divided into 5 subdivisions (dorsolateral, dorsal-intermediate, ventral-intermediate in the LEC; caudal and medial subdivisions in the MEC). In humans and higher primates the EC comprises 8 subdivisions (Olfactory, lateral rostral, rostral, medial intermediate, lateral caudal, caudal, and caudal limiting subdivisions (Insausti et al., 1995; Insausti et al., 1997; Dolorfo and Amaral, 1998a; van Groen, 2001; Canto et al., 2008). Functional implications of these subfields remain unclear, and therefore are generally accepted to divide the EC into LEC and MEC when characterising its anatomy and functional role.
1.1.2 Lamination and Cytoarchitecture

Like in other cortical areas, the lamination of EC is based on morphological characteristics of the resident neurones. In brief, EC is composed of six layers, of which layers I-III are regarded as superficial layers, while layers IV-VI are regarded as deep layers (Fig. 1.2). Both, superficial and deep layers contain a plexiform layer, which mainly consists of fibres (layer I in superficial layers and layer IV in deep layers) and two neuronal cell layers (layer II and III in superficial layers; layer V and VI in deep layers).

Layer I, also known as molecular layer, contains dense transversely-oriented afferent fibres, which terminate in layers II – VI. In spite of the fact that this layer contains relatively low amount of cells, two morphologically distinct types of neurones, horizontal and multipolar neurones (MPNs), are present (Finch et al., 1988; Araque et al., 1998; Wouterlood et al., 2000; Canto and Witter, 2012a, b). Most of the cells in layer I are inhibitory interneurones that provide feed-forward inhibition to principal neurones in layers II and III (Finch et al., 1988; Wouterlood et al., 2000; Canto and Witter, 2012a, b).
In layer II, the major and most abundant neurone is the stellate cell (or fan cell), which is characterised by a star-like shape and is distributed preferentially in the superficial and middle part of the layer II, being the main source of outward projections to the hippocampus (Klink and Alonso, 1997). Pyramidal-like neurones, which are distributed in the deep part of layer II, are also principal neurones and contribute to the outward projections to the hippocampus (Klink and Alonso, 1997; Tahvildari and Alonso, 2005). The size of the principal neurones in layer II is relatively large (with soma diameter of 20-25µm) that makes them different from neurones in layer II of neighbouring perirhinal and postrhinal regions of the cortex (Insausti et al., 1997; Rapp et al., 2002). Interneurones in the layer II appear in different shapes, such as MPN, bipolar, and chandelier cells (Hendry et al., 1989; Schwerdtfeger et al., 1990; Jones and Buhl, 1993; Soriano et al., 1993; Tahvildari and Alonso, 2005). Axons arising from these interneurones form local synapses within layer II as well as project to the hippocampus (Schwartz and Coleman, 1981; Schwerdtfeger et al., 1990; Tahvildari and Alonso, 2005).

Layer III is the widest layer of the EC that contain cells with various shapes and sizes. Large to medium size pyramidal neurones, which are loosely distributed, are the most abundant cell type in layer III (Fig. 1.2). The pyramidal neurones constitute the main source of projections to the hippocampus (Gloveli et al., 1997; Witter and Amaral, 2004; Tahvildari and Alonso, 2005). In addition, stellate cells and MPNs also appear in layer III and project to the hippocampus (Germroth et al., 1989). Interneurones, such as pyramidal-like and bipolar cells, form local network within superficial layers (I-III; (Gloveli et al., 1997; Kumar and Buckmaster, 2006).

Many fibres pass through in layer IV of the EC, which therefore is often regarded as “lamina dissecans” due to its cell-sparse feature. Layer IV is also regarded as the border between layer III and layer V (Canto et al., 2008). However, occasionally bipolar cell and pyramidal-like neurones are observed in layer IV of the EC (Kohler et al., 1986; Canto et al., 2008). The latter morphology is comparable to pyramidal neurones within layers III or V, and these cells send axons reaching the white matter, a characteristic of projecting neurones (Lingenhohl and Finch, 1991).

Layer V of the EC is a stratified layer and can be divided into superficial portion (Va)
containing large to medium-sized pyramidal neurones and deep portion (Vb/Vc) comprising smaller pyramidal cells (Hamam et al., 2000; Hamam et al., 2002). Axons of all the pyramidal neurones in layer V project towards the deep white matter and send collaterals to the superficial and deep layers (Gloveli et al., 2001; Witter and Amaral, 2004). Horizontal neurones, MPNs and bipolar neurones are also present in layer V, constituting principal neurone groups and extending their axons to the angular bundle (Lingenhohl and Finch, 1991; Gloveli et al., 2001; Hamam et al., 2002).

In the EC, layer VI is delineated by white matter and it is fused with layer V at the more caudal position. Two groups of neurones, MPN and pyramidal neurones are the principal neurones that are distributed throughout this layer (Canto and Witter, 2012a, b).

1.1.3 Differences and Similarities in Lamination between the LEC and MEC

There are distinct cell types with specific distribution within the LEC and MEC. In general, the most prominent differences in neuronal distribution between the LEC and MEC can be found in layer II. As for principal neurones, stellate neurones are most abundant in layer II of the MEC; however, they are much less common in the LEC. Instead, another cell type called “fan cell” is the most numerous in layer II of the LEC (Klink and Alonso, 1997). Fan cells in the LEC have similar morphology as stellate cells in the MEC, although the dendrites of fan cell only branch in ascending and horizontal directions; in contrast dendrites of stellate cell branch widely and radially. There is little difference in the morphology of neurones in layers III, V and VI between the LEC and MEC. As for interneurones, the morphology of them is very similar between the LEC and MEC, where these cells are represented by bipolar, MPN, basket and chandelier neurones as mentioned above. However, when labelled by calretinin, many immunoreactive cells are found in layers II and III of the LEC whereas these are rather rare in layers II and III of the MEC (Wouterlood et al., 2000; Witter and Amaral, 2004; Canto et al., 2008). Majority of principal neurones in both LEC and MEC are glutamatergic, whereas most of the interneurones contain gamma-aminobutyric acid (GABA) (Wouterlood et al., 2000; Witter and Amaral, 2004; Canto et al., 2008).
1.1.4 Entorhinal-Hippocampal Connections

The hippocampus has long been regarded as the memory centre in the brain (Eichenbaum and Cohen, 1988). The neighbouring cortical regions, which have dense connections with the hippocampus, received increasing attention because of their potential involvement in memory. Among these cortical areas, the EC contributes the major input to the hippocampus. In general, the superficial layers (mainly layers II and III) of the whole EC project to all fields of the hippocampal formation through the perforant pathway (PP). Substantial projections arising from layer II distribute to the outer two-thirds of the molecular layer in the DG (Fig. 1.1 and 1.3A; Witter et al., 1989; Witter and Amaral, 2004). These innervations form asymmetrical (presumed excitatory) synapses with principal neurones (Witter, 2007). According to the study by Tamamaki and Nojyo (1993), collaterals of the same projecting cells within the layer II of the EC reach and terminate in the outer portion of the stratum lacunosum-moleculare in CA3/CA2 field. There is no reciprocal projection from either the DG and/or CA3 to the EC (Tamamaki and Nojyo, 1993). Layer III of the EC is the main origin of entorhinal innervations to the CA1 subfield of the hippocampus and the subiculum (Fig. 1.3A). The majority of the entorhinal innervations mentioned above form asymmetrical synapses in the hippocampus, suggesting that EC provides excitatory input to the DG as well as to the CA1 subfield of the hippocampus (Tamamaki and Nojyo, 1993; Naber et al., 2001; Kloosterman et al., 2003). While superficial layers of the EC provide the main input to the hippocampus, deep layers (especially layer V) receive reciprocal projections from the CA1 and the subiculum. Some studies have reported that neurones in deep layers of the EC, including layer V and VI, send projections to the outer molecular layer where they form excitatory synapses and send collaterals to the inner molecular layer and granule layer of the DG (Fig. 1.3A; Deller et al., 1996; Gloveli et al., 2001).

→ Figure 1.3 Schematic illustration summarising the entorhinal projections to the hippocampus. (A) In general, the layer II (L II) of the EC projects to the DG, whilst layer III (L III) innervates the CA1 subfield of the hippocampus and the subiculum. Deep layers of the EC, layers V and VI (L V and L VI) also give rise projections to the DG. In addition, LV also projects to the parahippocampal area. (B) Along the dorsoventral axis, cells in the lateral band (LB) across the EC project to the dorsal part of the DG (dark aqua); the medial band (MB) projects to the most ventral part of the DG (mid-aqua); the intermediate band (IB) innervate the rest part of the DG (light aqua). (B modified from Kerr et al., 2007).
Along the dorsal (septal)-ventral (temporal) axis, three mostly non-overlapping bands (lateral, intermediate and medial) of interconnected neurones span the entire EC (both LEC and MEC) and target specific dorsoventral levels of the DG. The most lateral band connects to the dorsal half of the DG; the intermediate band preferentially innervates a more ventral part of the DG, whereas the medial band projects to the ventral quarter of
DG (Dolorfo and Amaral, 1998a). Therefore, projections from both the LEC and MEC target DG in the dorsal hippocampus more heavily than ventral hippocampus (Fig 1.3B; Kerr et al., 2007). Connectivity between the EC and CA1/subiculum exhibits similar topographical pattern.

When considering the different subdivisions of the EC, projections arising from neurones in the layer II of the LEC and MEC terminate in different dendritic regions of the DG. Specifically, the lateral perforant path (LPP) fibres originate from the LEC and innervate the outer one-thirds of the molecular layer of the DG. The medial perforant path (MPP) arises from the MEC and terminates in the middle one-third portion of the molecular layer of the DG (Witter, 1989). Unlike the terminal distribution of the layer II, projections from layer III of the LEC and MEC innervate distinct and non-overlapping sub-area of the CA1 subfield of the hippocampus and the subiculum. The LEC innervate the distal CA1 and proximal subiculum, whereas the MEC inputs reach the proximal CA1 subfield and the distal subiculum (Suzuki and Amaral, 1990). Reciprocal projections from the CA1 subfield and the subiculum, primarily terminate in the deep layer (mainly layer V) of the EC, although some minor innervations to the layer III of the EC may arise from the CA1 subfield of the hippocampus (Naber et al., 2001; Kloosterman et al., 2003). The distal CA1 and proximal subiculum project to the LEC deep layers, whereas the proximal CA1 and distal subiculum project to the MEC deep layers (Suzuki and Amaral, 1990; Naber et al., 2001).

1.1.5 Entorhinal-Cortical and Entorhinal-Subcortical Connections

1.1.5.1 Entorhinal-Cortical Connectivity

Generally, the superficial layers (layer I-III), and especially layer I, act as recipients of cortical sensory inputs. Two major sources of cortical signals are the perirhinal and the parahippocampal (postrhinal in rodents) cortices, which may relay association memory and visual information to the EC respectively (Suzuki and Amaral, 1994). Projections from those two regions mainly innervate layer III, and to a lesser extend
layer I of the EC (Burwell and Amaral, 1998a). Olfactory structures, such as the olfactory bulb and piriform cortex, also provide substantial inputs to the layer I of the EC (Burwell and Amaral, 1998b). Pre- and parasubiculum regions of the brain have dense projections to the EC with their terminals distributing widely in the superficial layers of the EC (Caballero-Bleda and Witter, 1994). While the cortical regions mentioned above send large projections to the superficial layers of the EC, other cortical regions such as the medial prefrontal, retrosplenial, insular and parietal (somatosensory) cortices give rise to projections that terminate in the deep layers of the EC (Insausti et al., 1997; Kobayashi et al., 2002; Kobayashi and Amaral, 2003; Jones and Witter, 2007; Kobayashi and Amaral, 2007; Fig. 1.4). It should be noted that cortico-entorhinal connections are mostly reciprocal (Fig. 1.4; Suzuki and Amaral, 1994). The dense projections from the EC to cortical regions primarily arise from neurones located in the layer V of the EC. Nevertheless, there are some exceptions such as entorhinal projections to infralimbic and olfactory areas that originate from neurones in layers II and III of the EC (Insausti et al., 1997).

The Perirhinal cortex forms the main input to the LEC, whereas parahippocampal cortex is the major input to MEC (Kerr et al., 2007). In addition, LEC is more heavily innervated by piriform and insula cortex, while MEC receives more inputs from occipital, cingulate, retrosplenial and parietal cortices. Moreover, inputs from pre- and parasubiculum are restricted to the MEC. (Fig. 4; Caballero-Bleda and Witter, 1994; Witter and Amaral, 2004; Kerr et al., 2007) Conversely, LEC innervates more heavily to the perirhinal, piriform and insular cortices whereas MEC give rise to projections preferentially terminating in the parahippocampal (postrhinal), temporal and parietal cortices (Kerr et al., 2007). Taken together, anatomical evidence indicate that LEC and MEC might be involved in different cognitive and memory functions by processing distinct information (Kerr et al., 2007; Canto et al., 2008).
1.1.5.2 EC Connections with Subcortical Structures

The EC has also bidirectional connections with subcortical structures such as the basal forebrain, amygdala, thalamus, hypothalamus, and the brainstem (Insausti et al., 1987; Pitkanen et al., 2000; Saunders et al., 2005; Kerr et al., 2007). It is suggested that the main innervations of the basal forebrain to EC originate from the medial septal nucleus and the substantia innominata (Insausti et al., 1987). These are mainly cholinergic projections that primarily terminate in the layer II of the EC (Insausti et al., 1987). The amygdala afferents arising from the lateral, accessory basal and the basal nuclei of the amygdala terminate in layers III and V of the EC (Pitkanen et al., 2000). Thalamic input, which mainly originates from the nucleus reuniens and the nucleus centralis medialis, mainly terminates in layers I and III of the EC (Van der Werf et al., 2002). Additionally, hypothalamic projections arising from the supramammillary nucleus and lateral hypothalamic area diffusely innervate layers III to VI of the EC (Burwell and Witter, 2002). Several brainstem structures also give rise to projections that innervate the EC. These structures include the raphe nuclei (especially the dorsal
and medial raphe nuclei), which provides the serotonergic input to the EC, mainly targeting layer III of the EC; noradrenergic afferents, originating from the locus coerulesus mainly innervate superficial layers (layer II and III), whereas dopaminergic innervations from the ventral tegmental area target both the superficial and deep layers of the EC (Wilcox and Unnerstall, 1990; Akil and Lewis, 1994; Rosenkranz and Johnston, 2006; Lei et al., 2007).

Entorhinal projections arising from layer V innervate different subcortical structures (Witter and Amaral, 2004). Fibres originating from the EC to septal regions preferentially terminate in the lateral septal complex (Alonso and Kohler, 1984). Entorhinal projections to the amygdala mainly target the central nucleus of the amygdala (Pitkanen et al., 2000); while inputs to the thalamus primarily terminate in the medial dorsal, lateral dorsal and the paraventricular nuclei (Van der Werf et al., 2002; Saunders et al., 2005). In addition, the EC sends dense projections to the striatum, in particularly to the nucleus accumbens (Totterdell and Meredith, 1997).

Like the EC connections to the hippocampus and cortical regions, subcortical innervations to the EC exhibit diversity between EC subdivisions (Kerr et al., 2007). The LEC receives heavy innervations from the amygdala whereas MEC has more substantial inputs from the dorsal thalamus and hypothalamic areas (Insausti et al., 1987; Kerr et al., 2007). In addition, cholinergic afferents from the septum and dopaminergic innervations from ventral tegmental area mainly terminate in the LEC (Insausti et al., 1987; Kerr et al., 2007). However, it is suggested that both LEC and MEC have comparable projections to subcortical structures, despite the fact that the former dominates entorhinal input to the amygdala (Pitkanen et al., 2000).

1.1.6 Intraentorhinal Connections

In general, intrinsic connections of the EC are arranged in three bands spanning the whole LEC and MEC, corresponding to its projections to the hippocampus along the dorsoventral axis (Fig. 1.3B; see section 1.1.4). Cells in the lateral band have intrinsic connections with cells in the lateral band whereas cells in the medial band have intrinsic projections mostly confined to the medial band (Dolorfo and Amaral, 1998b; Chrobak
and Amaral, 2007). Cells located in the intermediate band are not only interconnected heavily within the band but also have moderate projections to the lateral and medial bands (Dolorfo and Amaral, 1998b). The lateral band and medial band have very minor projections to the intermediate band while there is no direct connection between the lateral and medial bands (Dolorfo and Amaral, 1998b). Hence, connectivity between the bands is relatively sparse. It is clear that the intrinsic connections of the EC are not confined to the subdivisions (LEC and MEC) but rather to the rostrocaudally orientated bands (which cross LEC and MEC), indicating communications between the LEC and MEC bands (Dolorfo and Amaral, 1998b; Chrobak and Amaral, 2007). Connections within the lateral and medial bands originate from either superficial or deep layers. Fibres originating from superficial layers (I-III) mostly form synapses on neurones in the superficial layer, whereas fibres from the deep layers (IV-VI) terminate in both superficial and deep layers. As a result the superficial layers contain higher density of intralaminar associational fibres and terminals (Dolorfo & Amaral 1998a; Fig. 1.5).

Axons of layer I neurones travel either horizontally within layer I or towards layers II and III (Germroth et al., 1989; Canto et al., 2008). The majority of principal neurones in layer II send axon collaterals, which either ascend to layer I or descend to layer III of the EC. Axon collaterals from layer III pyramidal neurones spread within layer III as well as in the other superficial layers (Gloveli et al., 1997). Most of the interneurones within layers II/III are GABAergic and contribute to local inhibitory network in the superficial layers (Mikkonen et al., 1999; Wouterlood et al., 2000; Kumar and Buckmaster, 2006). Pyramidal neurones in layer V project axons with collaterals ascending to the superficial layers of the EC and mainly forming asymmetrical (excitatory) synapses. Among these synapses, 56% are formed between principal neurones, and 44% with inhibitory interneurones in the superficial layers (van Haeften et al., 2003). Thus, deep-to-superficial projections contribute to both excitation and feed-forward inhibition in the superficial layers. Moreover, it has been suggested that predominant associational connections arose from layer V and terminate in the superficial layers (more prevalent in layers I and III; Kohler, 1986; Dolorfo and Amaral, 1998b).
1.2 Functional Roles of the EC

1.2.1 EC as Information Transfer and Integration Hub

As mentioned above, the EC is heavily connected with cortices and the hippocampus. The superficial layers of the EC receive a variety of inputs from cortical areas and then transfer information to the hippocampus. The CA1 subfield and subiculum of the hippocampal formation send feedback output to the EC deep layers where the neurons project to the cortical regions. Thus, the EC serve as the interface between cortices and the hippocampus.

The two subdivisions, LEC and MEC, preferentially receive projections originating from different cortical/subcortical areas (Kerr et al., 2007; Canto et al., 2008). The LEC receives more substantial input from the piriform and insula cortices as well as the amygdala and perirhinal cortex (a region involved in polymodal sensory processing and object-related pathway), whereas the MEC receives heavy innervations from visual...
associated areas such as the occipital cortex, parietal cortex and dorsal thalamic nucleus as well as head-direction system in the presubiculum (Witter et al., 1989; Chrobak and Amaral, 2007; Kerr et al., 2007). Therefore, the LEC and MEC conduct different information to the hippocampus; the LEC transfers polymodal and non-spatial information, whereas the MEC conveys visual and spatial information to the hippocampus (Fig. 1.4 and 1.6; Kerr et al., 2007). These differences are further corroborated by functional tests. In particular increased c-fos expression in the LEC was detected during food preference task (Smith et al., 2007a; Smith et al., 2007b). On the other hand, a manifest elevation of c-fos in the MEC was observed during spatial learning (Vann et al., 2000). These data are in agreement with recent imaging studies showing that the perirhinal-LEC pathway is involved in memory retrieval of nonspatial object (faces) whereas signals in the parahippocampal-MEC are more related to space (Schultz et al., 2012). Taken together, these studies provide evidence supporting the functional division of the EC in both rodents and human (Vann et al., 2000; Kerr et al., 2007; Smith et al., 2007a; Schultz et al., 2012). The distinct information conducted by the LEC and MEC may be integrated within the EC due to the intraentorhinal connection (Dolorfo and Amaral, 1998b). As mentioned previously, these intraentorhinal connections are organised into three bands (the lateral, intermediate and medial bands) that correspond to projections to the hippocampus along the dorsoventral axis. These three bands span across the LEC and MEC (Fig. 1.3B; Dolorfo and Amaral, 1998b, a). Cells are interconnected with other cells within the same band, suggesting information is specifically integrated within the same dorsoventral band of the EC (Dolorfo and Amaral, 1998b, a; Kerr et al., 2007). There is little associational connection across different bands, suggesting separation of cortical input at the different dorsoventral levels of the EC (Dolorfo and Amaral, 1998b). Therefore, differential information processing in the EC may occur at the lateromedial (i.e. LEC and MEC) or at dorsoventral (the three bands) levels. Meanwhile, interconnections within the dorsoventrally arranged bands raise the possibility of information integration before their transmission to the hippocampus (Witter et al., 1989; Dolorfo and Amaral, 1998a; Chrobak and Amaral, 2007; Kerr et al., 2007; Canto et al., 2008).
Figure 1.6 Schematic diagram showing the non-spatial and spatial information streams through the EC and the hippocampus. The cortical nonspatial input (orange) reaches the LEC, whereas spatial information arrives to the MEC. The distinct types of information then pass to the DG and CA3 subfield of the hippocampus converge (green) at this level. Nonetheless, the LEC and MEC target different regions of the CA1 subfield and the subiculum, hence passing selective information to particular CA1/subiculum area. The reciprocal projections from subiculum terminate in the deep layers of the EC where the cells innervate the superficial layers as well as other cortices (modified from (Deshmukh and Knierim, 2011).

Inputs from layer II of both the LEC and MEC to the hippocampus target different dendritic areas of neurones throughout the DG or CA3 subfield, and thus being able to converge (Fig 1.6; Burwell and Amaral, 1998a; Deshmukh and Knierim, 2011). It is suggested that excitation from both the LPP and MPP synapses are required to fire the
granule cell in the DG, where both the non-spatial and spatial information merge at this level. Consequently, signals from either LPP or MPP are enhanced through the competition, which is caused by the distance between the LPP/MPP synapses and soma. Due to the fact that the LPP fibre profile is thick in suprapyramidal layer while the MPP fibre profile is thick in infrapyramidal layer, signals from LPP and MPP are enhanced in suprapyramidal or infrapyramidal blade, respectively (Hayashi and Nonaka, 2011).

Therefore, selection and integration of different inputs from the LEC and MEC may be achieved through synaptic cooperation, while synaptic competition supports gating for the non-spatial from the LEC and spatial information from the MEC (Hayashi and Nonaka, 2011). Unlike in the DG/CA3, due to the distinct distribution of terminals, information flow through the LEC and MEC layer III do not converge in CA1/subiculum (Fig. 1.6). Nonetheless, the projection from neurones in the layer III of the EC is critical for modulating synaptic activity in the CA1 subfield of the hippocampus (Levy et al., 1998). Synapses consisting of projections from EC layer III and pyramidal cells in CA1 exhibit both long-term depression and long-term potentiation, and thus can either enhancing or lowering the spiking of CA1 pyramidal neurones (Dvorak-Carbone and Schuman, 1999; Remondes and Schuman, 2002, 2004).

This modulation from entorhinal input seems to be critical for synaptic plasticity at the Schaffer collaterals synapses, which is a key component in memory formation (Levy et al., 1998; Remondes and Schuman, 2002; Ang et al., 2005). Moreover, it is further indicated that entorhinal input is sufficient to maintain cell firing in CA1 during spatial memory recognition process even after lesioning Schaffer fibres, suggesting an important involvement of the EC in the memory process (Brun et al., 2002). Additionally, electrophysiological recordings revealed that inputs from the EC to the subiculum activate specific type of cells, which exhibit intrinsically bursting property in the subiculum, while CA1 projections activate all types of neurones within the subiculum (Gigg et al., 2000). The subicular neurones that are excited by EC input can trigger activities of neurones within the same cell assemblies. Additionally, the reciprocal output from the subiculum targets a limited number of cells in the EC. Therefore, EC afferents serve for the selection force, and this “point to point” property of entorhinal-subiculum connection may be relevant for encoding spatial information (Gigg et al., 2000; Gigg, 2006).
According to electrophysiological studies, hippocampal output to the deep layers is able to activate the communication between the deep and superficial layers by back propagating action potentials to the distal dendrites, and thus hippocampal output, is to some extent, integrated with incoming cortical information in the EC (Kloosterman et al., 2003). Moreover, CA1 and subiculum innervations may target neurones in the deep layers that in turn project to superficial layers. Therefore, output from CA1/subiculum to the EC modulate entorhinal projections to hippocampus, forming a functional “re-entry loop” (Iijima et al., 1996; Naber et al., 2001; Kloosterman et al., 2003; Kloosterman et al., 2004). Activation of any component of this loop induces positive feedback via the interlaminar connections (deep to superficial) of the EC. The high specificity of this circuit enables selective information to be stored in the CA1/subiculum of the hippocampal formation (Naber et al., 2001). In addition, the projections from EC layer II to the DG and from layer III the CA1 are both involved in the re-entry loop of entorhinal-hippocampal system, suggesting two parallel but possibly functionally distinct pathways contribute to the persistent neuronal activity in this loop during cognitive process (Naber et al., 2001; Bartesaghi and Gessi, 2004; Kloosterman et al., 2004).

All in all, EC acts not only as a hub for information transduction but also as an active gatekeeper that selects processes and integrates information between cortices and the hippocampus, and thus contribute to memory and cognitive functions (Fig. 1.6).

1.2.2 Entorhinal Activation and Neuronal Activity in Cognition

As discussed in the previous section, EC is fundamentally involved in cognitive process due to its heavy connection with the hippocampi and cortices. The fMRI imaging and electrophysiology studies suggested that activation of the EC neurones is involved in several cognitive processes, including working memory (especially spatial learning and memory) and long-term memory (Ranganath et al., 2003; Craig and Commins, 2005; Fransen, 2005; Fyhn et al., 2008).
1.2.2.1 Working memory

Early studies have shown that animals with ablation of the EC and/or perirhinal cortex had poor performance in the delayed match-to-sample (DMS) and delayed non-match-to-sample (DNMS) tasks, in which working memory is required to remember the sample stimuli when the test stimuli is presented (Gaffan and Murray, 1992; Otto and Eichenbaum, 1992). In line with these studies, functional MRI revealed increased signals in the EC as well as parahippocampal regions during the establishment of working memory with novel stimuli (Ranganath and D'Esposito, 2001; Schon et al., 2004; Hasselmo and Stern, 2006). It is suggested, therefore, that the EC is involved in the formation of working memory (Hasselmo and Stern, 2006). Moreover, recordings of neuronal activity during behavioural tests showed sustained spiking activity in the EC of rats in DNMS tasks and of monkeys in DMS tasks (Suzuki et al., 1997; Young et al., 1997). This persistent activity was suggested to underlie the basis of working memory and rely on acetylcholine (ACh; Klink and Alonso, 1997b; Egorov et al., 2002; Fransen et al., 2002; Fransen, 2005). Systemic injections of muscarinic antagonist such as scopolamine impaired the performance of working memory in primates (Aigner et al., 1991). Selective lesion of ACh fibres in the EC impaired memory encoding during DNMS tasks with novel stimuli (McGaughy et al., 2005). Electrophysiological and pharmacological studies showed that activation of ACh muscarinic receptors resulted in sustained activity of neurones in the EC (Fransen et al., 2002; Shalinsky et al., 2002). Furthermore, it is suggested that tasks with higher memory demand were accompanied with stronger cholinergic activation of muscarinic receptors in the EC (Tahvildari et al., 2007). Computational modelling has demonstrated that in the presence of ACh, synaptic stimulation causes strong depolarisation of the cell and opening of voltage-sensitive calcium channels thus increasing intracellular calcium level. Increased cytosolic calcium in turn activates calcium-sensitive non-specific cation channels that cause more depolarisation and further spiking activities (Fransen et al., 2002; Fransen, 2005). Hence, sustained spiking activities in the EC, which are activated by ACh inputs, are required for working memory (McGaughy et al., 2005; Hasselmo and Stern, 2006).

1.2.2.2 Long-term Memory

The EC has been suggested to take part in various processes during the formation of
Chapter 1

long-term memory (Suzuki and Eichenbaum, 2000; Izquierdo et al., 2006; Hasselmo et al., 2010). Single unit recordings of human EC cell during the encoding and retrieval phases of a recognition task, of which the time gap can be up to ten hours between two phases, showed responses during both phases (Fried et al., 1997). The majority of the recorded neuronal responses in the retrieval phase were enhanced by familiar stimuli, suggesting that EC activity may participate in encoding stimuli into long-term storage (Fried et al., 1997). Neuroimaging studies also indicated that EC activity is predictive for long-term memory by showing that increased and sustained signals in the EC during the delayed period of DMS task correlate with subsequent recognition memory performance (Schon et al., 2004; Schon et al., 2005). Moreover, several reports indicated that EC is essential for storage of lasting non-spatial and spatial memory as well as memory consolidation thus being a principal player in the long-term memory (Ardenghi et al., 1997; Izquierdo et al., 1997; Hebert and Dash, 2002; Luft et al., 2004; Ross and Eichenbaum, 2006). Remondes and Schuman (Remondes and Schuman, 2004) demonstrated that lesions of input from layer III of the EC to CA1 24 hours after learning impaired long-term memory but spared short-term memory; suggesting that information transferred by the EC is essential for memory consolidation. Animal behaviour studies showed increasing expression of the immediate early gene c-fos in the EC from 1 day up to 21 days after memory task training, providing a direct evidence for the participation of the EC in long-term memory consolidation (Ross and Eichenbaum, 2006). Rossato and colleagues (Rossato et al., 2004) have shown that N-methyl-D-aspartate (NMDA) receptors in the EC are critical for the onset of memory consolidation, which might be inhibited by GABA_A receptors. Additionally, there is abundant evidence showing that post-training infusion of pharmacological agents into the EC enhances or suppresses memory consolidation (Ardenghi et al., 1997; Izquierdo et al., 1997; Hebert and Dash, 2002; Roesler et al., 2002; Hebert and Dash, 2004; Rossato et al., 2004). Administration of an analogue of cyclic adenosine monophosphate (cAMP) into the EC, immediately or several (3-6) hours after training enhances memory retention performance of animals, suggesting that activation of cAMP-dependent pathways in the EC is important for memory consolidation (Ardenghi et al., 1997; Roesler et al., 2002). The extracellular signal regulated kinase (ERK), one of the protein kinases that might be downstream of cAMP-dependent signalling, is required in the EC during memory consolidation as shown by ERK inhibitor-induced memory impairment (Hebert and Dash, 2002; Roesler et al., 2002; Hebert and Dash,
2004; Luft et al., 2004; Rossato et al., 2004). Similarly to other brain regions relevant for memory protein synthesis in the EC is required for memory consolidation, as indicated by impaired long-term memory retention after infusion of protein synthesis inhibitors, such as anisomycin, emetine and cycloheximide (Lima et al., 2009).

The EC is also involved in memory retrieval, a process allowing the recall and use of the stored information (Fried et al., 1997; Dolcos et al., 2004; Knake et al., 2007). Single unit recording in human brain displayed EC responses in the memory retrieval tasks, and 40% of the responses were influenced by an old (familiar) stimulus (Fried et al., 1997). Neuroimaging studies indicated increased blood flow in the EC, and multi-unit recording revealed an increase in neuronal firing and population excitatory postsynaptic potential in the EC during the retrieval of memory (Knake et al., 2007). Recent fMRI study indicated a functional dissociation within the EC for memory retrieval; the MEC exclusively contributing to spatial recollection, whereas the LEC being involved in non-spatial or object-related spatial memory (Doeller et al., 2010; Schultz et al., 2012).

1.2.2.3 Spatial Representation and Memory

Brun and colleagues (2002) found that EC input to the CA1 subfield of the hippocampus is sufficient for spatial recognition memory by demonstrating that animals with injection of neurotoxin in the CA3 subfield still displayed normal performance in spatial recognition tasks, and the CA1 neurones of these animals continued to express “places fields” (significant increase in the firing rate when the animal is in a specific position/direction in the environment) after lesion of CA3. In another study, damage to MPP, by which the MEC projects to the hippocampus, led to deficits in the place learning during water maze tasks corroborating the involvement of MEC in spatial learning and memory (Ferbinteanu et al., 1999). In concordance with these studies, direct lesions of the EC impaired the spatial learning as well as compromised spatial working memory (Kaut and Bunsey, 2001; Van Cauter et al., 2008). The same study (Van Cauter et al., 2008) also showed that the firing rate and the size of place filed of place cells in the CA1 is significantly reduced in EC-lesioned animals, suggesting that the EC is essential for maintaining stable spatial representation by CA1 place cells. A recent study indicated that MEC-lesioned rats failed to detect spatial changed and
impaired path integration, demonstrating an important role of MEC in spatial information processing and computation (Van Cauter et al., 2012). In fact, specific neurones in MEC layers II, III and V called “grid cells” are very similar to place cells in the hippocampus, exhibiting spatial firing properties and showing sharp and coherent firing field (Fyhn et al., 2004; Hafting et al., 2005). Nevertheless, each grid cell, unlike place cell, has multiple non-overlapping firing fields, which are equally spaced and are organised into a hexagonal patterns containing six equilateral triangles (Fyhn et al., 2004; Hafting et al., 2005; Moser et al., 2008). Different cells have grids at different spatial scale and orientation. Individual grid cell fires at different rate across the vertices. Due to these characteristics, the firing fields of grid cells are arranged to form a directional and topographical neural map (Hafting et al., 2005). The firing field of grid cell is rarely affected by the speed and direction of movement, suggesting this grid system must constantly correct for movement and thus being strongly linked with path integration (Fyhn et al., 2004; Fyhn et al., 2008). In addition to grid cells, the EC also contains head-direction cells firing only when the head is pointing to a specific direction as well as conjunctive grid x head-direction cells exhibiting characteristics of both grid and head-direction cells (Sargolini et al., 2006). Grid cells are predominant in layer II, while the ratio of head-direction cells and conjunctive cells are higher in layers III and V of the EC (Sargolini et al., 2006). Another type of cell, called border cell, was found in the MEC; these cells fire when the animal is moving along the border of an enclosed environment (Solstad et al., 2008). All these four types of cells are key elements for path integration and navigation (Fyhn et al., 2004; Sargolini et al., 2006; Fyhn et al., 2008; Solstad et al., 2008).

Spatial representation of grid cells, including average spacing and field size, increases progressively, whereas the spatial-modulated firing decreases from the dorsal to the ventral MEC, suggesting that the spatial modulation and precision of the firing field decreases along the dorsoventral axis of MEC (Fyhn et al., 2004; Brun et al., 2008). This phenomenon corresponds to the increasing size of firing field of place cell along the dorsoventral axis of the hippocampus and the exclusive involvement of the dorsal hippocampus (Deshmukh and Knierim, 2011). The hippocampal place map can be formed by linear converging spatial signals from the MEC (Fyhn et al., 2004; Moser et al., 2008). Spatial signals transferred from the MEC to place cells in the CA1 might reflect synchronisation of fast gamma oscillation, which is important for network
processing in cognition and memory, in the MEC and CA1 (Fries et al., 2007; Colgin et al., 2009). Recent neuroimaging study revealed the first evidence for human grid cells by displaying adaption of activation signals in the EC to running direction, again confirming that the MEC is crucial for spatial computation and spatial memory processes (Doeller et al., 2010).

1.3 Entorhinal Dysfunction in Neuropathology

1.3.1 EC in temporal lobe epilepsy and schizophrenia

Entorhinal dysfunction has been suggested to be involved in diseases that compromise cognitive functions, such as temporal lobe epilepsy (TLE) and schizophrenia (Schwarcz and Witter, 2002; Cunningham et al., 2006). Epileptic patients show higher interictal discharges (measured by intracranial electrophalography) in the EC than in other cortical regions, and animal models of the disease exhibit spontaneous recurrent epileptiform discharges in the EC. Both patients and animals demonstrate endogenous epileptiform activity in the EC (Goncharova et al., 2009; Carter et al., 2011). The studies of TLE by Du and colleagues (1993, 1995) reported preferential neuronal loss in layer III of the MEC in both TLE patients and in the rat model. In another study, neurones in the EC layer III of a rat model were shown to have higher susceptibility to epileptiform activity that subsequently propagates to the CA1 subfield of the hippocampus (Wozny et al., 2005). It was also suggested that the TLE animal model has less synaptic remodelling in EC layer II, which is caused by the loss of pyramidal neurones in layer III, therefore contributing to hyper-excitability in layer II of the EC (Kumar and Buckmaster, 2006). The higher excitability of neurones in the layer II of the MEC might be due to an increased expression of Na\(^+\) channels in this region; it however does not reflect increased synaptic activity (Hargus et al., 2011). In addition to neurones, activation of microglia and astroglial remodelling were found in the EC of rat epileptic model. The inability of astrocytes to maintain K\(^+\) homeostasis, in particular those expressing immature K\(^+\) channels, may account for hyperactivity in this area (Jung et al., 2009). Astrocytic damage was also found in the EC, this being accompanied with increases in CA1 field excitatory postsynaptic potential, suggesting that astroglial remodelling in the EC is involved in the generation of CA1 hyperactivity.
(Kim et al., 2008). According to Gomez-Gozalo et al. (Gomez-Gonzalo et al., 2010), astrocytes in the EC are also involved in epileptiform discharges; elevation in intracellular Ca$^{2+}$ in astrocytes was sufficient to shift neurones towards the discharge threshold in the EC.

In addition to epilepsy, the EC is also believed to contribute to schizophrenia (Schwarcz and Witter, 2002; Prasad et al., 2004; Cunningham et al., 2006). In vivo fMRI studies revealed decreased EC volume with asymmetric changes (more in the right EC) in schizophrenia patients (Prasad et al., 2004; Baiano et al., 2008). Reduction in EC volume was also related to psychotic symptoms, especially to delusions (Prasad et al., 2004). Combination of electrophysiology and immunohistochemistry found an increase of glutamatergic axons in the EC as well as disruption of gamma rhythms on inhibitory interneurones. This effect was mediated through NMDA receptors (Longson et al., 1996; Cunningham et al., 2006). Regarding the essential connections between the EC and the hippocampus, it was suggested that increased excitatory input to the DG and CA3 subfield from the EC, together with decreased inhibitory GABAergic activity in CA3, result in overall overexcitability of the CA1 region in patients with schizophrenia (Benes and Berretta, 2000). Post-mortem studies revealed cytoarchitectonic changes manifested by neuronal displacement and reduced neuronal size in the superficial layers of the EC (Arnold et al., 1995; Arnold et al., 1997). In addition, changes in synaptic-related marker mRNAs (such as synaptophysin) were also found in entorhinal layers II/III in post-mortem brains from schizophrenia patients (Hemby et al., 2002). Altered expression of microtubule-associate protein in the EC (Hemby et al., 2002) was also found in schizophrenia (Arnold, 1999). In addition, abnormally increased level of glial-extracellular matrix protein in the EC of post-mortem brains from schizophrenia patients suggested involvement of glia.

1.3.2 EC in non-AD Dementia and Ageing

Due to its key role in memory processing, the EC has been suggested to be involved in dementia, such as frontotemporal dementia (FTD), semantic dementia (SD, a variant of FTD that affects language associated memory), subcortical ischemic vascular dementia (SIVD) and Alzheimer’s disease (AD).
Several studies indicating alterations in EC are associated with the FTD (Laakso et al., 2000; Kril et al., 2005). Compared with AD, hippocampus showed less severe atrophy, which is restricted to the anterior part, in FTD, while the pattern and extent of structural atrophy in the EC was comparable in both FTD and AD patients (Laakso et al., 2000). This suggested a direct involvement of the EC and possible disruption of EC-cortical and EC-hippocampal connection in this type of dementia. In addition, hippocampal atrophy occurred earlier than entorhinal atrophy in FTD, therefore, the later volume loss of the EC can be used to distinguish FTD and AD (Kril and Halliday, 2004). To the contrary Mummery and colleagues (Mummery et al., 2000) reported no significant structural atrophy in the EC in SD, although other studies suggested the role for entorhinal atrophy in SD (Galton et al., 2001; Davies et al., 2004). It appears also that the left EC is more severely affected in SD (Chan et al., 2001; Galton et al., 2001; Good et al., 2002). This asymmetric atrophy of the EC may be due to the fact that the left EC has heavier relation with activation of anterior cingulate cortex, which is involved in associated memory (Braskie et al., 2009). The EC was also found to be affected in the SIVD, where entorhinal atrophy may result from de-afferentation of afferent pathways to the EC, rather than from direct vascular insults (Du et al., 2002).

It remains debatable whether the EC undergoes structural atrophy during ageing. Many studies reported only a little shrinkage of EC during normal ageing, however, the EC does exhibit atrophic tendency during ageing and late-onset age-related increase in entorhinal shrinkage was found in aged subjects (Shimada, 1999; Rodrigue and Raz, 2004; Raz et al., 2005; Raz et al., 2008). A recent study by Raz et al. (Raz et al., 2010) revealed apparent shrinkage of the EC in healthy elderly compared to young subjects; age was also found to be related to the shrinkage rate of the EC (Du et al., 2006). It is further suggested that the annual rate of structural atrophy in the EC is associated with the decline in memory and cognition as measured by Cattell Culture Fair Intelligence Test, Letter Sets Test, association memory tests and free-recall tests at the follow-up period (5 years), suggesting atrophy of the EC can predict lower fluid intelligence and poorer memory performance (Rodrigue and Raz, 2004; Raz et al., 2008).

In aged EC some neurones with neurofibrilary tangles (NFT), which is a pathological hallmark of AD, or neurones in transition to NFT in layer II were identified (Morrison
and Hof, 1997). It was suggested that NFT formed at this stage is associated with memory deficit in ageing (Mesulam, 1999). Nevertheless, there is no evident neuronal loss in the EC during ageing (Gomez-Isla et al., 1996). Due to the lack of evidence for neuronal degeneration in the EC during ageing, it is suggested that reduced synaptic connections may account for the cognitive deficits in senescence. Smith and colleagues (Smith et al., 2000) demonstrated that reduced presynaptic boutons in the hippocampal CA3 subfield, which is mainly innervated by neurones from layer II of the EC, are related to cognitive decline in aged rats. There are also studies reporting that the DG receives less entorhinal input in aged rats, which may contribute to age-associated spatial memory impairment (Geinisman et al., 1992; Nicolle et al., 1999). In addition, expression of NMDA receptors, including NR1 and NR2A subunits, decreases in the EC of aged animals, which might result in reduced synaptic efficacy and thus memory processing (Liu et al., 2008). Moreover, a study by Scheff et al. (Scheff et al., 2006) indicated a remarkable atrophy in the perforant path in elderly people with memory impairments. Stranahan et al. (2011) further suggested additional decrease in expression of reelin, a long-term potentiation (LTP) enhancing protein in the layer II neurones, which is associated with disturbed entorhinal-hippocampal connections, thus contributing to memory deficits during ageing.

Taken together, the reduction in EC volume and/or impairment in the perforant path is associated with cognitive and memory dysfunction in senescent brain.
2. Alzheimer's Disease Pathology in the EC

2.1 Overview of Alzheimer's Disease (AD)

Alzheimer’s disease (AD) is named after German psychiatrist Alois Alzheimer, who described the case of dementia praecox (Alzheimer, 1907). This was a 51-year-old female patient (Auguste D) who exhibited memory impairment, compromised ability of language and comprehension as well as paranoia (Alzheimer, 1907). After the death of Auguste D, Alois Alzheimer did a post-mortem study of her brain and found pathological alterations that are known as senile plaques (SP) and NFTs around the cortex (Alzheimer, 1907). In addition, he also provided the first evidence for glial changes in the plaque-containing area (Alzheimer, 1907, 1910).

AD is a progressive and irreversible neurodegenerative disease, in which neuronal/synaptic degeneration is found in several brain regions including hippocampus, EC, amygdala and nucleus of Meynert (nbM) (Whitehouse et al., 1982; Gomez-Isla et al., 1996; Kril et al., 2002). Among the affected brain regions, the nbM and the cholinergic neurones of this region are especially susceptible to AD (Whitehouse et al., 1981). Clinical symptoms associated with AD include impaired cognitive functions (such as memory deficit and language disabilities), behavioural changes and psychological symptoms (such as hallucination and depression) (Braak et al., 1999; Harciarek and Jodzio, 2005). The prevalence of AD is increased with age and the incidence of the disease positively correlates with increased life expectancy. Over 35 million people around the world are suffering from dementia associated with AD (Braak et al., 1999; Cumming and Brodtmann, 2010).

Sporadic AD (SAD) that accounts for the majority of cases is generally diagnosed in senior people aged over 65 years old (Garcia-Alloza et al., 2005; Small and Cappai, 2006). SAD is characterised by the late-onset of the clinical symptoms and progressive pathological development (Small and Cappai, 2006). Another form of AD, familial AD (FAD) accounts for less than 5% of all cases. The FAD is inherited through autosomal dominance (Garcia-Alloza et al., 2005; Tanzi and Bertram, 2005; Small and Cappai, 2006). Mutations in genes encoding for amyloid protein precursor (APP) and presenilins 1 and 2 (PS1 and PS2, components of the secretase complex that give rise to
amyloid β peptides) are involved in FAD (Tanzi and Bertram, 2005). FAD shows the same pathological hallmarks as SAD although with much earlier onset - FAD usually occurs in patients at the age of 40-65 and progresses very rapidly (Shastry and Giblin, 1999; Small and Cappai, 2006).

2.2 Pathological Hallmarks

Abnormal accumulation of two proteins, Aβ and tau, which are two major components of SP and NFT respectively, have been identified in AD brains and are regarded as the main pathological hallmarks of the disease (Braak and Braak, 1991). SP is used to describe complicated lesion that consists of extracellular deposits of Aβ and associated molecules as well as degenerating neuronal processes, reactive astroglia and activated microglia. The plaques appear not only in the brain neuropil but also in or around the blood vessels, which causes angiopathy. Tangles are formed within neurones and are recognised as threads in fine distal dendrites while regarded as NFTs when formed in somas and apical dendrites, which are found in extracellular space as ghost tangles when neurones die (Braak and Braak, 1997).

**Figure 1.7** Cartoon showing the process of APP cleavage. (A) When APP is processed sequentially by α - and γ -secretase, the end product is non-amyloidogenic. However, (B) APP cleaved by β-secretase results in C99, which contains Aβ sequence. A subsequent processing by γ-secretase releases Aβ peptide.
The amyloid hypothesis implies that Aβ depositions are the primary factors in AD pathogenesis. Aβ derives from APP, which is a transmembrane glycoprotein involved in synaptic formation and repair. The cleavage of APP is carried out by different secretases on the N- and C-terminals (Nunan and Small, 2000). The APP processed by α-secretase generates sAPPα and an 83-residue portion of C-terminal (C83). This is not pathogenic since α-secretase act within the Aβ sequence and Aβ is cleaved during the process (Fig. 1.7A). However, cleavage by β-secretase generates a shorter soluble fragment sAPPβ and a 99-residue portion on C-terminal (C99; Fig. 1.7B). The C-99 fragment is then processed by γ-secretase, resulting in neurotoxic Aβ peptides (Fig. 7B). The final products could be Aβ1-40 or Aβ1-42. Although the Aβ1-40 form is more common than Aβ1-42, Aβ1-42 is more hydrophobic and prone to aggregate in the neurones (Jarrett et al., 1993). Following intracellular aggregation of Aβ1-42, the neurone secretes Aβ1-42 into extracellular space which then forms the extracellular plaque (Selkoe, 2001). It is suggested that the extracellular aggregation of Aβ is neurotoxic and causes neuronal/synaptic degeneration.

NFT is primarily composed of paired helical filaments (PHFs), which are made of hyperphosphorylated tau. Tau is a member of microtubule-associated proteins that is predominantly expressed by neurones and is preferentially, albeit not exclusively, present in axons (Lee et al., 2001). As well as promoting the polymerization of microtubules, which are associated with material transport between cell body and axon, tau could bind to and stabilize microtubules when phosphorylated (Schneider and Mandelkow, 2008). Hyperphosphorylation of PHF-tau is the major event in the development of neurofibrillary pathology (Braak and Braak, 1991). Numerous phosphorylation sites, consisting of serine or threonine residues, have been identified (Hanger et al., 1998; Schneider and Mandelkow, 2008). The phosphorylation could be regulated by a variety of protein kinases and phosphatases (for review, see Billingsley and Kincaid, 1997). Hyperphosphorylation of tau protein results in the formation of PHFs, compromising the capacity of binding to microtubules as well as interrupting intracellular elements that are vital for cellular metabolism and physiology such as mitochondria and endoplasmic reticulum (Garcia de Ancos et al., 1993; Ebneth et al., 1998; Mandelkow et al., 2004). NFTs have been implicated in neuronal loss in AD and other brain diseases. There is evidence indicating that NFTs are correlated with cognitive impairment and compromised memory in AD (Lee et al., 2001). Those
findings suggested a primary role of Tau in pathology of AD neurodegeneration.

2.3 Neurochemical Changes

2.3.1 Cholinergic System

ACh plays an important role in cognitive processes (Fodale et al., 2006). In the 70’s, several studies revealed significant reduction in presynaptic ACh markers, such as choline acetyltransferase (AChT, which is responsible for the synthesis of ACh) in the cerebral cortex of AD patients at early stages of the disease (Perry et al., 1977). In the 80’s, the specific loss of ACh neurones in AD brains was identified (Bartus et al., 1982), leading to a huge interest in pharmacological studies and development of ACh drugs against AD. The “cholinergic hypothesis of AD” suggests that the loss of ACh neurones in the basal forebrain contributes to cognitive deficit (Birks et al., 2000). Recently, it was postulated that reduced synthesis of ACh by reduced activity of AChT caused AD pathogenesis in the cortex and hippocampus (Fodale et al., 2006). Serious damage to the ACh neurones in the nbM, which widely project to the hippocampus and is one of the most important areas of mnesic functions, indeed contributes to the cognitive dysfunction in AD patients (Winkler et al., 1998; Fodale et al., 2006). It was also suggested that ACh enhances LTP that underlies memory in CA1 region of the hippocampus, therefore ACh neuronal loss and changes in ACh neurotransmitter levels may in consequence lead to severe memory impairment (Auld et al., 2002).

Substitutive enhancement of ACh system is fundamental for modern AD therapy. A variety of acetylcholinesterase (AChE; a protease that hydrolyses acetylcholine, resulting in producing choline and acetate groups) inhibitors have been used for treatment of AD, including donepezil, galantamine and rivastigmine (Birks et al., 2000). The main therapeutic function of AChE inhibitors is to increase the availability of ACh level in the brain. These AChE inhibitors produce modest improvement in cognition in AD patients in mild to moderate stage of the disease (Birks et al., 2000). However, these drugs become inefficient during the disease progression and are incapable of reversing or halting AD progression (Oddo and LaFerla, 2006). Other drugs enhancing ACh transmission, such as muscarinic and nicotinic agonists, have also been used for AD
treatment (Caccamo et al., 2006). However, the involvement of different neurotransmitter systems in AD and the pathological mechanisms are very complicated, a growing body of researches have focused on different therapeutic targets for better treatment of AD.

2.3.2 Glutamatergic System

Glutamate is the principal excitatory neurotransmitter in the central nervous system (CNS). Glutamatergic neurotransmission is fundamental for neuronal development, neuronal physiological functions and cognition (Riedel et al., 2003; Perez-Otano and Ehlers, 2004). A growing body of evidence suggest that disturbance in glutamatergic system is involved in AD (Francis, 2003). The role of glutamate in the progression of AD is supported by the fact that the expression of glutamate transporters, which are responsible for re-uptake and homeostasis of glutamate, decreased in several brain areas including the hippocampus and EC of both animal models and AD patients (Li et al., 1997; Simpson et al., 2010). Excessive extracellular glutamate induces excitotoxicity, which is regarded as the mechanism underlies the neurodegeneration in AD (Hynd et al., 2004; Walton and Dodd, 2007). NMDA glutamate receptors play important role in mediating excitotoxicity because of high permeability of Ca\(^{2+}\) (Walton and Dodd, 2007). Elevated intracellular Ca\(^{2+}\) activates several Ca\(^{2+}\)-sensitive kinases, which may lead to hypophosphorylation of tau (Lee et al., 2001; Hynd et al., 2004). Moreover, it is suggested that A\(\beta\) exerts its neurotoxic effect through excitotoxic pathway via overactivation of NMDA receptors (Harkany et al., 2000b). Therefore, blocking NMDA receptor may ameliorate AD-associated degeneration. This is supported by the fact that MK-801, a non-competitive NMDA antagonist, inhibits A\(\beta\)-induced neurotoxicity (Harkany et al., 2000a; Harkany et al., 2000b). Nevertheless, MK-801 can also prevent NMDA receptor activation under physiological condition and thus cause serious cognitive deficits (Molinuño et al., 2005).

Another, somewhat milder and non-competitive NMDA receptor antagonist, memantine (1-amino-3,5-dimethyl-adamantane) has been has been shown to prevent over-activation of NMDA receptor by inhibiting prolonged Ca\(^{2+}\) influx, and thus prevent glutamate-induced excitotoxicity. However, it does not block receptors in physiological condition (Molinuño et al., 2005). In addition, memantine treatment
significantly attenuated neuronal damage and prevented memory deficits caused by injection of Aβ (Nakamura et al., 2006). Memantine has been approved to treat AD since 2003 (Scarpini et al., 2003; Molinuevo et al., 2005). Clinical trials also showed that memantine treatments improved cognitive functions in AD patients (Parsons et al., 1999; Kirby et al., 2006).

2.3.3 Serotonergic and Cannabinoid Systems

Significant reduction in serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoiatic acid in several regions of AD brains as well as cerebrospinal fluid have been reported (Garcia-Alloza et al., 2005). This decline may be due to the de-afferentation of 5-HT fibres since large NFTs presence and neuronal loss have been found in the raphe nuclei, which is an area with densely distributed 5-HT neurones (Curcio and Kemper, 1984). Additionally, there is also evidence for decreased expression of 5-HT receptors in cortical regions (reviewed by Meltzer et al., 1998) as well as reduction in the density of serotonin transporters (Cross, 1990), implying compromised reuptake of 5-HT in AD. There are also studies revealing correlations of altered 5-HT transmission not only with cognitive functions (measured by mini-mental state examination) but also with behavioural symptoms such as overactivity and psychosis (Lai et al., 2002; Garcia-Alloza et al., 2005). Corresponding to the findings mentioned above, our recent study showed abnormal serotoninergic fibre sprouting in the CA1 subfield of the hippocampus in triple transgenic (3xTg-AD) mouse model of AD, suggesting either increased reuptake or enhanced 5-HT synthesis in this region, therefore, implying that altered serotoninergic transmission may contribute to the memory deficit in AD (Noristani et al., 2010).

Post-mortem study has shown increased expression of cannabinoid (CB) receptors within plaque depositions of AD brains (Ramirez et al., 2005) as well as increased nitration of cannabinoid receptors, as consequence of the reaction of nitrogen oxide(NO) and superoxide to form the toxic peroxynitrite radical. In the same study, it is indicated that administration of synthetic cannabinoid prevented Aβ-induced toxicity and cognitive deficits (Ramirez et al., 2005). Study by Milton (2002) showed neuroprotective effects of cannabinoid agonists mediated through CB1 receptor. Moreover, inhibition of endocannabinoid uptake may reverse Aβ-induced memory
impairment (van der Stelt et al., 2006). Additionally, CB can reduce phosphorylation of glycogen synthase kinase-3β, which is responsible for hyperphosphorylation of tau (Esposito et al., 2006). In addition cannabinoids block activation of microglia and microglia-mediated neurotoxicity. Taken together, CB exerts neuroprotective effects through multiple means and thus preventing and ameliorating the neurodegenerative process in AD (Ramirez et al., 2005; Campbell and Gowran, 2007).

2.4 Affected Brain Regions

The EC is the first region affected in AD. EC undergoes serious structural atrophy as well as neuronal and/or synaptic loss and shows plaque deposition and tangles at early stage of AD (Braak and Braak, 1991). Limbic system also undergoes serious structural atrophy during AD progression, especially the hippocampus and amygdala (Braak et al., 1999; Du et al., 2001). Volume reduction in these regions may therefore result in compromised short-term memory and emotional problems. The prefrontal cortex, which is associated with higher order cognitive functions, is also altered in AD (Li et al., 2009; Zamboni et al., 2012). Neuronal loss in the basal forebrain and/or de-afferentation of ACh neurones in basal forebrain has been identified in AD brains (Yankner, 1996).

2.4.1 Nucleus basalis of Meynert

The nucleus basalis of Meynert (nbM) is located in the basal forebrain, providing ACh input to other cortical and subcortical areas since the majority of neurones (80-90%) within the nbM are cholinergic (Mesulam and Geula, 1988). The nbM is divided into 6 different sub-regions: anterior, anterointermediate, anterolateral, anteromedial, interomediodorsal and interomedioventral regions; each sub-region has its specific projecting targets (Mesulam et al., 1983). The anterointermediate area provides ACh projections to the medial portion of neocortices, such as cingulate gyrus; the anterolateral division is the major source of ACh innervations to the amygdala, frontal and parietal cortices; ACh neurones in the intermediate regions innervate the prefrontal, insula and temporal cortices (Mesulam et al., 1983). Degeneration of ACh neurones in the nbM of AD brains was revealed in early 80’s (Whitehouse et al., 1981; Whitehouse et al., 1982; Vogels et al., 1990). The fMRI evidence for decreased signal in the nbM might be related to the degeneration within this brain region (Teipel et al., 2005). In
addition to cell death, histological studies revealed significant reduction in the size of the remaining ACh neurones in the nbM of AD brains (Pearson et al., 1983; Rinne et al., 1987). It is further demonstrated that down-regulation of ChAT synthesis as well as activity in the remaining neurones of the nbM during AD progression (Strada et al., 1992).

2.4.2 Hippocampus

The hippocampus derived its name due to its seahorse-like shape. It is a curved structure located in the medial temporal lobe and is divided into four main subfields, namely CA1, CA2, CA3 and DG (Amaral and Witter, 1989). The CA subfields comprise several layers, including stratum oriens, stratum radiatum, pyramidal cell layer and stratum lacunosum moleculare (Amaral and Witter, 1989; Amaral et al., 2007). Among the layers, the pyramidal cell layer is the main layer in CA fields, consisting of pyramidal neurones as well as other neuronal types such as axo-axonic, basket, bistratified and radial trilaminar cells (Witter and Amaral, 2004). The DG is also a laminar structure, composed of molecular layer, granular cell layer and the hilus (Witter and Amaral, 2004). The granular cell layer contains cell bodies of different types of neurones, including granule cell, basket pyramidal cell, multipolar and fusiform cells (Witter and Amaral, 2004). The principal cell type in this layer is the granule cell, of which the dendrites extend to molecular layer and the axons travel through the hilus and reaches CA areas (Amaral et al., 2007). As mentioned in section 1.1.4, the hippocampus is heavily innervated by the EC, and these two areas, together with the subiculum, form the tri-synaptic circuit (Scharfman, 2007). Projections from the EC layer II form synapses on dendrites in the molecular layer of DG. In turn, the granule cells projects to the CA3 via the mossy fibres (Treves et al., 2008). Then CA3 pyramidal neurones innervate the CA1 through Schaffer collaterals (Amaral and Witter, 1989). This circuit is featured by activity-dependent synaptic change, LTP, which underlies the basis of cognitive process (Bliss and Lomo, 1973; Collingridge and Lester, 1989). Therefore, the hippocampus is generally regarded as the memory centre in the brain (Eichenbaum and Cohen, 1988; Squire, 1992).

The involvement of hippocampus in the AD progression has been well documented (Braak et al., 1999; Sperling et al., 2010). Post-mortem studies have indicated the NFTs
appearing at Braak stage I or II in the hippocampus while SPs occur at early and middle stages of AD (Braak and Braak, 1991, 1995). In addition, neuroimaging has demonstrated hippocampal atrophy in AD brains (Du et al., 2001; Kato et al., 2001). This shrinkage of the hippocampus might result from neuronal and synaptic loss in this area (Scheff et al., 2005; Zarow et al., 2005). Evident loss of neurones was found in CA1 of AD brains, which may be caused by NFT pathology in this region (Van Hoesen and Hyman, 1990; Braak and Braak, 1991; West et al., 1994; West et al., 2000). Several post mortem studies showed extensive loss of synapses, especially in the molecular layer of DG where is the terminating zone of PP, suggesting that disconnection between the hippocampus and EC contribute to the memory impairment in AD (Scheff and Price, 1998; Scheff et al., 2006). Moreover, many studies have demonstrated decreased activities in the hippocampus of AD subjects during episodic encoding tasks (Small et al., 1999; Kato et al., 2001; Golby et al., 2005). Taken together, the neurodegeneration in the hippocampus is critical for cognitive and mnesic deterioration in AD.

2.4.3 Prefrontal Cortex

The prefrontal cortex (PFC) is located in the anterior part of the frontal lobe. It is divided into six broad regions: dorsolateral (DLPFC), ventrolateral (VLPFC), dorsomedial (DMPFC), ventromedial (VMPFC), frontopolar (FP) and orbitofrontal (OFC) cortices (Barbas, 1988; Yeterian and Pandya, 1991). It is connected with primary and secondary association cortices, EC, hippocampus, thalamus and amygdala (Porrino et al., 1981; Ray and Price, 1993; Barbas, 2000; Ongur and Price, 2000; Fuster, 2002). Medial and orbital areas develop heavy connections with the limbic structures relatively early, these structures including the amygdala, hippocampus and EC, as well as subcortical structures such as the thalamus and hypothalamus (Porrino et al., 1981; Ray and Price, 1993; Ongur and Price, 2000). Thus, the medial and orbital regions are involved in emotional and visceral behaviour as well as the formation of memory (Jay et al., 2004; Funahashi, 2006; Phillips et al., 2008; Ray and Zald, 2012). The lateral regions (DLPFC and DMPFC) develop later and connect with the basal ganglia, hippocampus, premotor cortex and cingulated cortex (Fuster, 2002) and are believed to be involved in higher executive functions (Fuster, 2002; Phillips et al., 2008). In addition, animal lesion and infusion studies demonstrated poor performance of PFC-lesioned animals in memory tasks, suggesting that the PFC also takes part in working
memory (Goldman-Rakic, 1996; Petrides, 2000). In concordance with animal studies, neuroimaging evidence found increased signals in the PFC during working memory process (Blumenfeld and Ranganath, 2006). Furthermore, PFC is also involved in consolidation of long-term memory as supported by a variety of animal studies (Izquierdo et al., 2007; Takehara-Nishiuchi et al., 2011; Ray and Zald, 2012).

Decreased PFC activation in AD patients during cognitive tests shown by fMRI evidence suggested that dysfunction of the PFC in AD is associated with cognitive functions such as self-awareness and selective attention (Li et al., 2009; Zamboni et al., 2012). Additionally, by using molecular probes for plaque and tangles, a recent neuroimaging study indicated that increased NFT burden in the PFC is correlated with compromised cognitive functions (Braskie et al., 2008). The dysfunction of PFC might be a result of degeneration of synapses and neurones in the PFC, leading to subsequent cognitive deficits (Minger et al., 2001; Bussiere et al., 2003).

2.4.4 Entorhinal Cortex

2.4.4.1 Atrophy and Neuronal Alteration in the EC

The EC the earliest affected brain regions in AD (Braak and Braak, 1991). Post-mortem and in vivo fMRI measurements have shown significant reduction in EC volume (Braak and Braak, 1991; Kordower et al., 2001; Seahill et al., 2002; deToledo-Morrell et al., 2004). The EC shows greater reduction rate than the hippocampus, corroborating its earlier involvement in AD neuropathology (Du et al., 2001). Brain imaging revealed significant decrease in volume of the EC in patients with mild cognition impairment (MCI), which is featured by decline in cognitive function that does not meet the criteria for dementia. MCI patients are at high risk of developing AD, further emphasising that the shrinkage of the EC occurs before the onset of dementia (deToledo-Morrell et al., 2000). The reduced volume of the EC has been linked to compromised memory, so that shrinkage of the EC could be an indicator for a decline of memory function (Rodrique and Raz, 2004). There is also evidence indicating that the white matter in the parahippocampal area, including perforant path, undergoes a major decline in MCI (Stoub et al., 2006). The loss of hippocampal-entorhinal connections is
very likely to predict memory dysfunction (Stoub et al., 2006).

A post-mortem study carried out by Hyman et al. (Hyman et al., 1984) showed significant loss of neurones in layer II of the EC. As projections from layer II of the EC are the major input to DG of hippocampus, the authors suggested that the disconnection between the hippocampus and the EC caused by the neuronal loss in layer II may be responsible for memory deficit in AD (Hyman et al., 1984). Profound neuronal loss in layer II of the EC was also found in both MCI and mild AD patients (Gomez-Isla et al., 1996; Kordower et al., 2001). It was suggested that the onset of neuronal loss in the EC and the hippocampus are linked with the onset of cognitive decline (Mikkonen et al., 1999; Price et al., 2001). Mikkonen and colleagues (Mikkonen et al., 1999) observed that neurones in the layer II of the lateral, intermediate and caudal sub-regions of the EC are more vulnerable than those in the layer II of the rostral and medial areas of the EC. The former part receives more ACh input; the observed phenomenon may be linked with ACh pathology in AD (De Lacalle et al., 1994). Indeed, cholinergic de-afferentation in the EC is accompanied with a decrease in the postsynaptic cholinergic receptors, especially nicotinic cholinergic receptor (discussed later in section 2.4.4.3), of which activation may exert protective effect against amyloid pathology (Geula and Mesulam, 1995; Takada et al., 2003; Teaktong et al., 2004; Liu et al., 2007). In addition to subfield-specific loss of neurones, some plastic changes, either pre- or postsynaptic, in the dendritic regions of layer V pyramidal neurones of the EC were suggested. Calretinin staining was identified in the basal dendrite of layer V pyramidal neurones in AD whereas the staining is profound in the apical dendrites in healthy subjects (Mikkonen et al., 1999). It should not be overlooked that the EC is also heavily involved in cortico-hippocampal circuit and play an important role as information transfer/integrate centre, so that any pathology in the EC may lead to interruption of this signalling process and thus lead to cognitive deficits.

2.4.4.2 Tangles and Plaques in EC

According to Braak and Braak (Braak and Braak, 1997), neurofibrillary changes initially start in the transentorhinal regions (stage I/II), and then move to the layer II of EC and the hippocampus at limbic stages (III/IV). At the late stages (V/VI), tau pathology could be found in all subdivisions of the cerebral cortex. In the EC, NFTs are
formed in the pyramidal or stellate cells in layers II and V (Solodkin et al., 1996). The tau pathology in the EC shows specific spatio-temporal progression. It appears in the layer II earlier than in layer V, and the LEC is affected earlier than MEC in association with the ACh de-afferentation (Thal et al., 2000b). Tau pathology progresses from the EC to PP, targeting specific areas in the hippocampus (Thal et al., 2000b). Although NFTs are present in normal elderly people without cognitive deficit, the density of NFTs increases in dementia (Haroutunian et al., 1999). Similarly, the proportion of NFT-containing neurones in the EC increases as cognitive dysfunction proceeds (von Gunten et al., 2006). Neuronal loss in the EC strongly correlates with the formation of NFTs (Fukutani et al., 2000). The formation of NFTs also accounts for the neuronal loss in the EC of AD brains. Thus, NFT is likely to be linked with cognitive impairment through a mechanism of neuronal loss (Giannakopoulos et al., 2003). Conversely a large proportion of neurones in layer II remain unaffected by NFTs until the late stage of AD progression, suggesting that NFTs exert their effect on cognition dysfunction through another mechanism (Hof et al., 2003).

The initial Aβ plaque depositions, which occur before the formation of tangles, are found in the basal neocortex, especially temporal areas (i.e., rhinal cortices); then proceed into neighbouring cortical areas and the hippocampal formation (Braak and Braak, 1997; Thal et al., 2000a). The SPs in EC mainly occur in the layer III while only a few Aβ plaques are found in layer II of the EC (Mufson et al., 1999). Nevertheless, the correlation between Aβ burden and cognition is controversial. Many studies indicated that the level of amyloid deposit in the EC is not correlated with the cognitive decline (Mufson et al., 1999; Giannakopoulos et al., 2003; Guillozet et al., 2003). Nonetheless, one recent study using Cambridge Examination for Mental Disorders in the Elderly, of which ability in specific cognitive aspects (ex. Memory, executive function, language expression and comprehension) is derived, indicated that entorhinal amyloid aggregation is associated with memory but not general cognitive deficit, and thus, emphasising the importance of amyloid burden in memory deficits in AD (Thomas et al., 2005).

2.4.4.3 Neurochemical Alterations in the EC

As mentioned above, loss of ACh neurones in the basal forebrain results in selective
de-afferentation in several brain regions associated with cognitive functions such as the EC (Birks et al., 2000). The layer II of the EC receives dense ACh input, preferentially in the lateral division (De Lacalle et al., 1994). It has been shown that there is up to 80% reduction in the density of AChE-positive fibres in the EC in AD brains (Geula and Mesulam, 1996). More reduction of ACh afferent was observed in the EC of severe AD when compared to mild AD patients (Geula and Mesulam, 1996; Geula et al., 2008). Degeneration of ACh fibres in the EC is likely to occur on those fibres with abnormalities (thickened and swollen terminals), which is an effect of normal aging (Geula et al., 2008). The decline of ACh input to the EC has been suggested to disrupt working memory (McGaughy et al., 2005; Hasselmo and Stern, 2006). In addition to de-afferentation, ACh pathology in the EC involves decreased level of nicotinic cholinergic receptors (Teaktong et al., 2004; Oddo and LaFerla, 2006). Several studies have shown decreased nicotinic receptor subunit $\alpha$-immunoreactive neurones in the layer III of the EC as well as a reduction in the number of $\alpha$7-positive neurones in the layer II of the EC (Teaktong et al., 2004). However, increased $\alpha$7-positive neuropils and astrocytes have also been found in the EC, suggesting phagocytic behaviour of astrocytes in AD (Teaktong et al., 2003; Nagele et al., 2004).

There is also significant AD-related change in glutamatergic receptor expression in the EC. Several studies have revealed that $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit, GluR 1 and GluR2/3, decrease in the EC of AD brains (Wakabayashi et al., 1994; Yasuda et al., 1995; Thorns et al., 1997; Wang et al., 2000). Selective decline of GluR2/3 subunits has been reported to occur preferentially in layers II and III of the EC (Yasuda et al., 1995; Wang et al., 2000). In contrast to the consistent reduction of AMPA receptor subunits in AD, the change in the expression of NMDA receptors is unclear. According to Ulas and Cotman (Ulas and Cotman, 1997), the NR1 mRNA level decreased in entorhinal layer III; similar but not significant decline was also found in layer II and deep layers (Wakabayashi et al., 1994; Yasuda et al., 1995; Thorns et al., 1997) (IV- VI) of the EC in AD. The mRNA level of NR2A and NR2B in the EC are also reported to decrease (Bi and Sze, 2002). As for protein level, both non-phosphorylated and phosphorylated NR2A and NR2B subunits significantly decrease in the EC; this decline correlates with the loss of presynaptic vesicle proteins and poorer cognitive performance (Sze et al., 2001). However, in the same study, no significant changes in NR1 subunit (obligatory for receptor formation)
expression were found in the EC of AD vs. normal controls. Moreover, other post-mortem also indicated that the protein level of NMDA receptor subunits, including NR1, NR2A and NR2B, remain unaltered in the EC of AD brains (Thorns et al., 1997; Wang et al., 2000).
3. Astrocytic Involvement in AD within Cortical Regions

The CNS is composed of different cellular elements, of which the two main types are neurones and neuroglia. The term “neuroglia” was introduced by Rudolf Virchow as the brain connective tissue that fills “the space not occupied by neurone” (Virchow, 1856, 1858; Weigert, 1895; Somjen, 1988). Virchow also proposed that neuroglia holds the nervous elements together and gives the CNS the “shape of the whole” (Kettenmann and Verkhratsky, 2008).

Glial cells in the CNS are represented by several major subtypes broadly divided into macroglia (astrocytes, oligodendrocytes, NG2 cells and ependymal cells) and microglia, which form brain tissue immunity. Microglia derive from myeloid-lineage progenitors, whereas macroglia originates from neuroepithelium.

Astrocytes are a heterogeneous class of neuroglial cells with different anatomical, biophysical and antigenic properties (Kimelberg, 2004). There are specialised radial glia called Bergamann glia and Muller cell in the cerebellum and retina, respectively; pituicyte in neurohypophysis; protoplasmic in grey matter and fibrous in white matter (Palay and Chan-Palay, 1974; Andriezen, 1983; Hatton, 1988; Newman and
Reichenbach, 1996; Doetsch et al., 1997). Protoplasmic astrocytes have many branches and fine processes extending from the cell body and forming endfeet on blood vessels or contacts with neurones, fibrous astrocytes have longer but less prominent process, which cover axons at nodes of Ranvier or form endfeet on blood vessels.

![Figure 1.8](image)

**Figure 1.8** Fluorescent microphotograph showing the morphology of protoplasmic astrocytes from (A) GFAP-immunolabelling in the cortex or (B) Fluorescence Alexa 488-filling in the CA1. A (unpublished image), B from (Bushong et al., 2002). Scale bars = 10µm for A and B.

Protoplasmic astrocytes constitute the main and most important glial population in the grey matter (Rodriguez et al., 2009). During the evolution, the morphology of protoplasmic astrocytes became more complex and their relative number (when compared to neurones) increases (Nedergaard et al., 2003). These astrocytes are organised in anatomical domains, with minimal overlap (Bushong et al., 2002). Within the domain, individual astrocytes contact the blood vessels, neurones and synapses with its branching processes (Nedergaard et al., 2003). By using dye-filling, Bushong and colleagues (Bushong et al., 2002) indicated that rather than being star-shaped protoplasmic astrocytes possess numerous highly branched ramifications, being more spongiform (Fig. 1.8B). The author further indicated that GFAP-labelling only delineates 15% of the total astrocytic volume (Bushong et al., 2002).

### 3.1 Astroglial cytoskeleton

GFAP is the main IF, together with microfilament (such as actin) and microtubule, constitutes the cytoskeleton of astroglia. Vimentin, nestin and synemin are the other IF
proteins in astroglia. It is known that astrocyte precursor contain vimentin, nestin and synemin at early developmental stages (Lendahl et al., 1990; Sultana et al., 2000). As astrocytes differentiate, astrocytes start to express GFAP, which gradually replaces those IF proteins (Lendahl et al., 1990; Yachnis et al., 1993). Although vimentin is also expressed by mature astrocytes, the level is only low to moderate in the healthy CNS (Shaw et al., 1981; Pixley et al., 1984). Nonetheless, it is noteworthy that co-existence of GFAP and vimentin is required for normal IF assembly of astroglia (Pekny et al., 1995; Eliasson et al., 1999). Pekny group further indicated that devoid of both GFAP and vimentin or deficient in vimentin prevent astrocytes to be reactive (Eliasson et al., 1999; Wilhelmsson et al., 2004). In addition, to upregulation of GFAP and vimentin, accumulating evidence demonstrated that re-expression of nestin and synemin is also a hallmark for reactive astrocytes in response to CNS insults or pathology (Frisen et al., 1995; Lin et al., 1995; Jing et al., 2007).

Actin, a microfilament, and its associated proteins are also key elements of astroglial cytoskeleton. Actin is essential for cytoskeleton organisation, cellular signalling and motility of astroglia (Cotrina et al., 1998). The actin-associated proteins in astroglia mainly consist of ezrin, radixin and moesin (ERM). ERM are found in very fine perisynaptic processes of astrocytes where is devoid of GFAP. ERM is responsible for motility of astroglia and thus enable rapid structural changes under certain conditions (Derouiche and Frotscher, 2001).

3.2 Physiological role of astrocytes

Astrocytes have many functions in supporting neurones and maintaining brain physiological environment, such as neuronal development and synaptogenesis, induction of brain-blood barrier, homeostasis of extracellular ions and neurotransmitters (Wang and Bordey, 2008).

3.2.1 Regulation of Synaptogenesis and the Number of Synapses

Co-culturing neurones with astrocytes significantly increases the number of synapses, suggesting astrocytes are critical for the formation and maintenance of functional synapses (Ullian et al., 2001). Astrocytes produce and secrete cholesterol, which
promotes the synthesis of presynaptic components such as synaptic vesicles and associated proteins during the formation of synapses (Thiele et al., 2000; Lang et al., 2001; Mauch et al., 2001). Additionally, astrocytes release molecules such as laminin, N-cadherin and fibronectin, promoting and supporting synaptogenesis (Matthiessen et al., 1989; Smith et al., 1990; Shea et al., 1992; Kanemaru et al., 2007). Moreover, there is evidence that these are immature but not mature astrocytes that release thrombospondins, which is able to induce synaptogenesis in vivo, further confirming the astrocytic contribution to synaptogenesis in developing brain (Christopherson et al., 2005). Astrocyte-derived factors, such as tumour necrosis factor α and activity-dependent trophic factor increase the expression of postsynaptic density (ex. glutamatergic receptors); thus further supporting the maturation of synapses and enhancing the connection of synapses (Blondel et al., 2000; Beattie et al., 2002).

In spite of their role in facilitating synaptogenesis and maturation of synapses, astrocytes can also produce proteolytic enzymes, particularly the matrix metalloproteinase, which degrade the extracellular matrix and destabilise synaptic contacts (Muir, 1997; VanSaun and Werle, 2000). Consequently, the astrocytic processes would invade the synaptic cleft and replace the synapse.

3.2.2 Potassium Buffering

Neuronal activity results in the accumulation of K⁺ ions in the extracellular space. The excessive K⁺ leads to further depolarisation, causing hyperactivity and epilepticform activities of neurones (Blondel et al., 2000; Hinterkeuser et al., 2000; Beattie et al., 2002; David et al., 2009). Therefore, maintenance of K⁺ is very important for neuronal excitability and function. The responsibility of removing extracellular K⁺ mainly lies with astrocytes (Kofuji and Newman, 2004). The mechanism of K⁺ buffering by astrocytes can be categorised into local K⁺ uptake and spatial K⁺ buffering (Kuffler and Nicholls, 1966; Orkand et al., 1966; Somjen, 2002). Astrocytes can take up extracellular K⁺ passively by K⁺ influx through inward rectifying K⁺ channels (Kir) or K⁺/Na⁺ or K⁺/Cl⁻ transporters (Newman, 1986; Ballanyi et al., 1987; Karwoski et al., 1989). High K⁺ permeability of astrocytes gives them a negative resting membrane potential close to K⁺ equilibrium potential. Accumulation of intracellular K⁺ in astrocytes is followed by water influx, thus causing swelling of astrocytes (Kofuji and
Newman, 2004) Therefore, the capacity of local $K^+$ uptake is limited and a more powerful and sustained mechanism is required. In 1960s, “spatial $K^+$ buffering” was proposed that implied $K^+$ ions are taken up by a single astrocyte and are redistributed throughout the astrocytic syncytium, in which astrocytes are integrated into a continuous structure by gap junctions (Kuffler and Nicholls, 1966; Orkand et al., 1966). In this case, the local elevation of intracellular $K^+$ is kept minimal since $K^+$ ions are dispersed. This astrocytic control of $K^+$ homeostasis was already confirmed in rat cortical slices (Fig. 1.9; Holthoff and Witte, 2000).

3.2.3 Induction and Maintenance of Blood-Brain Barrier

The blood-brain barrier (BBB), which is formed by brain micro vascular endothelial cells and astrocytic endfeet, is the filter for substances entering the brain (Kacem et al., 1998; Abbott et al., 2006). The complex tight junctions between the endothelial cells strictly restrict the paracellular permeability, even for small ions. Only limited small water-soluble molecules and lipid-soluble agents can access the brain parenchyma via passing the tight junctions and the surface of lipid membranes of endothelium, respectively. Thus, molecules are forced to take a transcellular route, i.e. via various transporters, exchangers and receptors, to cross the BBB (Abbott and Romero, 1996). As a result, BBB protects the brain from detrimental substances from the bloodstream, while offering the nutrients to CNS (Abbott et al., 2006).

Astrocytes do not physically form the BBB in mammals; however, the presence of astrocytes is critical for the induction of BBB formation as well as maintenance of BBB (Rubin et al., 1991; Dehouck et al., 1994; Hawkins et al., 2002). The BBB tight junction proteins are up-regulated when co-cultured with astrocytes (Dehouck et al., 1994; Sobue et al., 1999; Bauer and Bauer, 2000). Astrocytes are also able to induce the expressions of the transporters in the endothelial cells, including glucose transporter 1 (GLUT 1) and P-glycoproteins (Schinkel, 1999; McAllister et al., 2001).

3.2.4 Metabolic Support

Astrogial endfeet cover the blood vessels with their endfeet and contact neurones
and synapses with their processes. Therefore, astrocytes are well positioned to offer metabolic support to neurones via a cascade known as astrocyte-neurone lactate shuttle (ANLS) hypothesis (Pellerin and Magistretti, 1994; Pellerin, 2003). Astrocytes take up glucose by GLUT 1 located on the endfeet membrane facing the blood vessels and convert it by aerobic glycolysis to lactate. Lactate is then released to the extracellular space and are transported to neurones, serving as energy substrate (Fig. 1.9; Pellerin and Magistretti, 1994; Pellerin, 2003; Pellerin et al., 2007). Hu and Wilson (Hu and Wilson, 1997) provided an indirect evidence for ANLS by demonstrating that sustained activation of PP led to a decrease in extracellular lactate concentration in the DG, which is followed by a subsequent increase. There are also reports indicating that metabolic responses (enhanced glucose utilisation) to persistent synaptic activation mainly rely on astrocytic glycolysis (Voutsinos-Porche et al., 2003; Pellerin and Magistretti, 2005). Activity-dependent increase in neuronal metabolism is supported by lactate from glycosis in a non-neuronal compartment, presumably astrocytes (Serres et al., 2003, 2004, 2005), corroborating lactate transfer between astrocytes and neurones when the energy demands increase. Nevertheless, it remained to be investigated whether astrocyte-neurone lactate shuttle serve as the main fuelling mechanism during normal neuronal activity.

3.2.5 Glutamate Homeostasis—Glutamate-Glutamine Cycle

As has been mentioned before, excessive accumulation of extracellular glutamate is detrimental for neurones (Lucas and Newhouse, 1957; Olney, 1969; Olney and Ho, 1970). High extracellular glutamate leads to over-activation of glutamate receptors, which depolarises cell membrane and activates voltage-gated ion channels allowing the subsequent cation influx, such as Na⁺ and Ca²⁺ (Choi, 1992). Together with Ca²⁺ release from endoplasmic reticulum, it results in a substantial increase in cytosolic Ca²⁺, which in consequence activates cell death cascades such as apoptotic pathway or induces mitochondria dysfunction and energy depletion (Tymianski and Tator, 1996; Duchen et al., 2008). As a result, neurones undergo either rapid necrotic cell death or apoptosis (Young et al., 2010).

Therefore, it is of extreme importance to maintain the low extracellular glutamate level. Unlike ACh system, there is no effective enzyme within synapses to break down
glutamate (Logan and Snyder, 1972). Diffusion of glutamate is not an efficient mechanism, either (Danbolt, 2001). Only 20% of glutamate released during synaptic transmission act on postsynaptic glutamatergic receptors, whereas the remaining 80% is taken up by astrocytes via glutamate transporters (Verkhratsky and Kirchhoff, 2007). Five subtypes of glutamate transporters, known as excitatory amino acid transporters (EAATs) have been cloned (Danbolt, 2001). Among them EAAT 2 (also know as glutamate trasnporter 1, GLT-1) and EAAT1 (also known as glutamate-aspartate transporter, GLAST) are the two principal glutamate transporters exclusively expressed by astrocytes (Danbolt, 2001) as corroborated by autoradiographic and immunohistochemical studies (Currie and Kelly, 1981; Danbolt, 1994; Chaudhry et al., 1995; Lehre et al., 1995; Wadiche et al., 1995; Danbolt, 2001). Astrocytic GLT-1 takes the main role in the majority of glutamate uptake (Danbolt, 2001; Kirischuk et al., 2007).

Glutamate transporters are driven by electrogenic gradient, coupling glutamate uptake with Na\(^+\) and H\(^+\) influx and K\(^+\) efflux (Danbolt, 2001). One glutamate molecule is cotransported with three Na\(^+\) ions and one H\(^+\) in exchange for one K\(^+\) (Zerangue and Kavanaugh, 1996; Levy et al., 1998a; Owe et al., 2006). Thus, the transporter creates net Na\(^+\) inward current, resulting in elevated intracellular Na\(^+\) concentration in astrocytes. This increase is counteracted by the Na\(^+\)/Ca\(^{2+}\) exchanger and/or Na\(^+\)/K\(^+\) pump (Verkhratsky et al., 2011). This device maintains low intracellular Na\(^+\) concentration and thus the continuous performance of glutamate transporters.

Once glutamate is taken up by astrocytes, it is converted to glutamine in an adenosine triphosphate (ATP)-dependent process by glutamine synthetase (GS), which is specifically expressed by astrocytes. The nontoxic glutamine is then released by astrocytes and transported back to neurones and serve as a substrate for glutamate synthesis through conversion by glutamate dehydrogenase (Fig. 1.9; Danbolt, 2001).
Figure 1.9 Schematic Illustration of Astroglial functions in the brain. Astrocyte together with microvascule and neurone for the neurovascular unit. Astrocyte forms endfeet surrounding the BBB as well as enwraps the synapse with perisynaptic processes. Astrocyte exters several function to support neurones and maintain the extracellular homeostasis (see text for details; modified from Abbott et al., 2006: Nedergaard and Verkhratsky, 2012).
Chapter 1

3.2.6 Bidirectional Communication between Neurone and Astrocyte

For the past 3 decades, emerging evidence has demonstrated that astrocytes are able to sense and respond to synaptic transmission (Cornell-Bell et al., 1990; Lehre et al., 1995; Porter and McCarthy, 1996; Pasti et al., 1997; Araque et al., 2002). Astrocytes express diverse neurotransmitters receptors, in particular purinoreceptors as well as ionotropic and metabotropic glutamate receptors (Jabs et al., 1994; Brand-Schieber et al., 2004; Fields and Burnstock, 2006; Lalo et al., 2006). Astrocytic expression of neurotransmitter receptors is associated with the local neurotransmitter environment. For example astrocytes in the area with dense 5-HT innervations contain 5-HT receptors while astrocytes in the region with ACh and dopaminergic terminals express nicotinic and dopamine D2 receptors (Carson et al., 1996; Sanden et al., 2000; Xu and Pandey, 2000; Negyessy and Goldman-Rakic, 2005; Duffy et al., 2011). Activation of these receptors may evoke transient increase in intracellular Ca\(^{2+}\) of astrocytes (Kirischuk et al., 1999; Araque et al., 2002; Zur Nieden and Deitmer, 2006; Verkhratsky et al., 2009). Astrocytic Ca\(^{2+}\) signals propagate to the neighbouring astrocytes through gap junctions creating Ca\(^{2+}\) wave. The mechanism of this long-range Ca\(^{2+}\) signalling throughout astrocytic syncytium is associated with intracellular endoplasmic reticulum (ER) Ca\(^{2+}\) stores (Verkhratsky et al., 2011). Receptor activation leads to activation of phospholipase C pathway and the subsequent generation of second messenger Inostitol 1,4,5-triphosphate (InsP\(_3\)), which binds to InsP\(_3\) receptors on the ER and thus triggers Ca\(^{2+}\) release from the ER (Verkhratsky et al., 1998; Scemes and Giaume, 2006). Intercellular diffusion of InsP\(_3\) via gap junctions as well as extracellular diffusion of neurotransmitters (ATP or glutamate) released by astrocytes contributes to the Ca\(^{2+}\) wave propagation (Charles et al., 1992; Arcuino et al., 2002; Scemes and Giaume, 2006).

Astrocytes can not only respond to synaptic transmissions but also modulate neuronal activities. The concept of “tripartite synapse” postulates that neurotransmitter released to the synaptic cleft might bind to the receptors on adjacent astrocytes, inducing
intracellular $\text{Ca}^{2+}$ signals in astrocytes and the subsequent release of so-called “neurotransmitters,” such as ATP and glutamate from astrocytes, which will in turn modulate neuronal activities (Araque et al., 1999; Fig 1.10). For instance, synaptic glutamate transmission evokes $\text{Ca}^{2+}$ elevation in astrocytes, leading to the release of glutamate from astrocytes, which enhances the synaptic activity and synchronisation (Araque et al., 1998; Fellin et al., 2004). Evidence showing the presence of vesicular glutamate transporter (VGLUT) and vesicle-associated protein in the presynaptic process of astroglia further supported the bidirectional communication between glia and synapses (Montana et al., 2004).

Figure 1.10 Schematic Illustration of tripartite synapse. Astroglia is able to sense synaptic activity, and in turn releases gliotransmitters in response (see text for detail; modified from Araque et al., 1999).
Chapter 1

Taken together, signalling between neurones and astrocytes plays an important role in information integration in the brain (Pereira and Furlan, 2010).

3.3 Astroglia in Neuropathology

Neurological diseases result from alterations in brain homeostasis and synaptic connections as well as the balance between destruction, neuroprotection and regeneration (Rodriguez et al., 2009a; Verkhratsky et al., 2012). The brain homeostasis is generally maintained by astrocytes, and hence astrocytes are critically involved in the progression and severity of various neurological diseases (Verkhratsky et al., 2010). Acute and chronic brain insults cause a specific astroglial reaction, which is known as reactive astrogliosis (Pekny and Nilsson, 2005). In this astrocytes undergo hypertrophy, featured by up regulation of GFAP expression and showing an enlarged cell body together with an increase of the number and extension of their branches (Wu and Schwartz, 1998). Reactive astrogliosis protect the brain by isolating the damaged area through formation of glial scar, reforming the blood-brain barrier and facilitating the remodelling of the neural circuitry in lesioned areas (Nishiyama et al., 2005; Pekny and Nilsson, 2005; Sofroniew, 2009). The reactive astrogliosis varies gradually with the severity of the insults (Sofroniew, 2009). Mild to moderate astrogliosis leads to the production of various molecules, such as thrombospondins and laminins that promote neurite growth and synaptogenesis and thus facilitate remodelling of synaptic networks (Sofroniew, 2009; Sofroniew and Vinters, 2010). Severe astrogliosis results in the formation of glial scar, which isolates the lesioned area and eventually substitute the previous structure, serving as a border of damages and inflammation (Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010).

Astrogliosis, however, could be deleterious. Reactive astrocytes are potentially detrimental in several ways (Sofroniew and Vinters, 2010), including secretion of cytokines to aggravate inflammation (Brambilla et al., 2005; Brambilla et al., 2009); becoming a source of glutamate thus exacerbating excitotoxicity (Takano et al., 2005); producing reactive oxygen species to increase the oxidative stress and neurotoxicity (Chen et al., 2001; Swanson et al., 2004; Hamby et al., 2006) and an increasing lactic acidosis that worsens the damage during ischemia (Rossi et al., 2007). Furthermore,
astrocytes could spread death signal molecules through gap junctions (Contreras et al., 2002; Thompson et al., 2006; Rossi et al., 2007).

3.4 Astroglial Alteration in the Cortex during ageing and AD

3.4.1 Effects of Ageing on Cortical Astroglia

Several reports indicated elevated GFAP mRNA and protein in the cerebral cortex and other brain regions (including hippocampus) of aged rodents (Goss et al., 1991; Kohama et al., 1995). In addition, increased number of GFAP-immunoreactive astrocytes accompanied by hypertrophic morphology has been found in aged rat cortex (Amenta et al., 1998). Therefore, an up-regulation of GFAP is associated with an hypertrophy and proliferative response (Cotrina and Nedergaard, 2002). It was estimated that a 20% increase in the number of astrocytes and pericytes occurs in the cortex and other brain regions of aged rats (Peinado et al., 1998; Rozovsky et al., 1998). It was also claimed that age-associated changes of astroglia are more evident in female than male (Mouton et al., 2002). There is also evidence indicating regional variations of markers of astrogliosis (Morgan et al., 1999). However, studies on human and nonhuman primates did not show age-associated changes in the profile or number of astrocytes (Pakkenberg et al., 2003; Pelvig et al., 2008; Peters et al., 2008). It requires further investigation to understand if the age-related changes in astrocytic population and morphology are dependent on region, gender and species.

It is of great interest to determine whether this morphological change in astrocytes induces altered signalling and functions of astrocytes in aged brains (Cotrina and Nedergaard, 2002). For instance, astroglia is essential for glutamate balance in the brain (Danbolt, 2001). Several studies focused on ageing affect and astrocytic contribution on glutamate homeostasis (Goss et al., 1991; Gottfried et al., 2002; Garcia-Matas et al., 2008). No changes in GS mRNA were found in spite of elevation in GFAP mRNA and protein, suggesting stable GS expression of aged astrocytes (Goss et al., 1991). Experimental studies showed controversial results of glutamate uptake of aged cortical astrocytes in vitro, which may be due to different species and preparation (Goss et al., 1991; Gottfried et al., 2002; Garcia-Matas et al., 2008). Astrocytes also provide protections for neurones from oxidative stress, which has been regarded as a feature in
ageing brains (Lee et al., 2000; Chen et al., 2001; Shih et al., 2003). Lin and colleagues (Lin et al., 2007) demonstrated that cultured astrocytes from old animals exhibited lower mitochondrial membrane potential and are more susceptible to oxidative stress thus providing less protection when co-cultured with NGF-differentiated PC12 cells with exposure to oxidant. Similarly, several reports indicated compromised neuroprotections from astrocytes in normal ageing animal as well as in spontaneously hypertensive rats (Deng et al., 2006; Ritz et al., 2009). In addition, there are also studies revealing age-dependent accumulation of tau in astrocytes in both human and baboon brains (Schultz et al., 2000; Yang et al., 2005). As a consequence, astrocytes may lose their functions, manifested by reduced expression of glutamate transport, BBB disruption and focal neurodegeneration (Forman et al., 2005; Dabir et al., 2006). Recent studies emphases on altered synaptic network rather than neurodegeneration in ageing brain (Stranahan and Mattson, 2010). This is therefore important to understand whether these represent an age-associated change in astrocytic support to synapses and communication with synapses. It was indicated by Sykova (Sykova et al., 1998) that the capabilities of astrocytes in ion and extracellular volume are compromised during ageing. This might cause consequent synaptic alteration (Adams and Jones, 1982). Electrophysiological evidence displayed decreased glial responses to synaptic activity due to the decline in the density of glutamate receptors and purinoreceptors in aged astrocytes (Lalo et al., 2011). This may imply a decrease in neuronal-glial signalling, which is involved in information processing and integration and thus reflect an age-dependent decline in brain cognition (Pereira and Furlan, 2010).

3.4.2 Astroglial Alteration in the Cortex during AD Progression

Alzheimer (Alzheimer, 1907) and Cajal (Ramon y Cajal, 1918) were the first to notice astroglial alteration in AD brains. Alzheimer found abundant glial cells with a clear hypertrophy in the vicinities of plaques (Alzheimer, 1907). Proteomic and histological studies revealed increased GFAP expression as well as the number of GFAP-positive astrocytes in the cortical regions including EC in AD (Muramori et al., 1998; Porchet et al., 2003; Kashon et al., 2004; Simpson et al., 2010). Surface density of GFAP in EC is significantly higher in AD than in healthy controls (Vanzani et al., 2005). Higher surface density of GFAP may represent hypertrophy of astrocytes, the feature of reactive astrogliosis in the EC of AD brains (Vanzani et al., 2005). Moreover,
this increment in GFAP initiates even at early Braak stages and correlate with the
degree of dementia and cognitive dysfunction in AD, suggesting that GFAP elevation
can be a marker for early AD diagnosis (Muramori et al., 1998; Kashon et al., 2004).

A growing body of evidence has shown profuse distribution of reactive astrocytes
around the Aβ plaques in both human autopsy tissues and animal models of AD
(Mandybur and Chuirazzi, 1990; Shao et al., 1997; Nagele et al., 2003; Nagele et al.,
2004; Rodriguez et al., 2009b; Olabarria et al., 2010). Additionally, *in vivo* experiments
have revealed that direct injection of Aβ into rat cortical regions induces astrogliosis in
local and adjacent areas (Sipos et al., 2007; Perez et al., 2010). Thus, it was suggested
that accumulation of Aβ protein may trigger astrogliosis via alterations in Ca^{2+}-
dependent cascade (Norris et al., 2005; Chow et al., 2011). Both *in vitro* and *in vivo*
studies have shown that Aβ may affect astrocytic Ca^{2+} signalling (Abramov et al., 2003;
Kuchibhotla et al., 2009). Application of Aβ to mixed cultures of neurones and glial
cells induced spontaneous Ca^{2+} influx and oscillations, which may be directly or
indirectly associated with Aβ-induced neuronal death (Abramov et al., 2003). In line
with this, another study performed on APP/PS1 transgenic mice showed Aβ initiated
Ca^{2+} transients in local astrocytes with subsequent Ca^{2+} propagating and spreading
through the astrocytic network, suggesting a general response of astrocytes to focal Aβ
pathology (Kuchibhotla et al., 2009).

In spite of the effects of Aβ on astrocytes, astrocytes have a controversial role in Aβ
processing, clearance and degradation (Verkhratsky et al., 2010). There is evidence
showing that astroglial processes surround Aβ plaques with some of them penetrating
the SPs (Kato et al., 1998). These cortical reactive astrocytes intimately surround the
plaques contain different forms of Aβ (Thal et al., 2000a; Nagele et al., 2004). It was,
therefore, suggested that astrocytes are able to take up Aβ42 through phagocytosis of
degenerated synapses and accumulate this substance. Some Aβ-containing astrocytes
then undergo lysis and atrophy, forming astrocytic amyloid plaques (Nagele et al.,
2003). An important evidence for the external source of the amyloid material is the co-
localisation of the Aβ42 in activated astrocytes with neuron-specific proteins, such as
the a7 nicotine acetylcholine receptor (a7nACHr) and ChAT(Nagele et al., 2003;
Teaktong et al., 2003; Teaktong et al., 2004). The existence of neurone-specific proteins
in astrocytes validated the astroglial phagocytosis and its cleaning function of
degenerated synapses (Nagele et al., 2004). On the other hand, there is evidence demonstrating up-regulation of neprilysin, an enzyme degrading $\text{A}\beta$, in reactive astrocytes surrounding the plaques in the cerebral cortex (Apelt et al., 2003). *In vivo* and *in situ* experiments showing decreased $\text{A}\beta$ after incubating with exogenous mature astrocytes from healthy brains supported the role of astrocytes in the clearance and degradation of amyloid (Wyss-Coray et al., 2003). However, in the same study, it was also indicated that endogenous astrocytes in contact with $\text{A}\beta$ deposits are unable to eliminate $\text{A}\beta$, implying that astroglia-mediated clearance of $\text{A}\beta$ may precede and/or account for the accumulation of $\text{A}\beta$ in AD (Wyss-Coray et al., 2003).

Meanwhile, astroglia itself may contribute to $\text{A}\beta$ production. Astrocytic expression of $\beta$-secretase 1 (BACE 1), which is confined to neurones, has been observed in several transgenic animal models of AD (Rossner et al., 2001; Heneka et al., 2005). Reactive astrocytes also exhibit prominent PS1 ($\gamma$-secretase) expression in AD brains (Huynh et al., 1997; Weggen et al., 1998). Since both secretases are critical in the formation of $\text{A}\beta$, it is suggested that astrocytes might be producers of $\text{A}\beta$. Nevertheless, the expression of APP in astrocytes is very low (Nagele et al., 2004) and there is no sufficient evidence showing direct correlation of astrocytic BACE 1 and PS1 with the presence of $\text{A}\beta$ deposits.

Astrocytes also display regressive changes, including fragmentation of processes as well as weak expression of GFAP in cell body (Kobayashi et al., 2002). The regressive changes of astrocytes have been observed in white and grey matter of AD brains (Kobayashi et al., 2002; Kobayashi et al., 2004). Decreased number of interlaminar astrocytes processes was detected in the cortex of AD brains, indicating degenerative process of astrocytes during the pathological progression (Colombo et al., 2002). Moreover, caspase-mediated cleavage of GFAP has been implicated in damaging astrocytic process and degeneration of astrocytes (Mouser et al., 2006). Correspondingly, microarray analysis revealed a decline in transcripts of astrocytic cytoskeleton proteins in AD, implying decreased expression of astrocytic cytoskeleton (Simpson et al., 2011). Data from our own laboratory (Olabarria et al., 2010; Yeh et al., 2011; Kulijewicz-Nawrot et al., 2012) demonstrated a cytoskeleton atrophy of astrocytes associated with reduced surface and volume of GFAP processes. Atrophic and hypertrophic astrocytes may, however, coexist. The proliferated astrocytes
accumulated in the vicinity of Aβ whereas atrophic astrocytes were present distantly to amyloid plaques (Olabarria et al., 2010). Therefore, astroglia undergoes different morphological alteration in response to AD pathology (Rodriguez et al., 2009b; Olabarria et al., 2010).

Astrocytic function in glutamate homeostasis is also considered as a key element in AD progression since disruption in glutamate-glutamine cycle and subsequent glutamate balance may contribute to synaptic and neuronal degeneration, as discussed previously (Tymianski and Tator, 1996; Hynd et al., 2004; Walton and Dodd, 2007; Duchen et al., 2008). Autoradiographic studies showed significant reduction in glutamate uptake in several cortical regions in AD brains (Greenamyre et al., 1985; Cross et al., 1987). Other reports also revealed decreased expression and activity of GS and glutamate transporters (Le Prince et al., 1995; Castegna et al., 2002; Hynd et al., 2004), suggesting impaired astrocytic glutamate balance during AD progression. Furthermore, presence of EAAT1 and EAAT 2, which is exclusively expressed in astrocytes in healthy brain, has been observed in neurones in AD cortices (Scott et al., 2002). Neuronal expression of astrocyte-specific GS was also found in the cerebral cortex of AD brains (Robinson, 2001). Therefore, it is suggested that this aberrant presence of astrocytic proteins in neurones may either compensate for astrocytic dysfunction or underlies the neurodegenerative mechanism in AD (Robinson, 2001; Scott et al., 2002).

However, there is also evidence pointing out unaltered glutamate level or glutamate uptake in cortical regions of AD patients (Mohanakrishnan et al., 1995; Beckstrom et al., 1999; Simpson et al., 2010). Increased GS immunoreactivity was also observed in the PFC of AD brains (Burbaeva et al., 2005). These different results may be due to antibody, epitopes and post-mortem intervals (Walton and Dodd, 2007). In particular, oxidation of GS can affect GS immunoreactivity by preventing antiserum binding to GS proteins (Hensley et al., 1995; Butterfield et al., 1997).
4. Animal Models of AD

Animals in the wild do not suffer from AD (Toledano and Alvarez, 2004). Various experimental animal models have been therefore developed (Cassel et al., 2008). Pharmacologically cholinergic-lesioned models were the first to be created due to the fact that cholinergic system is the specifically vulnerable in AD (Gotz et al., 2004; Toledano and Alvarez, 2004). Subsequently, many transgenic animal models have been developed (Gotz et al., 2004; Gotz and Ittner, 2008).

4.1 Lesion Model

The development of cholinergic-lesioned model has played an important role in evaluating the potential of cholinergic degeneration for cognitive deficit in AD (Winkler et al., 1998). Several animal models with deficits in cholinergic system have been created. Among these models, lesion in the nucleus basalis magocellularis (nbm), homologue to nbM in human, has been most frequently used (Toledano and Alvarez, 2004). A variety of methods have been applied to lesion the nbm, including injecting excitotoxins and more specific cholintoxin and immunotoxin. Excitotoxins, such as ibotenic acid, AMPA and NMDA, achieve the lesion by over-activating glutamate receptors and causing massive intracellular Ca\(^{2+}\) elevation, which causes cell death at the injection site (Dekker et al., 1991; Harkany et al., 2002). Other non-specific lesion agents including quinolic acid have also been used (Boegman et al., 1985). However, the nonspecific excitotoxins also affect other neurones, such as monoaminergic and glutamatergic neurones, and these lesioned animals showed contradictory results in cognitive tests. Ibotenic acid induced manifest impairments in several memory tasks whereas AMAP either induced mild memory deficits or had no effects on memory performance despite profound and specific loss of cholinergic neurones (Page et al., 1991; Muir et al., 1993; Auld et al., 2002). AF64A cholinotoxin, is a presynaptic neurotoxin and binds to the high affinity choline uptake system. The lesion effect of AF64A is both dose- and region-dependent (Hanin, 1996). When high doses are used (more than 2 nmol), all cholinergic cells are affected, whereas at low doses only cholinergic neurones with long projections are damaged, particularly those in the septum, nbM and the diagonal band of Broca (Wiley, 1992). Immunotoxin is another drug widely used for nbm-lesioned model. Cholinergic neurones in the basal forebrain
possess low-affinity nerve growth factor receptors (p75NGFR) in their plasma membrane (Gibbs and Pfaff, 1994). The immunotoxin, 192IgG-saporin, is a ribosome-inactivating protein conjugating with monoclonal antibody targeting at p75NGFR. 192 IgG-saporin enters the cell via endocytosis when binding with p75NGFR and is transported retrogradely from terminals to the cell body to interrupt the proteins synthesis of cholinergic neurones (Wiley, 1992). Selective lesion of cholinergic neurons in nbm does not resulted in consistent and clear correlation with memory impairment (Torres et al., 1994; Baxter et al., 1995; Baxter et al., 1996; Shen et al., 1996). Moreover, animals with infusion of non-selective or selective lesion agents into nbm did not show pathological hallmarks associated with AD that is critical for understating the relation between AD pathology and cognitive dysfunction (Gotz et al., 2004).

4.2 Single and Double Transgenic Animal Model

It is because mutations in APP, PS1, and PS2 have been shown to be associated with FAD, the majority of transgenic models are made through over expression of APP, PS1, or PS2 (Gotz et al., 2004). At first, transgenic PDAPP mice were created with APP mutations, which showed extracellular diffuse and neurite plaques, neuritic dystrophy, astrogliosis and synaptic loss (Games et al., 1995). Memory impairments were also observed in PDAPP animals; however, these were not correlated with Aβ burden (Gotz et al., 2004). Later, Hsiao and colleagues (Hsiao et al., 1996) developed another APP over expression model (Tg2576 mice) with double Swedish mutation APPswe (K670N and M671L). Tg2576 mice showed enhanced density of Aβ plaques in the brain areas involved in memory formation, such as hippocampus, as well as age-dependent deficits in LTP and memory (Hsiao et al., 1996). APP23 mice were developed at Novarits; this model over expressed human APP with Swedish mutation under alternative promoter. APP23 mice began to exhibit plaques, amyloid angiopathy as well as loss of CA1 neurones at a relatively early age (around 6 month) (Sturchler-Pierrat et al., 1997). Tg-CRND8, which was generated by combining of Swedish and V717F mutations, showed early and profound amyloid deposition associated with learning deficits (Chishti et al., 2001). Both PS1 and PS2 are involved in FAD, thus mutations in PS1 and PS2 may facilitate the generation of fibrillogenic Aβ42. Nevertheless, it was found that over expression of PS2 did not result in amyloid pathology (Herreman et al., 1999). At the same time, over expression of PS1 with M146L or M146V mutations resulted in
increased level of Aβ42 whereas over expression of wild type PS1 was ineffective (Duff et al., 1996). Co-expression of PS1 (M146L or PS1DE9) and APP_swe mutations increased the Aβ42/Aβ40 ratio and accelerated the formation of Aβ plaques (Borchelt et al., 1997). PSAPP mice were created by crossing Tg2576 mice with PS1 M146L mice, and showed an increase in Aβ42 level and early Aβ deposits (Holcomb et al., 1998). More recently, another double transgenic animal model was generated with over expression of both BACE 1 and APP_swe, these mice also exhibited accelerated plaques formation (Mohajeri et al., 2004).

Although tau mutation was not identified in FAD, it is involved in neurodegeneration in other forms of dementia (Gotz et al., 2004). The fist transgenic model harbouring Tau mutation was created in 1995. This model over expressed the longest isoforms of human tau (4 repeats) and displayed somatodendritic localisation of hyperphosphorylated tau with absence of NTF pathology (Gotz et al., 1995). After mutation of tau on FTD with Parkinsonism linked to chromosome 17 (FTDP-17) was identified, several transgenic animal models displaying NFT as well as reactive astrogliosis have been developed (Lewis et al., 2001; Gotz et al., 2001; Tatebayashi et al., 2002). Among them, the P301L is the most common mutation linked with FTDP-17 (Lewis et al., 2000). Expression of FTDP-17-associated mutation P310L induced NFT formation; nonetheless, the P301L transgenic mice also exhibited motor neuron degeneration, which is not found in AD (Lewis et al., 2000). A recently developed P301L transgenic model is under control of tetracycline-regulating operon so that the tau production can be reversed by doxycycline. This animal model displayed progressive neurofibrillary pathology as well as neuronal loss (Ramsden et al., 2005).

Unfortunately, all the animal models mentioned above failed to express both pathological hallmarks, Aβ plaques and NFTs, of AD (Gotz et al., 2004; table 1.1). They also failed to show similar spatiotemporal distribution of plaques and tangles in AD patients (Kurt et al., 2003). For this purpose and to improve AD animal model, LaFerla group developed triple transgenic animal model (Oddo et al., 2003a; Oddo et al., 2003b).
4.3 Triple Transgenic Animal Model

Triple transgenic animal model of AD (3xTg-AD) was developed by Frank Laferla’s lab (Oddo et al., 2003a; Oddo et al., 2003b). It is arguably one of the most relevant animal models of AD (Table 1.1). The 3xTg-AD mice harbour APPSWE, PS1M146V, and tauP301L transgenes. The 3xTg-AD mice exhibit progressive and spatiotemporal Aβ and tau pathology in relevant brain regions (Oddo et al., 2003a; Oddo et al., 2003b). Aβ deposition begins in the cortex and progresses to the hippocampus with ageing; however, NFTs are initiated in the hippocampus and then progresses to the cortex (Oddo et al., 2003a; Oddo et al., 2003b). Extracellular deposit of Aβ first appears in cortex at 6-month and progressed to CA1 region of hippocampus at 12-month (Oddo et al., 2003a). At 12 months Aβ pathology is evident within hippocampus and neocortical regions (Oddo et al., 2003a). To the contrary, tau pathology appears in CA1 of 3xTg-AD animals between 6 and 12 months. It should be noted that the 3xTg-AD mice develop Aβ deposit before NFT formation, consistent with the amyloid cascade hypothesis which suggests that Aβ accumulation being the earliest pathology of AD (Oddo et al., 2003a; Oddo et al., 2003b).

The 3xTg-AD model was generated by microinjections of two independent transgenes encoding human APPswe and human TauP301L into a single-cell embryo cell from homozygous PS1M146V knockin mice (Oddo et al., 2003a; Oddo et al., 2003b). This minimised genetic interactions and at the same time promoted maintenance of the mouse colony as well as reduced independent grouping of the mutations in the next generations (Oddo et al., 2003a; Oddo et al., 2003b; Gotz et al., 2004).

These mice also show age-related deficit in synaptic and cognitive functions (Oddo et al., 2003a). The 3xTg-AD mice exhibit deficits in basal synaptic transmission as well as LTP at the age of 6 months, and this synaptic dysfunction is correlated with intraneuronal accumulation of Aβ (Oddo et al., 2003a). In addition, the 3xTg-AD mice show selective loss of nicotinic receptors, corresponding to the findings in post mortem AD brains (Burghaus et al., 2000; Wevers et al., 2000; Oddo et al., 2005). Behavioural tests revealed that 3xTg-AD mice do not show cognitive deficits at 2 months of age, confirming that 3xTg-AD mice are not born with cognitive impairments (Billings et al., 2005). The earliest cognitive impairments in 3xTg-AD mice appear at 4 months, being
closely related with intraneuronal Aβ aggregates in the hippocampus and amygdala (Billings et al., 2005). Moreover, 3xTg-AD mice at 6 months show learning deficits in Morris water maze (MWM) task, and this impairment deteriorate as the disease progresses (Billings et al., 2005). Other behavioural analysis revealed significant impairment of spatial memory, objective memory, contextual memory and long-term memory (Billings et al., 2005; Caccamo et al., 2006; Clinton et al., 2007; McKee et al., 2008). Longitudinal studies using MWM demonstrated progressive and age-dependent decline in learning and memory from 6 to 18 months of age in 3xTg-AD mice, these impairments are closely related with an increase in Aβ and tau neuropathology (Billings et al., 2007; Clinton et al., 2007; Halagappa et al., 2007; Blurton-Jones et al., 2009; Martinez-Coria et al., 2010). Therefore, this model seems to be the most faithful in reproducing AD pathology.
Table 1.1 Summary of transgenic mouse models of AD

<table>
<thead>
<tr>
<th>Transgenic animal model</th>
<th>Neuropathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDAPP</td>
<td>Aβ deposit</td>
<td>Games et al., 1995</td>
</tr>
<tr>
<td>APPswe</td>
<td>plaque</td>
<td>Hsiao et al., 1996</td>
</tr>
<tr>
<td>APP23</td>
<td>plaque</td>
<td>Sturchler-Pierrat et al., 1997</td>
</tr>
<tr>
<td>Tg-CRND8 (APPswe/AppV717)</td>
<td>Plaque</td>
<td>Chishti et al., 2001</td>
</tr>
<tr>
<td>PS1M146L</td>
<td>Diffused plaque</td>
<td>Blanchard et al., 2003</td>
</tr>
<tr>
<td>PSAPP</td>
<td>plaque</td>
<td>Holcomb et al., 1998</td>
</tr>
<tr>
<td>APPswe x PS1A246E</td>
<td>plaque</td>
<td>Borchelt et al., 1997</td>
</tr>
<tr>
<td>BACE x APPswe</td>
<td>Plaque</td>
<td>Mohajeri et al., 2004</td>
</tr>
<tr>
<td>AIZ7 (4R tau)</td>
<td>Hyperphosphorylated tau</td>
<td>Gotz et al., 1995</td>
</tr>
<tr>
<td>Tau P301L</td>
<td>Tangle</td>
<td>Lewis et al., 2000</td>
</tr>
<tr>
<td>JNPL3 (4R+ P301L)</td>
<td>Tangle</td>
<td>Lewis et al., 2000; Arendash et al., 2004</td>
</tr>
<tr>
<td>rTg4510(P301L; Tet-off)</td>
<td>Tangle</td>
<td>Ramsden et al., 2005</td>
</tr>
<tr>
<td>Tg2576 x JNPL3</td>
<td>Plaque + Tangle</td>
<td>Lewis et al., 2001</td>
</tr>
<tr>
<td>Tg2576 x VLV (tauvlw)</td>
<td>Plaque + Tangle</td>
<td>Ribe et al., 2005</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td>Plaque + Tangle</td>
<td>Oddo et al., 2003a,b</td>
</tr>
<tr>
<td>(APPswe+PS1M146V + P301L)</td>
<td>Plaque + Tangle</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1

Scope and outline of the thesis

Entorhinal cortex (EC) is the hub for information transfer and integration between the memory centre, hippocampus, and other cortical regions. Therefore, EC participates actively in all cognitive and memory processes. Malfunction of the EC is observed and correlated with impaired cognition and memory deficits in ageing and age-related diseases such as Alzheimer’s disease (AD). AD is a progressive and irreversible neurodegenerative process characterised by Aβ deposition and abnormal hyperphosphorylation of tau protein as well as synaptic and neuronal degeneration. However, recently there is increasing evidence for a relevant and important glial involvement in the pathological progression of AD. Among the diverse groups of glial cells, astroglia is the main cellular constituent in the CNS responsible for the homeostasis as well as modulation of neuronal activity and synaptic network.

In this thesis, we focus on the astroglial alterations observed in the EC of the triple transgenic mice (3xTg-AD) model, which is the most relevant and closely resembles the spatiotemporal progression of human AD. We aim to answer the following research questions: (1) how astrocytes change, specifically their cytoskeleton, during the disease progression; (2) how the changes in astroglial cytoskeleton affects its capability of controlling homeostasis, in particular glutamate balance; (3) astroglial alterations in the EC and its major output target, the hippocampus, during normal ageing using control animals.

In the first chapter (introduction), we reviewed the recent and relevant literature on the anatomy and functions of the EC, EC dysfunction as well as its involvement in ageing and AD. Moreover, we review the role of astrocytes in brain physiology and pathology. Additionally, we briefly review the development of AD animal models, especially the 3xTg-AD.
Chapter 1

Chapter 2

Material and Methods

In this chapter, we describe the general procedure of tissue preparation and the methodology used to obtain the results presented in this thesis. We state the generation of 3xTg-AD animals, fixation and tissue processing, immunohistochemistry-fluorescence, immunohistochemistry-peroxidase, confocal imaging and morphological analysis of astrocytes, cell count and numerical density \((N_v; \text{ number of cells/mm}^3)\), Inverse optical density (IOD) measurement and statistical analysis.

Chapter 3

Astroglia is critical for brain homeostasis at different levels, from molecular to organ (Verkhratsky et al., 2010). Moreover, astroglia is one of the compartments of the so-called tripartite synapse, actively interacting with neurones (Araque et al., 1999; Hydon, 2001). Inevitably, astrocytes are involved in various processes of normal brain functions, and thus astrocytic malfunction may account for development and progression of neuropathology (Giaume et al., 2007). Several studies indicated reactive astrogliosis, featured by proliferation of GFAP and hypertrophy, in AD brains (Muramori et al., 1998; Porchet et al., 2003; Nagele et al., 2004; Vanzani et al., 2005). Other reports further indicated the presence of reactive astrocytes in relation to Aβ plaques, suggesting Aβ triggers astrogliosis (Nagele et al., 2003; Nagele et al., 2004; Rodriguez et al., 2009; Olabarria et al., 2010).

In chapter 3 we studied the alterations in astroglial morphology throughout the progression of AD in 3xTg-AD mice, at different ages (from 1 month till 18months). We also studied the \(N_v\) (\# cells / mm\(^3\)) of astrocytes in the EC. In addition, we examined the correlation between astrocytes and Aβ plaques presence. For this purpose, we used immunofluorescence and confocal microscopy to analyse the morphology of glial fibrillary acidic protein (GFAP) positive astrocytes. Surprisingly, our finding revealed a general atrophy, demonstrated by a significant reduction in the surface and volume of GFAP profiles without changes in the numerical density, starting as early as 1 month of age. We consider that this astrocytic astrophy might cause an
imbalance of neurotransmitters and ions as well as reduced support to neurones, leading to the disruption in synaptic connectivity, which underlie the basis of early cognitive and memory decline in AD. Moreover, we found that GFAP positive astrocytes rarely appear in the vicinity of plaques and when they do so they do not show any reactivity or hypertrophy. This peculiar behaviour of astrocytes may account for the vulnerability of the EC at the early stages and point out the very initial involvement of EC in AD, as happens in humans where is the first region affected.


My personal contribution to above manuscript includes performing experiments, data analysis, figure preparation, and writing the initial drafts of the manuscript, which later on was refined and ultimated with the support of my supervisors.

Chapter 4

Among the various functions, astrocytic maintenance of glutamate homeostasis is important for normal both brain functions and pathology (Danbolt, 2001; Riedel et al., 2003; Verkhratsky and Kirchhoff, 2007). By using the glutamate-glutamine cycle, astrocytes clean the synaptic glutamate by taking up glutamate with the glutamate transporters and convert it to glutamine via glutamine synthetase (GS), an enzyme specifically expressed in astroglia. Glutamine is then release by astrocytes and transported back to neurone for glutamate synthesis (Danbolt, 2001).

We reported astroglial atrophy in the EC of 3xTg-AD mice in chapter 3, thus we wanted to know if this cytoskeletal changes compromise astrocytic functions, and specifically astrocytic control of glutamate balance. Thus, in chapter 4, we analysed the changes in the GS labelled astrocytes within the EC of 3xTg-AD animals from young to later ages (1-18 months of age). Interestingly, we did not find significant difference neither in GS-positive astrocyte Nv nor in GS contents as determined by the IOD of GS immunoreactive (GS-IR) cells, when compared 3xTg-AD animals with Non-Tg control
group. Furthermore, by using dual immunofluorescence labelling and confocal microscopy, we analysed the co-localisation of GFAP-IR and GS-IR cells and the morphology of GS-IR astrocytes. In spite of the manifest GFAP atrophy, the surface and volume of GS-IR cells remain stable irrespective of their co-localisation with GFAP. Therefore, alterations in GFAP do affect neither GS expression nor the morphology of GS positive cells, suggesting that astroglial atrophy in the EC may affect the local and global synaptic network by other mechanisms different from the glutamate-glutamine shuttle. Finally the dual detection of these astrocytic markers indicates the existence of different astrocyte subpopulations in the EC, implying heterogeneity of astrocytic identity and functions which might be directly associated with the EC neuropathology and function impairments observed in AD.

**Yeh CY., Verkhratsky A., Rodriguez JJ. (2012). Astrocytes in the entorhinal cortex of the triple Transgenic Animal Model of Alzheimer’s Disease Show No Alterations in Glutamate-Glutamine Shuttle Components (manuscript in preparation).**

**My personal contribution to above manuscript includes performing experiments, data analysis, figure preparation, and writing the initial drafts of the manuscript, which later on was refined and ultimated with the support of my supervisors.**

**Chapter 5**

Due to the advance and development of medication, life span of human has extended so that the senescent population increases dramatically. However, ageing process is linked with cellular stress and bears the risk of several neurodegenerative diseases, including AD, which is accompanied by cognitive and mnesic impairments (Garcia-Alloza et al., 2005; Small and Cappai, 2006). Therefore, accumulating efforts and studies have been devoted to brain ageing and associated alterations. In fact, several studies have indicated structural and cellular changes in the brain during the ageing process which results in functional alterations and subsequent cognitive declines (Hof and Morris, 1997; Smith et al., 2000; Rodrigue and Raz., 2004; Liu et al., 2008; Raz et al., 2008).
Emerging evidence indicates that compromised synaptic connectivity underlies the cognitive decline during ageing (Geinisman et al., 1992; Nicolle et al., 1999; Smith et al., 2000; Scheff et al., 2005). It is to note that astroglia, being the major cellular responsible for the maintenance of brain physiological environment, plays an important role in modulation synaptic networks also during the ageing process (Fellin et al., 2004; Fellin et al., 2009). However, astroglial changes and behaviour during ageing have not been thoroughly studied. In chapter 5, we analyse the morphology of GFAP-IR astrocytes in both the hippocampus and the EC, the two areas heavily involved in cognition and memory and deeply affected during ageing, in normal old animals (12 to 24 months of age) in order to determine and assess the age-related remodelling of astrocytes.

Interestingly, our finding revealed heterogeneous alterations and behaviour of GFAP- and GS-positive astrocytes in these two areas. In the hippocampus, and more specifically in the DG and CA1, GFAP-positive astroglia underwent profound hypertrophy at advanced ages (18 and 24 months of age) whereas entorhinal astrocytes had evident atrophy in senescent brains. On the other hand, the cellular domain of GS-positive astrocytes in the DG and CA1 of senescent animals decreased markedly whereas there is no apparent change in GS-positive astrocytes in the EC.


My personal contribution to above manuscript includes participation in the experiments, data analysis, and figure preparation and writing the first draft of the manuscript, which later on was refined and ultimated with the support of my supervisors.

Chapter 6
General Discussion

In chapter 6, we initially include an overview of the findings from chapters 3-5, and discussed our results according to the actual literature and point out future directions
which will clarify the astroglial changes observed in AD and ageing, which hopefully could open potential new insights in therapeutical approaches. In summary, GFAP-IR astrocytes exhibited general atrophy, shown by evident reduction in the surface and volume of GFAP-IR profiles, in the EC of 3xTg-AD mice throughout the development and progression of AD. The rare existence of astrocytes in the vicinity of Aβ deposition, suggest that EC astrocytes do not respond to amyloid pathology in this animal model. Moreover, consistent GS expression may indicate that alterations in the main cytoskeleton protein of astrocytes have, at least, no direct influence on glutamate-glutamine cycle and thus glutamate balance in the EC is not affected through this process; which in addition to the presence of 2-3 different populations of astrocytes (see GS positive, GFAP positive and GFAP/GS positive) further suggest differential functional roles in neurotransmitter homeostasis.

Finally, age-dependent changes in astrocytic morphology indicate regional heterogeneity of astroglial responses during ageing.

Further investigation is required to have a better understanding of astrocytic subsets and alterations in the EC of AD. Investigation of S100β containing astrocytes in relation to Aβ will be fundamental for knowledge of EC astrocytes in response to AD pathology. It will be also interesting to investigate if environmental enrichment and voluntary wheel running have beneficial effect on EC astroglia in AD. To study the changes in neuronal plasticity by determining the expression of polysialylated neuronal cell adhesion molecule the EC neurons and their neurites as well as their relationship with astroglial changes and alterations will decipher the deleterious effects that we observed in synaptic connectivity and plasticity in AD. All this studies are currently being developed in our Laboratory.
Chapter 2

Material and Methods
1. Animals

*In all result chapters*

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

Experiments described in *Chapter 3 and 4* were performed on male 3xTg-AD mice and their background matching controls; experiments described in *Chapter 5* were performed on non-transgenic (non-Tg) control mice only. The procedures for generating 3xTg-AD mice have been described previously (Oddo et al., 2003a; Oddo et al., 2003b; Rodriguez et al., 2008; Rodriguez et al., 2009a; Rodriguez et al., 2009b) The human APP cDNA harbouring the Swedish double mutation (KM670/671NL, APPswe) and human four repeat Tau, harbouring the P301L mutations (tauP301L), both of which were under the control of mouse Thy1.2 regulatory element, were co-microinjected into a single-cell embryo derived from a homozygous presenilin 1(PS1M146V) knock-in mice (Oddo et al., 2003b). This procedure minimised the genetic interactions and facilitated the maintenance of the mouse colony as well as reduced the independent grouping of the mutations in the successive generations (Oddo et al., 2003a; Oddo et al., 2003b; Gotz et al., 2004). The background of the PS1 knock-in mice is a hybrid 129/C57BL6; the non-Tg control mice were from the same strain and genetic background as the PS1 knock-in mice, but they harbour the endogenous wild-type mouse PS1 gene. Transgenic mice were identified by southern blot analysis of tail DNA (Sugarman et al., 2002). All 3xTg-AD and non-Tg control littermates were obtained by crossing homozygous breeders. Animals were grouped by gender and genotype and housed under controlled temperature and light-dark cycles (12:12 h) with ad libitum access to food and water.
2. Fixation and Tissue Processing

*In all result chapters*

Anaesthetisation and perfusion of mice were carried out by the licensee (José Julio Rodríguez Arellano, JJRA). Following perfusion, the post-fixation, sectioning of the brain sections and all tissue processing steps were carried out by Chia-Yu Yeh (CYY). Male 3xTg-AD and non-Tg control mice were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg) at 1, 3, 6, 9, 12 and 18 months of age. 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 2% paraformaldehyde (60 – 100ml). Brains were then removed from the skull. The obtained brains were cut into 4 – 5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the lateral entorhinal cortex (LEC; Yeh et al., 2011). The brain slabs were then post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1 M 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 0.1 M PB, pH 7.4 were collected and stored in a cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4 at -20 °C. Coronal brain sections at levels -2.30 mm/-3.88 mm (LEC) posterior to bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).
3. Immunohistochemistry

3.1 Antibodies

In all result chapters

Different antibodies were used to label astrocytes and β-amyloid (Aβ) either in the neuropil of neurones or occasionally in astrocytes. A monoclonal mouse antiserum against glial fibrillary acid protein (GFAP; Sigma-Aldrich, UK; G3893) was used to label astrocytes in the EC. To detect intraneuronal Aβ and extracellular Aβ plaques and aggregations as well as their relation with astrocytes, we used a monoclonal mouse antibody against the amino acid residues 1 – 16 of Aβ (Covance, Emeryville, CA, USA; SIG-39300). For detection of neurofibrillary tangles, we used a monoclonal mouse antiserum against paired helical filament (PHF)-tau, which recognises the phosphorylated serine 202/threonine 205 amino acid residues of tau protein (Innogenetics, Zwijndrecht, Belgium; BR-03). To study the glutamate-glutamine cycle in the EC, a monoclonal mouse anti-glutamine synthetase was used (Millipore; MAB 302). Table 2.1 summarises all the primary antibodies and their dilutions used:

Table 2.1 Summary of primary antibodies used and their respective sources

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Type</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ (6E10)</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:2,000</td>
<td>Covance, USA; SIG-39300</td>
<td>Oddo et al., 2003a</td>
</tr>
<tr>
<td>PHF</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:1,000</td>
<td>Innogenetics, Zwijndrecht, Belgium; BR-03</td>
<td>Oddo et al., 2003a Rodriguez et al., 2008</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:5,000</td>
<td>Sigma-Aldrich, UK; G3893</td>
<td>Wilhelmsson et al., 2004; Olabarria et al., 2010; Yeh et al., 2011</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>IgG fraction</td>
<td>1:5,000</td>
<td>Sigma-Aldrich, UK; G9269</td>
<td>Wilhelmsson et al., 2004; Olabarria et al., 2010; Yeh et al., 2011</td>
</tr>
<tr>
<td>GS</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:2,000</td>
<td>Millipore; MAB301</td>
<td>Wilhelmsson et al., 2004; Olabarria et al., 2011</td>
</tr>
</tbody>
</table>
3.2 Immunoperoxidase Labelling

*In Chapters 3, 4*

To minimise methodological variability, sections through the LEC (-2.3 mm/ -3.80 mm posterior to Bregma) containing both hemispheres of all animals were processed at the same time using exactly the same experimental conditions. The brain sections were incubated for 30 minutes in a solution containing 30% methanol in 0.1 M PB and 3% hydrogen peroxide (H$_2$O$_2$, Sigma, Gillingham, UK) to eliminate the endogenous peroxidase. The sections were then thoroughly rinsed with 0.1 M PB for 5 minutes. To remove the excess of the aldehyde groups, we incubated the sections in 1% sodium borohydride (Sigma, Gillingham, UK) in 0.1 M PB for 30 minutes. After washing them profusely with 0.1 M PB, brain sections were then rinsed with 0.1 M Tizma base saline (TS) for 10 minutes (TS, Sigma-Aldrich, UK), followed by incubation in 0.5% bovine serum albumin (BSA, Sigma-Aldrich, UK) and 0.25% Triton X-100 (Sigma, UK) for 30 minutes to prevent unspecific bindings. Subsequently, the sections were incubated in 0.1% BSA in 0.1 M TS at pH 7.6 and 0.25% Triton X-100 (facilitating permeabilization) containing the primary antibodies (Table 2.1) for 48 hours at room temperature. After the primary antibody incubation, the sections were rinsed with 0.1 M TS for 15 minutes twice and incubated with the secondary biotinylated horse anti mouse IgG antibody (1:200; Vector laboratories, Peterborough, UK) at room temperature for 1 hour. Then they were rinsed with 0.1 M TS for 30 minutes followed by an incubation in avidin-biotin peroxidase complex (Vector Laboratories, Peterborough, UK). Visualisation of this peroxidase complex was carried out by incubation in a solution containing 0.022% of the 3,3’diaminobenzidine chromogen (DAB; Aldrich, UK) in 0.003% H$_2$O$_2$, or SG peroxidase substrate kit (Vector Laboratories, Peterborough, UK) for 1.5-5 minutes. Sections were then dehydrated in ascending concentrations of ethanol (50, 70, 80, 90, 95 and 100%) followed by xylene and were then permanently coverslipped with Entellan (Merck KGaA, Germany).
3.3 Immunofluorescence Labelling

*In all result chapters*

As mentioned above, after reducing the excess of aldehyde groups and the potential non-specific labellings, sections were incubated in primary antibody solution (mouse anti GFAP; rabbit anti S100β; see Table 2.1) at room temperature for 48 h for the single immunofluorescence. The sections were rinsed in 0.1 M TS for 30 minutes and incubated in corresponding fluorochrome-conjugated secondary antibody solution for 1 h at room temperature (see Table 2.2 for the details of secondary antibodies used) and finally rinsed in 0.1M TS for 30 minutes. For dual immunofluorescence labelling, the sections were incubated in primary antibody cocktails containing (1) rabbit anti GFAP and mouse anti Aβ; or (2) rabbit anti GFAP and mouse anti GS 1:2,000; or (3) rabbit anti S100 and mouse anti Aβ (see Table 2.1) for 48 hours at room temperature. Subsequently, each antigen was detected in a sequential manner on the same brain sections by incubation with their correspondent fluorochrome-conjugated secondary antibodies (see Table 2.2).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Type</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Goat</td>
<td>FITC-Conjugated IgG</td>
<td>1:100‡</td>
<td>Jackson</td>
<td>Olabarria et al., 2010;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yeh et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Jackson Immunoresearch, USA;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>115-096-146</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Goat</td>
<td>Alexa Fluor® 594</td>
<td>1:400</td>
<td>Invitrogen, Paisley, UK;</td>
<td>Olabarria et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A11005</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Goat</td>
<td>Alexa Fluor® 568</td>
<td>1:100‡</td>
<td>Invitrogen, UK; A11011</td>
<td>Yeh et al., 2011</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Donkey</td>
<td>Alexa Fluor® 488</td>
<td>1:400</td>
<td>Invitrogen, UK; A21206</td>
<td>Yeh et al., 2011</td>
</tr>
</tbody>
</table>

| ‡ Dilution used for detection of GFAP in Chapter 3. |

Afterwards, in both single and dual of immunoflorescence labelling, the sections were rinsed with 0.1M TS for 30 minutes and then in 0.1M PB for 15 minutes. Finally, the sections were permanently mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).
4. Morphological Analysis of GFAP-IR and/or GS-IR Astrocytes

In all result chapters

GFAP- and/or GS-IR cells throughout all the layers of the EC were randomly selected for analysis (Fig. 2.1; for area definition, see Chapter 1). To analyse morphology of GFAP-positive astrocytes, GFAP-IR cells (n=30 in each animal, 3-7 animals in each genotype and age group) were imaged using confocal scanning microscopy (Leica SP5 upright), with Z-step size at 0.2 µm. Parallel confocal planes were superimposed by the Leica LAS AF (for images obtained by SP5) or Leica LCS Lite software (for images obtained by SP2) for a flat 3D reconstruction. The series of images were converted from TIFF to GIF format for morphological analysis. Morphological analysis of astrocytes was performed using Cell analyst programme as previously described (Chvatal et al., 2007b; Chvatal et al., 2007a). By using 5 digital filters (average 3x3, convolution, gauss 5x5, despeckle, simple objects removal), we determined the surface (S) and the volume (V) of astrocytes, both in the vicinity of and distant to Aβ plaques. When analysing astroglial morphology in relation to Aβ plaques, all cells with somata within 50 µm from the plaque border were considered as plaque-associated, and cell with somata located more distantly (> 50 µm) were considered as not associated with plaques (Olabarria et al., 2010). To analyse the cellular profiles of GS-IR (labelled by Rhodamine; red) and GFAR-IR astrocytes (labelled by FITC; green), cells showing co-localisation of GS and GFAP (n=15 in each animal) and GS-IR cell, which was not co-localised with GFAP (n=15 in each animal) were randomly chosen for imaging. In the former case, both GS-IR and GFAP-IR cells were imaged at the same time. Cells were imaged by using confocal microscopy (Leica SP2, Mannheim, Germany), with recorded sections taken at every 0.6 µm. Parallel confocal planes were superimposed and Cell analyst (Chvatal et al., 2007b; Chvatal et al., 2007a) was used for morphological analysis as mentioned above.
Figure 2.1 Diagram illustrating a representative level of the LEC (-3.28 mm posterior to Bregma) selected for analysis (A). Brightfield micrograph of Touluidine blue stained section showing the corresponding tissue at the same level (B). A (modified from Paxinos and Franklin, 2004), B (personal unpublished image).
5. Cell Count in the EC

In Chapters 3, 4

In order to minimise counting bias, cell counting of specific group of astrocytes was performed blindly by a single investigator (CYY). All slides were coded prior to the counting by other lab member (Markel Olabarria or Harun Noristani) and the codes were not revealed until the analysis was completed. To determine the numerical density ($N_v$; # of cells / $\mu m^3$) of GFAP-IR and GS-IR astrocytes in the EC, we counted the total number of GFAP-IR and GS-IR astrocytes throughout all layers of the whole LEC (Fig. 2.1; see level selection and layer definition in section 3.2 and 4), an volume of 1,740,000 - 260,000,000 $\mu m^3$ in 3 - 4 non-consecutive coronal brain sections. Confocal stack images were used in the case of fluorescent labelling of GFAP. GFAP-IR astrocytes were intensely labelled against dark background whereas GS-positive astrocytes were intensely labelled with brown colour and against light background, making them easy to identify with equal chance of being counted. All the analyses were performed blindly with regards to mouse genotype and age.
6. Optical Density (OD) Measurement

In Chapter 4

All optical density (OD) measurements were conducted by a single investigator (CYY). All slides were coded prior to the counting by another lab member (Harun Noristani) and the codes were not revealed until the analysis was finished. To analyse the expression of GS immunoreactivity, GS-labelled sections from both 3xTg-AD and non-Tg control mice from 1 to 12 months of ages were selected for OD measurements, as described previously (Noristani et al., 2010; Olabarria et al., 2011). The appropriate LEC level was identified and obtained at bregma level -2.30 mm/-3.88 mm according to the mouse brain atlas of Paxinos and Franklin (Paxinos & Franklin 2004), as described previously (Section 2.4.). The intensity of the OD of GS-IR was quantitatively measured using a Nikon Eclipse 80i microscope coupled with 8001 MicroFIRE camera and computer-assisted imaging analysis (Image J 1.32j, NIH, USA).

Generally, the processes of measuring OD includes the following steps: (1) Identifying the region of interest (ROI); (2) acquiring image with constant light intensity and saving images for OD measurement; (3) calibrating the optical density (for details, see chapter 2 supplementary material); (4) measuring OD; and (5) performing the subtraction of background and calculating the final Inverse optical density (IOD).

6.1 Identifying the region of interest (ROI)

The size and shape of the ROI (LEC) were determined carefully. LEC is dorsally bordered with perirhinal cortex and laterally bordered with external capsule, at bregma level – 2.30 mm/-3.88 mm (see Fig. 2.1 for a representative section). GS-IR cells were distributed evenly throughout the entire measured area (Fig. 2.2).

Figure 2.2 Brightfield micrograph showing GS-labelling within the LEC (the big area with black outline). The OD is measured in the whole LEC. External capsule (Ext Cap) was used for background OD measurements (small square) (personal unpublished image).
6.2 Acquiring and saving images for OD measurement

All images were taken using a Nikon Eclipse 80i microscope with a x4 objective. The ND8 and NCB11 optical filters were used to ensure consistent quality of all the images for analysis as well as to avoid optical artefacts such as incorrect colour balance and lamp voltage fluctuations. All the optical filters were kept the same to ensure the specificity of the signal recorded by the camera. Shift in light intensity can also affect the brightness of the image, hence affecting the obtained value of OD measurement. To exclude this experimental bias, all images were taken at a constant light intensity and magnifications.

All images were saved in TIFF format with a resolution of 1600 X 1200 pixels. These images were used to measure the OD.

6.3 Calibrating the optical density

Before measuring the OD with Image J, it is important to do calibration to adjust the difference in the level of black and incident light. The calibration was carried out by using standard step-tablet. By using a constant rectangular selection without overlapping between adjacent bands, specific mean gray value of each band was measured (Fig 2.3). Nineteen bands were measured for this purpose and the values obtained were at the range between 252.24 to 4.88, with the higher values indicating lighter areas (white), whilst the lower values indicating the darker areas (black). By saving all the values into calibration file, a calibration curve was generating automatically (see supplementary material for details; Fig. 2.4).

Figure 2.3 Standard step-tablet used for calibration.
6.4 Measuring the OD

The values obtained from OD measurements represent the mean gray values of all pixels in ROI. For colour images (RGB), the mean value was calculated by converting each pixel to gray scale using the formula \( \text{gray} = (\text{red} + \text{green} + \text{blue})/3 \), which was directly performed by the ImageJ programme. GS expression of the entire EC was measured independently each hemisphere.

6.5 Performing the subtraction of background staining

The background OD was determined from the external capsule (Ext Cap) that was considered as blank since GS labelling in this part is nearly absent (Fig. 2.2; Miyake and Kitamura, 1992). All images used for the background staining were obtained in parallel with the specifically labelled areas using an identical light intensity and exposure. The OD measurement of control region was applied with the same calibration curve for ROI.

To analyse the change in GS density against constant control, 255 (which is the digital component value that a single 8-bit bite photography can offer) was divided by control region (Ext Cap) and the obtained factor was multiplied by the OD value of ROI in every given section (Olabarria et al., 2011). Thus, the OD measurements were rescaled against the background, avoiding bias due to different immunoreactivity in control regions between different brain sections (Noristani et al., 2010; Olabarria et al., 2011).
OD_{constant control} = (255/Ext Cap) \times ROI

Where Ext Cap (external capsule), ROI (region of interest) and OD (optical density).

IOD was obtained by subtracting from the obtained background level, which was set at 255. Measurement of mean density were taken and averaged, after background subtraction, from both the left and the right hemisphere of each section. The final results are shown as inverse GS density (IOD/pixel).

IOD = 255 - OD_{constant control}

Where OD (optical density) and IOD (inverse optical density).
7. Cell Surface Measurement

In Chapter 6

To analyse the domain of GS-IR astrocytes, the cell surface area of GS-IR astrocytes (n=30 in each animal; 4-5 animals in each age group) were imaged with light microscopy using ROI Nikon Eclipse 80i microscope (x63 objective) coupled with a 8001 MicroFIRE camera. All GS-IR cells in molecular layer of DG and all the layers of CA1 subfields of the dorsal hippocampus at the level of -1.58 mm/-2.46 mm posterior to bregma were selected randomly for imaging and analysis (Fig 2.5A and B). The GS-IR cells throughout the entire LEC of animals in each age group were also chosen indiscriminately for analysis. The surface area of every single GS-IR cell was measured using a computer-assisted Image J 1.32j (NIH, USA; Fig. 2.5C).

Fig 2.5 Diagram illustrating a representative level of the dorsal hippocampus (-1.70 mm posterior to Bregma) chosen for analysis (A). Brightfield micrograph of Touluidine blue stained section showing the corresponding tissue at the same level (B). Brightfield micrograph showing a typical GS-IR and the its cell surface area (C; enclosed by black dots). A(modified from Paxinos and Franklin, 2004), B (adapted unpublished image from J.J. Rodríguez), C (unpublished image) Key:DG: dentate gyrus; GrL: granular cell layer; Lac Mol: lacunosum moleculare; Mol: molecular cell layer; PyL: pyramidal cell layer; SO: Stratum oriens; SR: stratum radiatum.
Chapter 2

8. Statistical Analysis

Student’s $t$ distribution, which is similar to normal distribution and adopted for small sample size, is used to determine sample size. Minimal $n=3$ were used in an experiment group (e.g., a specific genotype at a particular age) to have the degree of freedom larger than 5, resulting in proximate normal distribution. In Chapter 3, paired or unpaired $t$-tests were used to determine the differences in the $N_v$, surface, volume, and soma volume of GFAP-IR astrocytes in the EC between each experimental group (3xTg-AD vs. age-matched non-Tg). In Chapter 4, unpaired $t$-tests were used to determine the differences in the $N_v$ and IOD of GS-IR cells between 3xTg-AD and the age-matched non-Tg controls as well as for the surface and volume of GS-IR cells between the two genotypes at 12 months. Finally, in Chapter 5, unpaired $t$-tests were used to examine the morphological changes of GFAP-IR cells in the hippocampus as well as in the EC of non-Tg animals at two different ages. Unpaired $t$-tests were also used to examine the differences in GS-IR cell surface area in the hippocampus and EC of non-Tg animals between 3 months and 24 months of age. All data are expressed as mean ± standard error of the mean (SEM). All statistical analysis was performed with Graphpad Prism (Graphpad Software, USA). Significance was accepted at $p \leq 0.05$. 
Chapter 2S Supplementary Materials

1. Measuring Optical Density Using ImageJ

1.1 Open ImageJ:

File>
Open>
Desktop>
Graticules folder>
“Step tablet”

![Image of Step Tablet]

**Figure 2.S.1** Standard step-tablet for calibrating the system before OD measurement

1.2 Select rectangular tool

- Measure each band on the step tablet without overlapping the adjacent bands (Fig. 2.S.1).

- Analyse>
  
  Measure (or Ctrl + M)

- On approx band 10 adjust brightness to visualise remaining bands.

1.3 Open Excel:

File>
Open>
Chapter 2

Desktop>
Graticules folder>
“Calibration for density”.

- Copy column B.

In ImageJ:
Analyze>
Calibrate.

**Figure 2.S.2** Standard OD values obtained from calibration

- Paste data from column B into blank column on calibration table.
- Select global calibration to apply to all images.
- Save as calibration.
- Select OK. This opens a graph. Save as GRAPH.

**Figure 2.S.3** Standard OD calibration curve
1.4 Close graph and step tablet.

1.5 Measure samples.

**It is important to keep constant calibration density to prevent experimental variability. To reuse the same calibration:**

1) Copy your measurements into an Excel file. Delete excess info making sure that your values are in column A.

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

3) Save as your 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 images.
Chapter 3

Early Astrocytic Atrophy in the Entorhinal Cortex of a Triple Transgenic Animal Model of Alzheimer’s Disease

ABSTRACT

The entorhinal cortex (EC) is fundamental for cognitive and mnesic functions. Thus, damage to this area appears as a key element in the progression of Alzheimer’s Disease (AD), resulting in memory deficits arising from neuronal and synaptic alterations as well as glial malfunction. In this chapter, we performed an in depth analysis of astroglial morphology in the EC by measuring the surface and volume of the GFAP profiles in a triple transgenic (3xTg-AD) mouse model of AD. We found a significant reduction in both surface and volume of GFAP-labelled profiles in 3xTg-AD animals from very early ages (1 month) when compared to non-transgenic (non-Tg) controls (48% and 54%, reduction, respectively), which was sustained for up to 12 month, (33% and 45% reduction, respectively). Appearance of β-amyloid depositions at 12 month of age did not trigger astroglial hypertrophy; neither had it resulted in close association of astrocytes with senile plagues. Our results suggest that the AD progressive cognitive deterioration can be associated with an early reduction of astrocytic arborisation and shrinkage of astroglial domain, which may affect synaptic connectivity within the EC and between EC and other brain regions. In addition the EC seems to be particularly vulnerable to AD pathology because of absence of evident astrogliosis in response to β-amyloid accumulation. Thus, we can consider that targeting astroglial atrophy may represent a potential therapeutic strategy, which might slow down the progression of AD.
INTRODUCTION

The entorhinal cortex (EC), part of the temporal cortex, is involved in mnesic processes by establishing the cortico-hippocampal circuits. The EC is divided into superficial (I-III) and deep layers (IV-VI) that show differential anatomical and functional organisation (Suzuki and Amaral, 1994; Witter and Amaral, 2004). The superficial layers being the main recipient of intracortical information and the major output source to the hippocampus (HC), whereas the deep layers are mainly responsible for projections to cortical regions (Fig. 3.1; Suzuki and Amaral, 1994; Witter and Amaral, 2004). Neurones from the EC layer II terminate in the middle and outer molecular layer of the dentate gyrus (DG) and send collaterals to the hippocampal CA2 and CA3 subfields (Witter et al., 1989; Tamamaki and Nojyo, 1993; Suzuki and Amaral, 1994). Layer III neurones project mainly to the CA1 and the subiculum, which in turn, feedback to the layer V of the EC (Naber et al., 2001). Functionally, activation of the EC and persistent neuronal activity in the EC are involved in the process of working memory (Ranganath et al., 2003; Fransen, 2005; McGaughy et al., 2005). The EC is also essential for memory consolidation (Remondes and Schuman, 2004; Craig and Commins, 2005), spatial navigation and memory (Fyhn et al., 2004; Moser et al., 2008).

Entorhinal dysfunction is involved in several brain diseases that compromise cognitive and memory functions, including Alzheimer's disease (Braak and Braak, 1991; Kril et al., 2002; Schwarcz and Witter, 2002; Cunningham et al., 2006). Alzheimer's disease (AD) is an irreversible neurodegenerative disease accompanied with cognitive/memory impairments, which is the most common cause for progressive dementia in the elderly people (Braak and Braak, 1991; Braak et al., 1999; Harciarek and Jodzio, 2005). AD is characterised by appearance of senile plaques (SPs) representing extracellular amyloid β-peptide (Aβ) depositions (Nagele et al., 2004) and neurofibril tangles (NFTs) consisting of abnormally hyperphosphorylated tau protein, the latter being the major microtubule associated protein (Schneider and Mandelkow, 2008). The EC is affected very early by AD pathology: it is arguably the first brain region experiencing Aβ accumulation (Braak and Braak, 1991, 1997; Thal et al., 2000). Similarly NFTs appear in the transentorhinal region and in the EC at the early AD stages (Braak stages I/II) and are widespread in the EC at middle Braak stages (limbic
stages III/IV) (Solodkin et al., 1996; Braak and Braak, 1997). Concomitant with Aβ and NFTs presence in the EC, this cortical region undergoes severe volume decrease that also affects its major projection target, the HC (Du et al., 2001; Halliday et al., 2003; Whitwell et al., 2008). Profound neuronal loss, mainly in layer II, also occurs in AD concomitantly with mild cognitive impairment (Gomez-Isla et al., 1996; deToledo-Morrell et al., 2000; Du et al., 2001; Rodrigue and Raz, 2004).

**Figure 3.1** Illustration scheme of entorhinal inputs to hippocampus. In brief, the superficial layers (I-III) project to the hippocampal formation and subiculum while the deep layers (V/VI) receive reciprocal inputs from these areas. Meanwhile, deep layers also innervate to parahippocampal areas and hippocampus.

The AD-related neuronal loss and atrophy of the EC are well documented in human patients (Gomez-Isla et al., 1996; Calhoun et al., 1998; deToledo-Morrell et al., 2000; Du et al., 2001; Kordower et al., 2001; Ribe et al., 2005). However, little is known
about AD-associated changes in EC astroglia. Post-mortem studies have revealed an increase in GFAP protein levels as well as in the number of GFAP-positive astrocytes within the EC, which seems to be associated with the Aβ load (Muramori et al., 1998; Porchet et al., 2003). Recently we reported the concomitant occurrence of astroglial atrophy and astrogliosis in the hippocampus of transgenic mice model of AD. The atrophy appears as a generalised process, whereas the astrogliosis was triggered by developing SPs and Aβ aggregates (Rodriguez et al., 2009b; Heneka et al., 2010; Olabarria et al., 2010; Verkhratsky et al., 2010; Rodriguez and Verkhratsky, 2011). In the present chapter, we extended previous analysis of AD associated changes in astroglia to the EC.
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 3R’s.

Animal models

The generation of 3xTg-AD mice has been well described (Oddo et al., 2003a; Oddo et al., 2003b; Billings et al., 2005; Rodriguez et al., 2008; Rodriguez et al., 2009a; Rodriguez et al., 2009b). The 3xTg-AD mice are derived from mixed 129/C57BL6 mice, which harbour APPswe, PS1M146V, and TauP301L mutations, and closely resemble the same spatiotemporal progression of amyloid and tau pathology in human AD. The non-Tg control mice were from the same strain and the same genetic background. All 3xTg-AD and non-Tg littermates were from homozygous breeders. Mice were grouped by gender and genotype; housed under control of temperature and light-dark cycle (12:12 h) with given ad libitum access to food and water.

Fixation and Tissue Processing

As previously described (Olabarria et al., 2010; also see Chapter 2, section 2), male 3xTg-AD at 1, 3, 6, 9, 12 months of age (n = 5, 4, 4, 5, 5; respectively) and their age-corresponding non-Tg controls (n = 4, 4, 4, 5, 5; respectively) were anesthetised with sodium pentobarbital (50 mg/kg) intraperitoneally. Brains were fixed by aortic arch perfusion of 3.75% acrolein (25ml, TAAB, UK) in 2% paraformaldehyde (Sigma, UK) and 0.1 M phosphate buffer (PB) at pH 7.4 followed by 75 ml of 2% paraformaldehyde. Brains were removed and cut into 2 - 3 mm coronal slabs of tissue containing the lateral EC and was post-fixed in 2% paraformaldehyde for 24 hours and sectioned at 40 – 50 µm with a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating sections in 0.1 M PB 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 -2.30 mm / -3.88 mm (lateral entorhinal cortex, LEC) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).
Antibodies

To detect and determine the changes in astrocytic cytoskeleton in the EC, a monoclonal mouse antiserum against GFAP (anti-GFAP; Sigma-Aldrich, UK; #G3893) was used. For the identification of Aβ aggregation as well as the relation of astrocytic cytoskeleton alteration with Aβ, a polyclonal rabbit anti-GFAP antiserum (Sigma-Aldrich, UK; #G9269) and a monoclonal mouse antiserum against amino acid residues 1-16 of Aβ, which reacts with abnormally processed isoforms and precursor form of Aβ (EFRHDS; anti-Aβ 6E10 [SIG-39320], Signet Laboratories, Dedham, MA) were used. For detection of NFTs, a monoclonal mouse antiserum against paired helical filament (PHF)-tau, which recognises phosphorylated serine 202/threonine 205 of tau protein (Innogenetics, Zwijndrecht, Belgium) was used. The immunolabelling pattern obtained with this antibody is equivalent to that obtained previously in different brain regions (Oddo et al., 2003a). Positive and negative control immunohistochemistry was to test non-specific labelling and/or cross reaction between antisera derived from different host species, showing no immunoreactivity (data not shown). The specificity of these antisera was confirmed by immunohistochemistry and western blot in previous reports (Goedert et al., 1995; Halliday et al., 1996; Rodriguez et al., 2008).

Immunocytochemistry

The procedure for immunohistochemistry has been described previously (Olabarria et al., 2010; Noristani et al., 2010, see also Chapter 2, Section 3). All brain sections from both non-Tg and 3xTg-AD groups from different ages were processed at the same time using precisely the same experimental conditions to minimise methodological variability. Sections were pre-treated with 30% methanol, 3% hydrogen peroxides in 0.1M PB for 30 minutes and subsequently 1% sodium borohydride (Sigma-Aldrich, UK) solution for 30 minutes. After washed with PB profusely, sections were then rinsed with 0.1M Tiszma base saline (TS, Sigma-Aldrich, UK), followed by incubation in 0.5% bovine serum albumin (BSA, Sigma-Aldrich, UK) and 0.25% Triton X-100 (Sigma, UK) for 30 minutes. The brain sections were then incubated in 0.1% bovine serum albumin in 0.1 M TS at pH 7.6 and 0.25% Triton X-100 containing following antibodies: monoclonal mouse anti GFAP (1:5000; Sigma, Saint Loise, Missouri, USA), monoclonal mouse anti- Aβ (1:1000; Emeryville, CA, USA), or monoclonal mouse anti paired helical filament (PHF) tau (1:1000; Innogenetics, Zwijndrecht, Belgium) at room temperature (RT) for 48 hours (see also Chapter 2, Table 2.1). The section were rinsed
with 0.1M TS and incubated in biotinylated horse anti mouse IgG (1:200; Vector laboratories, Peterborough, UK) at RT for 1 hour. After rinsed with 0.1M TS for 30 minutes, sections were incubated in avidin-biotin peroxidase complex (Vector Laboratories, Peterborough, UK). The peroxidase reaction was carried out by incubation in a solution containing 0.022% 3,3′diaminobenzidine (DAB; Aldrich, UK) or 0.003% H$_2$O$_2$, or SG peroxidise substrate kit (Vector Laboratories, Peterborough, UK) for 5 min. Brain sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and subsequently xylene; and then permanently coverslipped with usage of Entellan (Merck KGaA, Germany).

For single fluorescent labelling, the brain sections were incubated for 48 hours at RT in the primary antibody solution as mentioned above. Sections were then rinsed with 0.1M TS and incubated in secondary solution containing fluorescein (FITC) conjugated goat anti-mouse IgG (1:100; Jackson Immunoresearch, Baltimore Pike, PA, USA) for 1 hour at RT.

For dual labelling, brain sections were incubated for 48 hours at RT in primary antibody cocktail containing: (1) monoclonal mouse anti- Aβ antibody (1:1000; Covance, Emeryville, CA, USA) and (2) rabbit anti-GFAP (1:5000; SIGMA, Saint Louise, Missouri, USA) simultaneously. Sections were washed with 0.1M TS and incubated in Rhodamine (TRITC)-conjugated goat anti-rabbit for the detection of GFAP, followed by rinsed with 0.1M TS. Subsequently, the sections were incubated in FITC-conjugated goat anti-mouse IgG (Invitrogen, Paisley, UK) for the detection of Aβ. All the brain sections were mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).

**Morphological analysis of astrocytic cytoskeleton**

GFAP-positive astrocytes (n = 30-35 in the single fluorescent labelling experiments) throughout all the layer of the LEC were imaged by using confocal microscopy (Leica SP5, Mannheim, Germany) with 0.2 µm z-step size. Parallel confocal planes were superimposed and the Cell analyst program (Chvatal et al., 2007) was used for analysis of astrocytic cytoskeleton morphology. 5 digital filters (average 3x3, convolution, gauss 5x5, despeckle, simple objects removal) with threshold at 50 were used to determine the surface and the volume of the GFAP-positive astrocytic cytoskeleton (see Chapter 2,
Section 4). When analysing astroglial morphology in relation to Aβ plaques, all GFAP-positive astrocytes with somata within 50 µm from the plaque border were regarded as plaque-associated, whereas cell with somata located more distantly (> 50 µm) from Aβ plaques were considered as cells not associated with plaques (Olabarria et al., 2010).

GFAP-Immunoreactive (GFAP-IR) cell count in the EC

To determine the numerical density (Nv) of GFAP-IR astrocytes in the EC, we counted the number of GFAP-IR astrocytes throughout the layers of the whole LEC, an area of 435,000 µm² in coronal sections with 40µm thickness in 3-4 representative non-consecutive sections. Confocal stack images were used for this purpose. GFAP-IR astrocytes were intensely labelled against dark background, which made them easy to identify with equal chance of being counted. The analysis was performed blindly by a single observed (Chia-Yu Yeh) with regards to mouse genotype.

Statistical analysis

Data are expressed as mean ± SEM. Paired or unpaired t-test was used to examine differences in the Nv, surface, volume, and somata volume of GFAP-labelled cells between the 3xTg-AD and non-Tg control animals, using Graphpad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Significance was considered at p ≤ 0.05.
RESULTS

GFAP immunoreactive (GFAP-IR) cells were widely distributed throughout the whole entorhinal cortex of both non-Tg and 3xTg-AD mice (Fig 3.2A-F) being layers I and VI the ones showing more GFAP-IR cells and stronger immunoreactivity (Fig. 3.2A-B); GFAP-IR showing multiple branched processes extending in different directions from elongated cell bodies (Fig 3.3D-G).

Astrocytic cytoskeletal atrophy in 3xTg-AD mice

From 1 month of age we observed morphological remodelling of EC astrocytes in 3xTg-AD mice when compared to non-transgenic animals. The astrocytes in the 3xTg-AD animals had less primary branches (on average by 26%) originating from the soma, markedly reduced presence of secondary processes extending from the primary branches (reduced by 30% on average), as well as distal ramifications (reduced by 45%; Fig 3.3D-G). These changes indicate a morphological atrophy which was further confirmed by a significant decrease in both surface and volume of GFAP profiles in the EC of 3xTg-AD animals when compared to non-Tg controls (Fig. 3.3A-B). At younger ages (1 month), astrocytes of 3xTg-AD mice showed a significant decrease in GFAP surface when compared to control. The surface area of GFAP-IR profiles was reduced by 48% (378.45 ±64.56 µm² vs. 728.47±96.67 µm²; p=0.017; Fig. 3.3A). The volume of GFAP-IR cells in 3xTg-AD was reduced by 54% (115.38±25.82 µm³ vs. 252.94±32.73 µm³; p=0.012; Fig. 3.3B). This atrophic glial appearance was sustained through all ages up to 12 months, (Fig. 3.3A-B). This reduction was significant at 3 and 6 months of age: surface area was decreased by 44% and 39% (378.56 ± 64.53 µm² vs. 680.20 ± 53.70 µm², p=0.036; 354.68 ± 67.74 µm² vs. 581.26 ± 70.13 µm², p=0.025 respectively; Fig. 3.3A). The volume of GFAP-IR cells decreased by 42% and 42% (130.25 ± 25.82 µm³ vs. 224.32 ± 18.80 µm³, p = 0.0490; 116.15 ± 25.63 µm³ vs. 200.64 ± 31.07 µm³, p=0.025, respectively; Fig. 3.3B). However, from 9 months of age onwards the degree of atrophic changes became somewhat less pronounced because of a parallel atrophic changes developing in healthy aged controls. At 9 months of age the surface and volume of astrocytes in 3x-Tg-AD animals was smaller compared to control by 26% (391.04 ± 30.75. µm² vs. 529.47 ± 48.21 µm², p=0.008) and 25% (130.92 ± 12.40 µm³ vs. 174.71 ± 15.41 µm³, p = 0.014), respectively (Fig. 3.3A-B). Likewise, at 12 months
of age the decrease in surface and volume was 33% (387.73 ± 47.75 µm² vs. 578.16 ± 84.46 µm²; p=0.021; Fig. 3.3B) and 45% (130.73 ± 14.93 µm³ vs. 200.13 ± 25.18 µm³, p =0.017) respectively (Fig. 3.3A-B).

These generalised atrophy was also confirmed by a parallel and significant decrease in the somata volume of GFAP-IR astrocytes at very early age (1 month) (41.63 ± 7.43 µm³ vs. 71.69 ± 7.68 µm³, p =0.027; Fig. 3.3C) when compared to control mice. However, at older ages, this decrease in somata volume persisted but became insignificant when compared with non-Tg due to a parallel decrease in non-Tg control animals.
Figure 3.2 Brightfield micrographs showing the distribution of GFAP-IR astrocytes in the EC of non-Tg and 3xTg animals (A and B respectively). Confocal Imaging showing organization of GFAP-IR astrocytes in the EC of non-Tg and 3xTg-AD animals at 1 Month (C and D respectively) and 12-months (E and F respectively) at higher magnification. Bar graph showing the numerical density (number of cells/mm³) of GFAP-IR cells in the EC of 3xTg-AD and non-transgenic controls (n=5,4,4,5,5, for 3xTg-AD mice at 1,3,6,9,12-months of age; n=4,4,4,5,5 for control at 1,3,6,9,12 months of age). (G). Bars represent mean ± SEM.
Figure 3.3 Bar graphs showing comparison of GFAP (A) surface (B) volume and (C) body volume in global EC at the age of 1, 3, 6, 9 and 12 months between 3xTg-AD (n=5,4,4,5,5, respectively) and non-transgenic animals (n=4,4,4,5,5, respectively). Bars represent mean ± SEM (*p ≤ 0.05 compared with age matched non-transgenic control); Confocal micrograph showing astrocytic atrophy in 3xTg-AD at 1 month (E) and 12 months (G) compared with control animals (D and F).
Layer specific astrocytic atrophy during AD

We found a significant decrease in GFAP-IR cell surface areas in layers II, III and VI of 3xTg-AD animals when compared to non-Tg controls from 1 month of age by 57% (323.57 ± 53.89 µm² vs. 769.28 ± 180.91 µm²; p= 0.035), 45% (305.36 ± 70.05 µm² vs. 564.42 ± 62.21 µm²; p= 0.031) and 56% (416.65 ± 87.62 µm² vs. 964.04 ± 118.84 µm²; p=0.007; Fig. 3.4A), respectively. This decrease in GFAP surface was also paralleled with a reduction of cell volume in all layers, a reduction in volume with 66% in layer II (84.78 ± 17.82 µm³ vs. 251.43 ± 65.01 µm³, p =0.028), 68% in layer III (59.57 ± 16.16 µm³ vs. 187.70 ± 26.64 µm³, p =0.004) and 71% in layer VI (132.08 ± 44.52 µm³ vs. 360.13 ± 38.71 µm³, p =0.0072 Fig. 3.4B). But we did not find any significant difference in other layers I and V.

However, at middle age (6 months), these atrophic changes in surface and volume occurred only in layers II and V. The surface area decreased by 38% (320.53 ± 71.52 µm² vs. 520.03 ± 79.62 µm², p=0.021) and 41% (386.35 ± 97.00 µm² vs. 650.50 ± 70.60 µm², p=0.004) in layer II and V respectively (Fig. 3.4C); the volume decreased by 45% (93.74 ± 24.85 µm³ vs. 169.19 ± 38.39 µm³, p =0.023) and 42%(130.43 ± 37.92 µm³ vs. 224.85 ± 34.16 µm³, p=0.011) in layer II and V respectively (Fig. 3.4D). However, no significant decline in either surface or volume was found in layer I, III and VI.

Finally, at older ages (12 months) the decrease in either surface or volume was restricted to the layer VI but had a similar significance. 3xTg-AD animals showed decreased GFAP surface by 43% (418.81 ± 53.84 µm² vs. 739.91 ± 56.20 µm², p=0.007) and volume by 49% (149.03 ± 23.85 µm³ vs. 290.03 ± 29.20 µm³, p=0.004; Fig. 3.4E-F).
Figure 3.4 Bar graphs showing decreased GFAP surface (A, C, E) and volume (B, D, F) within specific layer between 3xTg-AD animals at the ages of 1, 6, and 12 months respectively. Bars represents mean ± SEM (*p ≤ 0.05; **p ≤ 0.01 compared with age matched non-transgenic control; n=5,4,4,5,5, for 3xTg-AD mice at 1,3,6,9,12-months of age; n=4,4,4,5,5 for control at 1,3,6,9,12 months of age).
Chapter 3

Astrocytic atrophy in the EC is not associated with loss of GFAP-IR astrocytes

The density of astrocytes in the EC and in its different layers of 3xTg-AD mice when compared to non-transgenic control mice was constant at all ages (Fig. 3.2E). The distribution of GFAP-IR cells throughout the EC was also similar at all ages (Fig. 3.2).

Rare association of astrocytes with Aβ plaques and absence of hypertrophic reaction

The presence of intraneuronal Aβ accumulation in EC is evident from 9 months of age although plaques and extracellular aggregates are not present until 12 months of age (Fig. 3.5). GFAP-positive astrocytes with somata within 50 µm from the border of Aβ labeling border were regarded as around the Aβ burden (Fig. 3.5; Olabarria et al., 2010). However, notwithstanding Aβ accumulation, GFAP-IR astrocytes rarely appeared to be associated with Aβ-positive neurones, extracellular Aβ aggregates or SPs in 3xTg-AD animals. GFAP-IR cells located within the areas with 50- and 150-µm radius to the border of Aβ were randomly selected for studying the association of astrocytes and SPs. All in all, less than 5% of astrocytes (1 of 24 GFAP-IR astrocytes) were located at a distance < 50 µM to Aβ (Fig. 3.5). Most importantly, however we failed to observe any signs of hypertrophy in EC astrocytes in 3xTg-AD animals at all ages; astroglial cells always demonstrated atrophic morphology.

Figure 3.5 Confocal images of dual labelling of GFAP (red) and β-amyloid (green) showing GFAP-IR astrocytes are distant from around intracellular Aβ deposits (with a distance of 86 µm) at 12 months; and distant from the Aβ plaques (with a distance of 125 µm) at 18 months. (n=3 for transgenic animals at 12 months and n= 3 for transgenic animals at 18months) Arrowheads indicate astrocytes.
DISCUSSION

The EC is regarded as a relay station (sometimes identified as a funnel) for cortico-hippocampal information transfer and integration, playing an essential role in cognition and memory (Kerr et al., 2007; Canto et al., 2008; Coutureau and Di Scala, 2009). Here, for the first time we present the evidence indicating an early (from 1 month of age) atrophy of astrocytes in the EC of 3xTg-AD mice; this atrophy being sustained through more advanced ages (up to 12 months). This atrophic remodelling occurred in the superficial and deep layers at early and middle age (1 to 6 months), although at the later age (12 months) it remained only in the deeper layers of the EC. The morphological atrophy of astroglia is not accompanied by an astrocytic loss at all ages.

The generalised atrophy characterised by a major reduction of astrocytic primary branches and massive reduction of secondary and distal processes in the EC appears in very young 3xTg-AD animals (1 months old), which did not have yet any signs of AD pathology. This atrophic appearance remains in later ages (12 months) distinguished by developed Aβ plaques and NFTs, which are confirmed by immunoreactivity of Aβ and phosphorylated tau (See Supp. Fig. 3.1; (Oddo et al., 2003b; Rodriguez et al., 2009b). Consistent reduction in GFAP expression is in agreement with our previous observations of generalised atrophy of astrocytes in the hippocampus, which similarly appear before the neuropathological marks (from 9 months) and are sustained in later ages (up to 18 months; Rodriguez et al., 2009b; Olabarria et al., 2010). Although some post-mortem studies have revealed hypertrophic astrocytes, characteristic of astrogliosis in the EC of AD brains (Muramori et al., 1998; Porchet et al., 2003; Vanzani et al., 2005), this is just related to intralaminar astrocytes since interlaminar astrocytes also showed disrupted processes and dynamic properties (Colombo et al., 2002). Furthermore, microarray analysis also showed a decrease in gene transcription of astrocytic cytoskeleton proteins in AD, implying down-regulation of astrocytic cytoskeleton (Simpson et al., 2011). At the same time, the density of GFAP-IR cells remains constant at all ages in both non-Tg control and 3xTg-AD mice. This indicates that neither ageing nor AD pathology trigger astrocytic cell loss. These data again correspond to our previous study and argue against the prominent astrogial proliferative response in the 3xTg-AD mice (Olabarria et al., 2010).
Astrocytic atrophy in the EC occurred very early, at 1 month of age. This is somewhat similar to the general observation that the AD pathology as manifest by Aβ accumulation, which begins in the neocortex and rhinal cortices including the EC, subsequently progressing to the hippocampus and eventually appearing in all subcortical areas (Braak and Braak, 1997; Thal et al., 2000). The formation of neurofibrillary tangles initiates in the transentorhinal as well as entorhinal cortices, advancing to the hippocampus through the perforant path and neighbouring cortical regions (Braak et al., 1999). The spatiotemporal occurrence of astroglial atrophy is therefore consistent with pathological hallmarks of AD, indicating the EC is the region first affected by AD pathology. The evident changes in entorhinal astrocytes appear within layers II, III and VI at early age (1 month); layers II and V are affected at middle age (6 months), whilst astrocytic atrophy is restricted to the layer VI of the EC at late ages (12 months). Nevertheless, it should be noted that there is no recovery of atrophic astrocytes in transgenic mice in the superficial layers at later age but a decrease in GFAP in non-Tg controls, implying an aging effect on control animals (Fig. 3.3).

Astroglia sustains multiple functions, including balance of neurotransmitters, release of trophic factors, metabolic support, extracellular ion buffering all of which maintain brain physiology and support neuronal connectivity (Danbolt, 2001; Nedergaard et al., 2003; Wang and Bordey, 2008; Verkhratsky et al., 2010; Verkhratsky et al., 2011). In addition, astrocytes are able to sense synaptic activity, regulate synaptic plasticity and synchronise neuronal networks; being thus involved in conscience and cognitive processes (Araque et al., 1999; Fellin et al., 2004; Lalo et al., 2006; Henneberger et al., 2010; Lalo et al., 2011). Finally by virtue of astrogliosis, astrocytes form the innate brain defence system localising CNS lesions and assisting in pathological remodelling of affected circuitry (Sofroniew, 2009). Early astroglial atrophy observed in the EC may result in reduced astroglial coverage of synapses and thus may appear a key factor in altered synaptic connectivity (Rodriguez et al., 2009b; Verkhratsky et al., 2010). In consequence, the synaptic condition and/or activity may be affected, causing synaptic remodelling, altered synaptic connections and network activity. It is likely that this early astrocytic alteration leads to compromised EC output to other areas, especially the hippocampus, which receives dense innervations from both the superficial and deep layers of the EC (Fig. 3.1). In fact, there is no significant neuronal loss in memory-associated-areas, such as the EC and the hippocampus, in 3xTg-AD mice (Oddo et al.,
2003a; Oddo et al., 2003b; Rohn et al., 2008; Bittner et al., 2010). Nonetheless, loss of synapses and/or dendritic spines as well as compromised long-term potentiation have been detected in several brain regions, suggesting impaired synaptic connectivity and functions in this animal model (Oddo et al., 2003a; Bertoni-Freddari et al., 2008; Bittner et al., 2010; Noristani et al., 2011). Therefore, the astrocytic atrophy in the EC may underlie synaptic pathology being the basis of cognitive and memory impairment at the early stages of the AD.

In contrast to the hippocampus (Olabarria et al., 2010), arrival of Aβ depositions and senile plaques in the EC does not trigger astrogliotic reaction at least from the morphological criteria. We did not observe a close association of astrocytes with senile plaques, nor did we find any signs of astroglial hypertrophy. This peculiar indifference of EC astrocytes to the AD specific lesion may underlie the particular vulnerability of the entorhinal cortex to the AD-like pathology and provide an explanation of why EC is the first brain region to be affected in the course of AD. Obviously further studies are required to extend the findings from AD animal model to human pathology.
Supplementary Figure 3.1 Brightfield micrographs showing that there is no Aβ-IR in non-Tg mice at both 3 and 12 months (A and C) but an initial and gradual Aβ deposition in the EC of 3xTg-AD at 3 months, which further increases at 12 months (B and D, arrowheads). There is no neurofibrillary tangles (PHF-tau) in either non-Tg and 3xTg-AD mice at 3 months (E and F) or non-Tg at 12 months (G), however, an incipient presence of PHF-tau appears in the EC of 3xTg-AD at 12 months (H, arrowheads).
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REFERENCES


Chapter 3

Craig S, Commins S (2005) Interaction between paired-pulse facilitation and long-term potentiation in the projection from hippocampal area CA1 to the entorhinal cortex. Neuroscience research 53:140-146.


Naber PA, Lopes da Silva FH, Witter MP (2001) Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. Hippocampus 11:99-104.


Chapter 4

Astrocytes in the Entorhinal cortex of the Triple Transgenic Animal Model of Alzheimer’s Disease Show No Alterations in Glutamate-Glutamine Shuttle Components

ABSTRACT

Astrocytes are fundamental for brain physiology and pathology, including Alzheimer’s disease (AD). Among their functions, the maintenance of glutamate balance via the glutamate-glutamine (Glu-Gln) cycle is critical for both normal cognitive functions and excitotoxicity relevant for AD progression. Astroglial glutamine synthetase (GS), converting glutamate to glutamine, is a key element in the Glu-Gln cycle. The entorhinal cortex (EC) is the brain area earliest affected in human AD. We have recently reported an early astrocytic atrophy in the EC in triple transgenic animal model of AD (3xTg-AD). Here, we studied and analysed whether the changes in astrocytic morphology coincides with alterations of the Glu-Gln cycle by determining astrocytic GS. We found that the numerical density of GS-immunoreactive (GS-IR) cells as well as GS content (measured by optical density, OD) remained constant and unaltered from 1 to 12 months of age, independent of the presence of plaques and initial tangles. Dual labelling images revealed GS-IR, GFAP-IR, GS/GFAP-IR subsets of astroglia. In spite of the evident decrease in GFAP-IR surface and volume, the surface and volume of GS-IR cells were unchanged regardless of its co-localisation with GFAP. Therefore, astrocytic atrophy, which is critical in the progression of AD from early stages, does not affect the glutamate homeostasis in the EC of 3xTg-AD mice, suggesting distinct functional populations of astrocytes, which may show distinct responses during AD progression.
INTRODUCTION

Astroglia, among other glial cells, are the main cells responsible for CNS homeostasis, including neurotransmitter balance (Araque et al., 1999; Danbolt, 2001; Halassa and Haydon, 2010). In this sense, glutamate, the major excitatory neurotransmitter in the CNS, plays a fundamental role in synaptic formation, stability and plasticity, which are fundamental for cognitive and memory processes (Bliss and Collingridge, 1993; Riedel et al., 2003; Robbins and Murphy, 2006). However, anomalous accumulation of extracellular glutamate might induce excitotoxicity and therefore neurodegeneration (Olney and Ho, 1970; Arias et al., 1998). To prevent this, astrocytes take up the glutamate through glutamate transporters and convert glutamate to glutamine via the glutamate synthetase (GS) (Danbolt, 2001). Glutamine is then released from astrocytes and is retrieved by neurones for de novo synthesis of glutamate. This glutamate-glutamine (Glu-Gln) cycle makes astrocytes essential not only for glutamate balance but also for glutamatergic neurotransmission (Danbolt, 2001).

Disruption of glutamate-glutamine cycle has been suggested to cause synaptic dysfunction and neuronal loss in several neurodegenerative diseases, including Alzheimer’s disease (AD; (Walton and Dodd, 2007). AD is a progressively and irreversibly neurodegenerative disease, accompanied by cognitive and memory decline, being the major cause of elderly dementia (Braak and Braak, 1991, 1997). Abnormal accumulations of β-amyloid and tau protein in the brain parenchyma are the main pathological hallmarks associated with AD and show a specific temporospatial evolution in AD brains (Braak and Braak, 1991, 1997; Braak et al., 1999). The entorhinal cortex (EC), among the multiple brain regions with AD pathology, is the first area affected by AD (Braak et al., 1999).

The EC locates between the neocortex and the hippocampus. The EC functionally plays an important role in the integration and transfer of information flow to those cognitive regions. The superficial layers (I-III) of the EC receive highly processed sensory input from cortical regions, constituting the major input to the hippocampus, whilst the deep layers (V/VI) project heavily to cortical regions. The EC layer II neurones project to the middle and the outer molecular layer of the dentate gyrus (DG);
sending collaterals to the hippocampal CA2 and CA3 subfields (Witter et al., 1989; Tamamaki and Nojyo, 1993; Suzuki and Amaral, 1994; Yeh et al., 2011). Layer III neurones project mainly to the CA1 and subiculum, which in turn feedback to the layer V of the EC (Naber et al., 2001). In addition to its functionally anatomical role and organisation, EC neuronal activity is critical in spatial memory acquisition and consolidation (Fyhn et al., 2004; Hafting et al., 2005; McGaughy et al., 2005; Moser et al., 2008).

We have recently reported a concomitant atrophy and hypertrophy of astrocytes in the hippocampus at middle and late stages, whilst early and sustained astrocytic atrophy in the EC and the medial prefrontal cortex (mPFC) (Olabarria et al., 2010; Yeh et al., 2011; Kulijewicz-Nawrot et al., 2012b) in a triple transgenic (3xTg-AD) animal model of AD, which mimics the temporal and spatial progression of AD pathology (Oddo et al., 2003a; Oddo et al., 2003b). Therefore, it is of extreme importance to determine whether morphological alterations of astrocytes lead to functional disruption, especially changes in the Glu-Gln cycle, which is an essential component in the neuronal degeneration and synaptic dysfunction associated with AD. In this Chapter, we analysed general expression of GS, the key player in the Glu-Gln cycle in the EC during the progression of AD pathology. We also studied the association of GS with astrocytic atrophy by dual labelling of GS and GFAP and analysed the morphology of GS-IR cells with and/or without co-expression of GFAP.
MATERIAL AND METHODS

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

Animal models

The generation of 3xTg-AD mice has been well described previously (Oddo et al., 2003a; Oddo et al., 2003b; Billings et al., 2005; Rodriguez et al., 2008; Rodriguez et al., 2009; see also Chapter 2, Section 2.1). The 3xTg-AD mice are derived from 129/C57BL6 hybrid, with APPswe, PS1M146V, and TauP301L mutations, resembling human AD pathology. The non-Tg mice were from the same strain with same genetic background. All 3xTg-AD and non-Tg littermates were homozygous. Mice were grouped by gender and genotype; and housed under controlled temperature with a light-dark cycle of 12:12h having ad libitum access to food and water.

Fixation and Tissue Processing

As previously described (Olabarria et al., 2010), male 3xTg-AD at 1, 3, 6, 9, 12 months of age (n =5, 4, 5, 5, 5; respectively) and their age-matched non-Tg controls (n =4, 4, 4, 4, 5; respectively) were anesthetized with sodium pentobarbital (50 mg/kg) i.p. The brains were trans-cardially fixed with 3.75 % acrolein (25ml, TAAB, UK) in 2 % paraformaldehyde (PFA, Sigma, UK) and 0.1 M phosphate buffer (PB) at pH 7.4, followed by 75 ml of 2 % paraformaldehyde. Brains were removed and cut into 2 - 3 mm coronal slabs containing the entire rostro-caudal extension of the EC. The tissue was post-fixed in 2 % paraformaldehyde overnight and further sectioned in 40 – 50 µm with a vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating sections were collected in 0.1 M PB and stored in a cryoprotectant solution containing 25 % sucrose and 3.5 % glycerol in 0.05 M PB at pH 7.4. Coronal sections at levels -2.30 mm / -3.88 mm (lateral entorhinal cortex, LEC) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004).
Antibodies

A mouse antiserum generated against GS (anti-GS; Millipore, UK: MAB302) was used for the detection, determination and distribution of astrocytes containing GS. To detect and determine the changes in astrocytic cytoskeleton in the EC, we used a rabbit anti-GFAP IgG fraction of antiserum (sigma-Aldrich, UK; G9269) (See also Chapter 2, Table 2.1). The specificity of these antisera was confirmed by immunohistochemistry and western blot in previous reports (Eng et al. 2000; Rodriguez et al. 2008; Olabarria et al. 2010). Negative controls, either by omission of primary (or secondary antibodies) or specific peptide pre-incubation, showed complete absence of GS labelling (Olabarria et al., 2011).

Immunohistochemistry

All the sections were processed at the same time using precisely the same experimental conditions to minimize methodological variability (Noristani et al., 2010; Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2012a). Briefly, after pre-processing, the sections were incubated with primary antibody solution containing mouse anti-GS (1:2000, cat# MAB302, Millipore, UK) for 48 hours at room temperature (RT). The sections were then rinsed in 0.1 M TS and incubated in 1:200 dilution of biotinylated horse anti-mouse IgG (Vector laboratories, Peterborough, UK) for 1 hour at RT. After rinsed with 0.1 M TS, the sections were incubated for 30 minutes in an avidin-biotin peroxidase complex (Vetor Laboratories Ltd, Peterborough, UK). The peroxidase reaction product was visualized by incubation in a solution containing 0.022% of 3,3’diaminobenzidine (DAB, Aldrich, Gilligham, UK) and 0.003% H2O2 for 1.5 minutes as described previously (Olabarria et al. 2011). EC sections were dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and subsequently xylene; and permanently coverslipped with Entellan (Merck KGaA, Germany).

For dual fluorescent labelling, to detect GS and GFAP co-existence, the sections were incubated for 48 hours at RT in a primary antibody cocktail containing: (1) monoclonal mouse anti- GS antibody (1:2000; cat# MAB302, Millipore, UK) and (2) rabbit anti-GFAP (1:5000; SIGMA, Saint Louise, Missouri, USA) simultaneously. Sections were washed with 0.1M TS and incubated in Rhodamine (TRITC)-conjugated goat anti-mouse IgG (1:200) for the detection of GS, followed rinses with TS. Subsequently, the sections were incubated in FITC-conjugated goat anti-mouse IgG (1:100; Invitrogen,
Paisley, UK) for the detection of GFAP. All the sections were mounted in an aqueous medium (Vectashield; Vector laboratories, Peterborough, UK).

Optical Density (OD) measurement

To analyze the level of expression of GS-immunoreactive (GS-IR), we measured the OD of GS-IR using Image J (NIH, USA), as previously described (Noristani et al., 2010; Olabarria et al., 2011). Briefly, The staining was observed throughout the full thickness of the section (40 µm) using light microscopy (Nikon Eclipse 80i). No differences were observed in GS-IR throughout the thickness of the sections (Olabarria et al. 2011). The OD was determined from a relative scale of intensity ranging from 0 to 255, with a measurement of 255 corresponding to very low GS-IR and 0 corresponding to the maximum labelling. The calibration density was constant for all measurements to avoid experimental variances. Sections background OD was determined from the external capsule (Ext Cap) that was considered as blank since GS labelling in the Ext Cap is nearly absent (Miyake and Kitamura, 1992). GS density of the entire LEC was measured independently and blindly by single observer. To analyze the change in GS density against constant control, the 255 was divided by control region (Ext Cap) and the obtained factor was multiplied by the region of interest in every given section. Inverse optical density (IOD) was obtained by subtracting from the obtained background level (set at 255). The results are shown as inverse GS density (IOD/pixel).

GS-IR cell count in the LEC

To determine the numerical density (Nv; # of cells/ mm³) of GS-IR astrocytes in the EC, we counted the number of GS-IR astrocytes throughout the layers of the whole LEC, for a proximate volume of 260,000,00 µm³ in 3-4 representative non-consecutive sections. GS positive astrocytes were intensely labelled against lighter background that made them easy to identify with equal chance of being counted. The analysis was performed blindly with regards to mouse genotype and age.

Colocalisation and Morphological Analysis of GS-IR and GFAP-IR astrocytes

GS-IR and GFAP-IR astrocytes throughout all the layers of the entire LEC from non-Tg (n=5) and 3xTg-AD animals (n=4) at the age of 12 months were imaged by using confocal microscopy (Leica SP2, Mannheim, Germany) with 0.6 µm z-step size for
determination of co-localisation of GS-IR (in red) and GFAP-IR (in green) as well as the following morphological analysis. To analyse the cellular profiles of GS-IR and GFAR-IR astrocytes, GS/GFAP positive cells and GS positive cells (n=15 in each subset of each animal) were randomly chosen for imaging. In the former case, both GS-IR and GFAP-IR cells were imaged at the same time. Parallel confocal planes were superimposed to construct their 3-dimensional structures and the Cell analyst program (Chvatal et al., 2007) was used for analysis. 5 digital filters (average 3x3, convolution, gauss 5x5, despeckle, simple objects removal) with threshold at 50 were used to determine the surface and the volume of both GS-IR and GFAP-IR astrocytes.

**Statistical analysis**

Unpaired t-test was used to examine differences in the Nv, surface, volume, and somata volume of GS and/or GFAP labelled cells between the 3xTg-AD and non-Tg animals, using Graphpad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Significance was considered at p≤ 0.05. Data are expressed as mean ± SEM.
RESULTS

In both non-Tg control and 3xTg-AD animals, at different ages, GS-IR astrocytes were extensively present throughout the entire EC being evenly distributed in all of its layers (Fig. 4.1). The GS-IR cells displayed a round soma with some primary branches and few secondary processes extending radially (Fig. 4.2A-D).

Figure 4.1 Light photomicrograph showing similar distribution of GS-IR astrocytes in the EC of non-Tg control and 3xTg-AD groups at 3 months (A and B respectively) and 12 months (C and D respectively). Light phoromicrograph of toluidine blue staining showing lamination of the EC (insert A).

Comparable Astrocytic GS N_v and content in the EC of 3xTg-AD and non-Tg animals

The N_v of GS-IR cells in the EC of 3xTg-AD mice was similar to non-Tg control mice throughout different ages (Fig. 4.2A-E; 5856 ± 681.9 cells/µm³ vs. 7958 ± 903.6 cells/ µm³, p= 0.10 at 1 month; 7053 ± 643.0 cells/µm³ vs. 6831 ± 540.0 cells/ µm³, p= 0.080 at 3 months; 6595 ± 392.5 cells/µm³ vs. 5583 ± 370.5 cells/ µm³, p= 0.11 at 6 month; 6255 ± 453.1 cells/µm³ vs. 5601 ± 422.7 cells/ µm³, p= 0.34 at 9 months; 5646 ± 210.8 cells/µm³ vs. 5365 ± 786.1 cells/ µm³, p= 0.74 at 12 months). Similar to N_v of
GS-IR cells, the general GS expression, which was measured IOD, was stable in the EC when compared 3xTg-AD mice with non-Tg mice at all ages (Fig. 4.2A-E; 50.64 ± 6.93 vs. 60.23 ± 1.91, p=0.27 at 1 month; 66.83 ± 4.19 vs. 57.63 ± 0.73, p=0.07 at 3 months; 59.20 ± 5.13 vs. 70.90 ± 5.90, p=0.18 at 6 months; 64.53 ± 2.37 vs. 64.08 ± 3.82, p=0.92 at 9 months; 66.35 ± 4.95 vs. 50.48 ± 6.51, p=0.08 at 12 months).

Different populations of astrocytes in EC

Based on the immunoreactivity, we observed three subpopulations of astrocytes: GS-IR, GFAP-IR and GS/GFAP-IR cells (Fig. 3); GS-IR cells also showed a distinct morphology from GFAP-IR cells. The former showed intense labelling which clearly shaped the somata and processes whereas the later displayed the extension of primary and secondary processes from a less distinct cell body (Fig 3A, B, C and D). The GS/GFAP-IR cells exhibited co-localisation of GS and GFAP mainly in the primary process and to less extent in the somata (Fig 3E-F). GS-IR cells outnumbered GFAP-IR and GS/GFAP-IR cells in the EC (Fig 3G). We observed that GS-IR cells accounted for 54.4% of entire astrocytic population, GS/GFAP-IR for 23.4% and GFAP-IR cells for 22.0% in the EC of Non-Tg control animals (n = 4; Fig 3G). In the EC of 3xTg-AD animals, approximately 60.5% of the cells were GS-IR, 20.3% were GS/GFAP-IR and 19.2% were GFAP-IR astrocytes (n=3; Fig 3G).
Figure 4.2 Light micrograph illustrating similar GS-positive astrocytes in the EC of non-Tg controls and 3xTg-AD animals at 1 month of age (A and B, respectively) or 12 months of age (C and D, respectively). Higher magnification showing similar morphology of GS-IR astrocytes between non-Tg and transgenic animals at both 1 and 12 months of age (inserts A-D). Bar graphs illustrating GS-IR Nc (number of cells/µm, E) and GS-IR IOD (D). Bar represents mean ± SEM (n=5, 4, 5, 5, 5; respectively for 3xTg-AD at 1,3,6,9,12 months; n=4,4,4,4,5, respectively for non-Tg at 1,3,6,9,12 months).
Figure 4.3. Confocal micrographs showing three subsets of astrocytes, being GS positive (red; A-B), GFAP positive (green; C-D) and GS/GFAP positive (E-F) astrocytes in the EC of non-Tg control and 3xTg-AD animals (E-F). (G) Bar Graph showing estimated percentage of different astrocytic population in the EC expressing GS (red), GFAP (green) or GS/GFAP (yellow) in non-Tg (n=4) and 3xTg-AD (n=3) animals (G).
GS in GS- and GS/GFAP-IR astrocytes remain unchanged, whereas GFAP in GS/GFAP-IR astrocytes undergo atrophy in the EC of 3xTg-AD animals.

At the age of 12 months, GFAP-IR astrocytes in the EC of 3xTg-AD mice had evident signs of morphological atrophy, indicated by significant reduction in GFAP-IR surface and volume when compared to age-matched non-Tg control mice. The surface and volume were reduced by 50.6% and 41.8%, respectively (224.10 ± 23.16 vs 337.50 ± 22.44 µm², p=0.02; 68.69 ± 6.99 vs 97.39 ± 7.71µm³, p=0.03; Fig 4 A and C; Yeh et al. 2011). However, GS-IR cells remained unchanged, showing comparable surface (1611.4 ± 429.8 vs. 1742.2 ± 344.4 µm², p=0.81; and 1281.0 ± 371.3 vs 1702.6 ± 487.1 µm², p=0.51; respectively for single GS-labelling cells and GS/GFAP-IR cells; Fig. 4A-B) and volume (361.6 ± 148.6 vs 481.6 ± 107.5 µm³, p=0.52; and 474.5 ± 129.5 vs 496.5 ± 100.0 µm³, p=0.90; respectively for single GS-labelling cells and GS/GFAP-IR cells; Fig 4 C-D) when compared to non-Tg control mice.
Figure 4.4 Bar graphs illustrating comparison of GS or GFAP surface (A) and volume (C) of cells express both GS and GFAP; comparison GS surface (B) and volume (D) of cells only expressed GS.  Bar represents mean ± SEM (* p<0.05 compared to age-matched non-Tg controls; n=4 for 3x-Tg and n=5 for non-Tg controls).
DISCUSSION

In this Chapter, we confirmed a significant reduction in the GFAP surface and volume in the EC of 3xTg-AD mice, corresponding to our results reported in Chapter 3 revealing a generalised astrocytic atrophy in this region. In contrast GS-IR astrocytes have a relatively stable general expression and morphology, shown by unaltered Nv and IOD of GS-IR. Nevertheless, we found different subsets of astrocytes by dual immunofluorescent staining, suggesting their functional variability. Moreover, morphological analysis revealed comparable surface and volume of GS in GS- and GS/GFAP-IR cells in the EC of both 3xTg-AD and non-Tg groups.

Astrocytes are involved in the progression of AD (Rodríguez et al., 2009; Verkhratsky et al., 2010). Both astrocytic hypertrophy and atrophy have been observed in brain regions of AD patients and animal models (Muramori et al., 1998; Colombo et al., 2002; Porchet et al., 2003; Olabarria et al., 2010; Yeh et al., 2011; Kulijewicz-Nawrot et al., 2012b). These morphological changes may be associated with a decline of GS expression as well as deficiency in glutamate transporters due to the loss of astrocytic processes and inability to traffic glutamate transporters, and thus leading to impaired glutamate uptake by astrocytes and the consequent glutamate imbalance (Robinson, 2001; Hughes et al., 2004; Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2012a). However, our results showed comparable distribution and Nv of GS-IR cells as well as general GS expression (shown by IOD) in the EC of 3xTg-AD mice, suggesting preserved Glu-Gln cycle in the EC. This is in agreement with the reports indicating constant level or glutamate uptake in cortical regions of AD when compared with non-demented subjects (Mohanakrishnan et al., 1995; Beckstrom et al., 1999).

Surprisingly, by dual labelling of GS and GFAP we observed different subsets of astrocytes in the EC, being GS-IR, GFAP-IR and GS/GFAP-IR astrocytes. Our results correspond to a study suggesting GS- and GFAP- positive astrocytes could be complementary populations of astroglia (Robinson, 2000), although we did also reveal astrocytes expressing both GS and GFAP. This reflects astroglial heterogeneity within the same area, in accordance with the reports demonstrating heterogeneity of astrocytic identity and functions (Walz and Lang, 1998; Holthoff and Witte, 2000; Emsley and Macklis, 2006; Simpson et al., 2010; Verkhratsky, 2010; Verkhratsky et al., 2011)
This morpho-functional heterogeneity was further corroborated by in-depth confocal image analysis of GS, which showed unchanged surface and volume of GS-IR astrocytes. To further study whether the expression and intracellular distribution of GS are affected by changes in GFAP expression, we analysed the morphology of GS/GFAP-IR astrocytes. We found a significant reduction in GFAP surface and volume; again confirming the general atrophy of astrocytic cytoskeleton in the 3xTg-AD mouse model of AD (Olabarria et al., 2010; Yeh et al., 2011). However, there was no significant difference in GS surface and volume of GS/GFAP-IR astrocytes between transgenic and non-Tg groups. Our results indicate that there is, at least, no direct effect of GFAP atrophy on intracellular distribution of GS in GS/GFAP-IR astrocytes in the EC.

In addition to intra-regional heterogeneity in astrocytes, astrocytes from different regions are similarly heterogeneous in their response to disease (Emsley and Macklis, 2006; Zhang and Barres, 2010). For instance, in the same 3xTg-AD animal model, most of the hippocampal astroglia express both GS and GFAP and show reduction in these two proteins at late age. Three subsets (GS-IR, GFAP-IR and GS/GFAP-IR) of astrocytes were also reported in the mPFC with GFAP atrophy throughout all stages of AD with decreased GS expression at early and middle ages (Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2012a). The differences in the subpopulation based on the immunolabelling and changes of GS-IR and GFAP-IR astrocytes in distinct areas correspond to the study by Hill and colleagues (Hill et al., 1996) that demonstrated astrocytes in different regions had different temporal responses to traumatic brain injury by displaying distinct morphology and gene expression at different time points. Therefore, heterogeneity of astrocytic responses in different brain regions may define the susceptibility and vulnerability of neurones in pathology (Zhang and Barres, 2010).

As mentioned above, there are different populations of astrocytes with distinct functional properties; it is important to study what the consequent effects of GFAP atrophy may bring. Each single astrocyte in rodent cortex covers around 90,000 synapses, hence decreased GFAP-IR level in our study may suggest reduced astrocytic processes and the synaptic coverage leading to compromised ability of astrocytes to control extracellular level of K+ and/or neurotransmitters as well as diminished support to local active synapses (Walz, 2000; Oberheim et al., 2006; Nedergaard and
Indeed, there is evidence revealing association of decreased GFAP labelled endfeet with decreased $K^+$ channel expression of astrocytes in transgenic animal models of AD (Oberheim et al., 2006; Wilcock et al., 2009). Recent studies also indicated that reduction in astrocytic cytoskeleton may be accompanied with a decrease in gap junction proteins, such as Cx43, suggesting a decline in astrocytic connectivity and compromised function for spatial ion buffering (Walz, 2000; Simpson et al., 2011). The decreased astrocytic communication and ion buffering ability might cause neuronal hyperactivity in the EC in 3xTg-AD mice (Arsenault et al., 2011). Therefore, alteration in astrocytic cytoskeleton might result in decreased astrocytic connection and maintenance and subsequent modulation on synaptic and network activity, which are critical for information processing in cognition (Rodriguez and Verkhratsky, 2011).

Different subsets of astrocytes in the EC implicate heterogeneous astrocytic identity and functional properties within this region. Moreover, these astrocytes may react distinctly to the disease. The stable expression of the critical component of Glu-Gln cycle, GS, in the EC features differences from mPFC and hippocampus, implying distinct regions exhibited diverse astrocytic responses during AD progression. Furthermore, due to the capability of astrocytic coverage over numerous synapses, it is likely the astrocytic atrophy in the EC might cause dysregulation of astrocytes on synaptic network, underlying the basis of cognitive deficit in AD.
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REFERENCES


Naber PA, Lopes da Silva FH, Witter MP (2001) Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. Hippocampus 11:99-104.


Chapter 5

Region Specific Ageing of Astrocytes: Hippocampal Hypertrophy and Enthorhinal Cortex Atrophy.

ABSTRACT

This chapter presents the studies of morphological ageing of astrocytes in entorhinal cortex (EC) and dentate gyrus (DG) and Cornu Ammonis 1 (CA1) regions of hippocampus of male SV129/C57BL6 mice of different age groups (3, 9, 18 and 24 months). Astroglial profiles were visualised by glial fibrillary acidic protein (GFAP) or glutamine synthetase (GS) staining, which were imaged using confocal and light microscopy for subsequent morphometric analysis. GFAP positive astrocytes in the DG and the CA1 of the hippocampus showed progressive age-dependent hypertrophy. At 24 months, astrocytes in both hippocampal areas were markedly hypertrophic, as indicated by a great increase in surface, volume and somata volume when compared to 3-month-old mice. However, GS positive astrocytes displayed smaller cellular surface areas at 24 months when compared to 3 month old animals. In contrast to the hippocampal regions, ageing induced a morphological atrophy of GFAP positive astrocytes in the EC: the surface, volume and cell body volume of astroglial cells at 24 months of age were decreased significantly when compared with 3 month group. Nonetheless, GS positive astrocytes in the EC of old mice did not undergo significant morphological change, as shown by constant GS cellular area when compared with 3-month-old mice. Based on the result of morphological analysis of two astroglial markers, we conclude that astrocytes undergo age-dependent morphological remodelling in a region-specific manner, without any change in the glutamate-glutamine shuttle.
INTRODUCTION

Astrocytes are the main homeostatic cells of the central nervous system. Astroglia control brain homeostasis at all levels including for example organ homeostasis (astrocytes control the emergence and maintenance of the blood-brain barrier), cellular homeostasis (astroglia/radial glia are stem elements for embryonic and adult neurogenesis), morphological homeostasis (astrocytes define nervous system micro-architecture and control synaptogenesis), molecular homeostasis (astrocytes regulate ion, neurotransmitter and neurohormone concentrations in the nervous tissue), metabolic homeostasis (astrocytes supply neurones with energy substrate), and defensive homeostasis (represented by astrogliosis) (see Kimelberg and Nedergaard, 2010; Parpura et al., 2012 for recent reviews). Astrocytes also play a central role virtually in all forms of neuropathology, determining, to a large extend, the progression and outcome of neurological diseases (Giaume et al., 2007; Rodriguez et al., 2009; Verkhratsky et al., 2012).

It is a general consensus that physiological brain ageing is not associated with substantial changes in the number of neurones in the most of the brain areas (Turlejski and Djavadian, 2002; Toescu and Verkhratsky, 2007). Much less is known about ageing of astroglia; it is almost universally believed that ageing is associated with astroglial proliferation and increase in expression of glial fibrially acidic protein (GFAP), which both are considered to be signs of reactive astrogliosis (Schipper, 1996; Unger, 1998; Cotrina and Nedergaard, 2002; Lynch et al., 2010). Morphological analysis of aged astrocytes however is mainly confined to hippocampal regions; in other parts of the CNS both increases (Cotrina and Nedergaard, 2002; Wu et al., 2005) and decreases (Nishimura et al., 1995; Cotrina and Nedergaard, 2002; Wu et al., 2005; Lasn et al., 2006; Mansour et al., 2008) in astroglial density at old ages were documented. In addition, to cytoskeletal changes, ageing effects on astrocytic functions in glutamate homeostasis such as glutamate uptake and/or expression/activity of glutamate synthetase (GS) were also studied. However, there were no consistent due to different experimental designs and methodology (Goss et al., 1991; Gottfried et al., 2002; Wu et al., 2005; Garcia-Matas et al., 2008). Astrocytes are highly heterogeneous in their morphological features and physiological properties and therefore they may react differently to the ageing process. In this Chapter, we report differential effects of ageing
on morphological characteristics of GFAP –positive and GS-positive astrocytes in the hippocampus and in the entorhinal cortex (EC) of mice.
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. Experimental techniques have been described elsewhere (Rodriguez et al., 2008; Olabarria et al., 2010, 2011; Yeh et al., 2011). Briefly, experiments were performed on male SV129/C57BL6 mice of different age groups (3, 9, 18 and 24 months; n = 3 - 7), which were anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Mice were perfused through the aortic arch with 3.75% acrolein (25 ml, TAAB, UK) in 2% paraformaldehyde (PFA, Sigma, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% PFA (75 ml). Brains were removed and cut into 4 - 5 mm coronal slabs consisting of the entire rostrocaudal extent of the hippocampus and the EC, and were then post-fixed in 2% paraformaldehyde for 24 hours and kept in 0.1 M PB, pH 7.4 (for details, see chapter 2, section 2). Coronal sections of the brain were cut into 40 – 50 µm with vibrating microtome (VT1000S, Leica, Milton Keynes, UK). Free floating 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 sections at levels -1.58 mm/-2.46 mm (hippocampus) and -2.30mm/-3.88mm (EC) posterior to Bregma, were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2004). GFAP and GS immunostaining was performed using a specific monoclonal mouse antisera against GFAP (anti-GFAP; Sigma-Aldrich, UK; #G3893) and GS (mouse anti GS; Millipore, UK; MAB302) respectively.

GFAP positive astrocytes were imaged using confocal microscopy (Leica SP5 upright). Parallel confocal planes were superimposed and morphological analysis was carried out by Cell analyst (Chvatal et al., 2007) using digital filters (average 3x3, convolution, gauss 5x5, despeckle, simple objects removal) to determine the surface (S), volume (V) and somata volume of GFAP positive astrocytes in the DG and CA1 subfields of the hippocampus as well as the entire EC. GS positive astrocytes were imaged using light microscopy (Nikon Eclipse 80i microscope) coupled with a 8001 microFIRE camera. The cellular areas of GS positive astrocytes were measured using Image I 1.32j (NIH, USA).
Unpaired $t$-test was used to examine differences in surface, volume and somata volume of GFAP-labelled cells at different age groups as well as the differences in surface area of GS-positive cells at 3- and 24-months old. Data is expressed as mean ± SEM.
RESULTS

GFAP positive astrocytes showed a characteristic stellate shape and multiple branched morphology (Fig. 5.1A-1L), although there are clear differences between the hippocampal and EC. Hippocampal astrocytes produce many primary processes with numerous secondary processes, all of them extending radially (Fig. 5.1A-1H). Entorhinal astrocytic GFAP profile is less branched showing very little secondary processes (Fig. 5.1I-1L). Similarly, distribution of astrocytes differed between the two brain regions. Astrocytes in the hippocampus covered the whole parenchyma with the pyramidal cell layer and granular cell layer being the only exception, where fewer astrocytes are present. In the EC the presence of GFAP positive astrocytes was less prominent.

Figure 5.1. Representative confocal 3-dimensional reconstructed images showing GFAP-IR astrocytes in the DG, CA1 and EC of animals at 3 months (A, E and I), 9 months (B, F and J), 18 months (C, G and K) and 24 months of age (D, H and L), respectively.
Chapter 5

GS positive astrocytes displayed small round cell bodies with primary branches and few secondary processes extending randomly and radially (Fig 5.2). In hippocampal subfields, DG and CA1, the distribution of GS positive astrocytes was similar to GFAP positive astrocytes, being widely present in the whole regions despite few GS positive astrocytes were found in the pyramidal and granular cell layers. In the EC, GS positive astrocytes were evenly and extensively distributed throughout the entire region whilst only partial GS-IR cells co-localised with GFAP labelling (see Chapter 4 for detail).

Astrocytes in the DG showed a progressive age-related hypertrophy

GFAP-positive astroglial profiles in DG were similar at 3 and 9 months of age (Fig. 5.1A-B; 5.3A-C). At 18 months of age we detected a significant increase in GFAP surface by 78.56% (1793.84±138.53 µm² vs. 1004.64±174.88 µm², p=0.0228), volume by 95.89% (665.95±64.18 µm³ vs. 339.96±70.83 µm³, p=0.0225), somata volume by 128.14% (274.03±25.90 µm³ vs. 120.11±28.19 µm³, p=0.0107) when compared to mice at 3 months (Fig. 5.1A and C; Fig. 5.3A-C). The increment was still evident when compare with 9 months of age, being the increase in GFAP surface by 83.41% (1793.84±138.53 µm² vs. 978.03±165.58 µm², p=0.0178), volume by 104.99% (665.95±64.18 µm³ vs. 324.87±68.97 µm³, p=0.0184) and cell body volume by 115.25% (274.03±25.90 µm³ vs. 127.31±31.32 µm³, p=0.0222) (Fig. 5.1B-C; Fig. 5.3A-C). At 24 months of the age a marked hypertrophy developed as evidenced by a manifest increase of surface by 390.70% and 404.05% (4929.75±1353.56 µm² vs. 1004.64±174.88 µm², p=0.007; 4929.75±1353.56 µm² vs. 978.03±165.58 µm², p=0.0034), volume by 404.43% and 427.87% (1714.89±492.87 µm³ vs. 339.96±70.83 µm³, p=0.0088; 1714.89±492.87 µm³ vs. 324.87±98.97 µm³, p=0.0044) and somata volume by 291.16% and 269.06% (469.85±130.96 µm³ vs. 120.11±28.19 µm³, p=0.0127; 469.85±130.96 µm³ vs. 127.31±31.32 µm³, p=0.0092) when compared to 3- and 9- month-old, respectively (Fig. 5.1A-B, C; Fig. 5.3A-C). When compared with later age, 18 months, there was also a clear but statically insignificant increase in surface by 174.81%, volume by 157.51%, and somata volume by 71.46% (Fig. 5.1C-D; Fig. 5.3A-C).

Astrocytes in CA1 showed a progressive age-related hypertrophy

An increase in astroglial GFAP profiles in the CA1 region was detected throughout the ageing process. At 9 months of age there was already an increase in GFAP surface
by 206.27% (1278.63±236.02 µm² vs. 417.48 ±57.48 µm², p=0.0098), volume by

286.56% (428.65±98.27 µm³ vs. 110.89 ±18.49 µm³, p=0.0141) and cell body volume

Figure 5.2 Light micrograph showing morphology and cell area of GS positive astrocytes in the DG, CA1 and EC of 3-month-old mice (A, C and E, respectively) and 24-month-old (B, D and E, respectively).
by 266.96% (165.54±40.22 µm³ vs. 45.11 ±5.46 µm³, p=0.0174) when compared to 3 months of age (Fig. 5.1F; Fig. 5.3D-F). At 18 months of age GFAP surface, volume and somata volume increased by 75.75% (2247.19 ±120.76 µm² vs. 1278.63±236.02 µm², p=0.0217), 113.32% (914.41 ±58.88 µm³ vs. 428.65±98.27 µm³, p=0.0133), and 108.29% (344.81±7.26 µm³ vs. 165.54±40.22 µm³, p=0.0118) respectively when compared to 9 months of age (Fig. 5.1F-G; Fig. 5.3D-F). The difference between 18 months and 3 months was more prominent; increased in surface by 438.27% (2247.19 ±120.76 µm² vs. 417.48 ±57.48 µm², p<0.0001), volume by 724.62% (914.41 ±58.88 µm³ vs. 1278.63±236.02 µm³, p<0.0001), and cell body volume by 664.34% (344.81±7.2 µm³ vs. 1278.63±236.02 µm³, p<0.0001) (Fig. 5.1E and G; Fig. 5.3D-F). The most significant change was detected at 24 months of age with an increase of surface by 1203.91% (5443.59 ±1641.09 µm² vs. 417.48 ±57.48 µm², p=0.0338), volume by 2209.98% (2561.52 ±843.14 µm³ vs. 1278.63±236.02 µm³, p=0.0407), and cell body volume by 1580.31% (758.03± 234.81 µm³ vs. 45.11 ±5.46 µm³, p=0.0349) (Fig. 5.1E and H; Fig. 5.3D-F). When compared mice at 9 and 18 months, the increase in GFAP surface, volume, and somata volume, was also apparent but not statistically significant (Fig. 5.1F-H, Fig. 5.3D-F).

Astrocytes in EC showed progressive age-dependent atrophy

In contrast to the hippocampus astrocytes in the EC showed a slight decrease in GFAP profiles already at middle age (9 months) (Fig. 5.1J, Fig. 5.3G-I). The entorhinal astrocytes in aged animals (18 and 24 months) display a morphological atrophy, corroborated by reduction in GFAP surface and total and somatic volume (Fig. 5.1K-L, Fig 5.3G-I). At 18 months of age, the astrocytes showed a significant decline in GFAP surface by 40.28% (680.2±53.70 µm² vs. 406.3 ±9.30 µm²; p=0.0076), volume by 37.76% (224.3.32±18.80 µm³ vs. 139.6±4.06 µm³; p=0.0131), and body volume by 55.24% (60.25±4.60 µm³ vs. 38.81±1.17 µm³; p=0.0117) when compared with the EC of young animals (3 months) (Fig. 5.11 and 1K, Fig. 5.3G-I); at 24 months of age, astrocytes in the EC show further reduction in GFAP surface by 40.68% (680.2±53.70 µm² vs. 403.6 ±9.31 µm²; p=0.0121), volume by 42.22% (224.3.32±18.80 µm³ vs. 129.6±12.15 µm³; p=0.0149), and body volume by 47.99% (60.25±4.60 µm³ vs. 31.33±2.63 µm³; p=0.0044) when compared with mice at 3 months (Fig. 5.1I and 1L; Fig. 5.3G-I); meanwhile, when compared with mice at 9 months, the aged mice (18 and 24-months) also showed a significant reduction in GFAP body volume by 27.24% (53.34±3.67 µm³ vs. 38.81±1.17 µm³; p=0.0265) and 41.26% (53.34± 3,67um³ vs. 31.33±2.63 µm³; p=0.0044).
31.33±2.63 µm³; p=0.0058) respectively (Fig. 5.1J-L; Fig. 5.3G-I).

Figure 5.3. Bar graphs showing the regional comparisons of GFAP surface, volume, and soma volume in the DG (A-C), CA1 (D-F) and EC (G-I) across ages. Bars represent mean ± SEM (*p ≤ 0.05; **p≤0.01; ***p≤0.001 compared with 3 months of age; † p≤0.05; †† p≤0.01 compared with 9 months of age; in DG, N=6, 7, 3 and 4 for 3, 9, 12 and 18 months, respectively; in CA1, n=4, 3, 3, 4 for 3, 9, 12 and 18 months, respectively; in EC, n=4,5, 3, 3 for 3, 9, 12 and 18 months, respectively).
Astrocytes in the DG and CA1 showed age-dependent decline in GS domain

GS-positive cell area was significantly decreased by 6.64% in the DG at 24 months when compared with 3 months (82.19±18.21 µm² vs. 916.97 ±18.23 µm²; p=0.0471; Fig. 5.2A and 2B; Fig. 5.4A). Similarly, a manifest decline in GS-positive cell area by 6.2% in the CA1 was detected at 24 months when compared with 3 months (1053.70±6.25 µm² vs. 988.73 ±19.26 µm²; p=0.0235; Fig. 5.2C and 2D; Fig. 5.4B).

Astrocytes in the EC showed constant GS domain during ageing

In contrast to the hippocampal regions, entorhinal astrocytes displayed constant GS domain, shown by similar GS-positive cell areas at 24 months when compared to 3 months (851.52±22.90 µm² vs. 806.27 ±14.28 µm²; Fig. 5.2E and 2F; Fig. 5.4C)
Figure 5.4. Bar graph showing regional comparison of GS-positive cell area in the DG (A), CA1 (B) and EC (C) at 3 and 24 months of age (* p \leq 0.05 compared with 3 months of age; in DG, N=4 for both 3 and 24 months; in CA1, n=4 and 5 for 3 and 12 months, respectively; in EC, n=4 for both 3 and 12 months).
There is a general belief that ageing coincides with an increased astroglial reactivity manifested by up-regulation of GFAP expression and astroglial hypertrophy (Kohama et al., 1995; Nichols, 1999; Cotrina and Nedergaard, 2002). This is regarded as a sign of astrogliosis and is often interpreted as a manifestation of mild neuro-inflammation that compromises the cognitive performance in old age. This, however, can be an oversimplified point of view. Astrogliosis is essentially a defensive reaction, represented by complex continuum of morpho-functional remodeling of astroglia aimed at localising the brain damage and assisting neuronal survival and regeneration of connectivity in neuronal networks (Sofroniew, 2009; Verkhratsky et al., 2012). The opposite reaction, the astroglial degeneration and atrophy can bear significant pathological relevance, as atrophic astrocytes reduce support of neuronal networks and may be fundamental for reducing neuronal connectivity. Indeed, astroglial atrophy has been detected at the early stages of various neurodegenerative diseases including for example Alzheimer’s disease, amyotrophic lateral sclerosis and Wernicke encephalopathy (see Rossi et al., 2008; Rossi and Volterra, 2009; Heneka et al., 2010; Verkhratsky et al., 2010; Rodriguez and Verkhratsky, 2011). In this Chapter we report reduced astroglial appearance in aged EC, which may indicate reduced astroglial support for neuronal networks. Ageing is associated with cognitive decline and it correlates with hippocampal alterations (Barnes, 1979, 1988) and structural atrophy of the EC (Rodrigue and Raz, 2004; Du et al., 2006; Raz et al., 2008; Raz et al., 2010). In addition, several studies reported atrophy in perforant path and reduced synaptic connections between EC and hippocampus play the key role in memory impairment in during ageing (Scheff et al., 2006; Stranahan and Mattson, 2010). These changes may reflect this reduced astroglial support.

Astrocytes are known to play the major role in glutamate homeostasis via glutamine-glutamine cycle. GS is specifically expressed in astrocytes, and covert glutamate, which is taken up by astrocytes, to glutamine. Glutamine is then released by astrocytes and transported to neurons, serving as a material for glutamate synthesis. Thus, GS is essential in the glutamate balance. In this Chapter, we report a decreased GS-positive cellular domain in the DG and CA1 whereas a constant GS-positive cellular areas in the EC. Together with GFAP hypertrophy, our results in the hippocampus are consistent
with the study indicating coincident increase in GFAP and down-regulation in GS (Ortinski et al., 2010), and thus implying an inverse correlation of GFAP and GS expression (Weir and Thomas, 1984). On the contrary, the unaltered GS-positive cellular domain in the EC during ageing is in agreement with the evidence revealing constant GS expression in the cortex of aged brain (Goss et al., 1991; Wu et al., 2005). It is of note that most of the hippocampal astrocytes express both GS and GFAP (Olabarria et al., 2011). Nevertheless, three subsets of astrocytes were identified by dual labeling of GFAP and GS (GS-positive; GFAP-positive; GS- and GFAP-positive) in the EC (see Chapter 4 for details). The regional-dependent alterations in GS-positive cell areas during ageing in this Chapter further suggest different ageing effects on the role of astroglia in glutamate-glutamine cycle.

We demonstrated here that age-dependent remodelling of astroglia and functional alterations are very different in different brain regions. This further confirms astroglial heterogeneity and calls for detailed regional mapping of age-dependent morphological and functional changes in astroglia.
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Disclosure Statement
All the authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence the present work. We also confirm that all animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the license from the Home Office.
REFERENCES


Chapter 5


Chapter 6

General Discussion
1. General Overview

As introduced in the previous chapters, the EC has a fundamental role in brain mnesic and cognitive functions due to its anatomical connections with the hippocampus and other cortices as well as its cellular activities during memory processes (Ranganath et al., 2003; Fyhn et al., 2004; Craig and Commins, 2005; Fransen, 2005; Kerr et al., 2007; Canto et al., 2008). For instance, sustained spike activity in the EC underlies the basis of theta rhythm, which may reflect the interaction between the EC and hippocampus, and thus is correlated with working memory (Egorov et al., 2002; Fransen et al., 2002; Fransen, 2005). Furthermore, the discovery of grid cells, resembling place cells in the hippocampus and exhibiting spatial firing properties in the EC, corroborates the central role of the EC in spatial computation and spatial memory (Fyhn et al., 2004; Hafting et al., 2005; Moser et al., 2008). Thus, damage to the EC instigates impaired memory and cognition in ageing and in age-related neurodegenerative disease, such as AD (Gomez-Isla et al., 1996; deToledo-Morrell et al., 2000; Sze et al., 2001; Rodrigue and Raz, 2004; Scheff et al., 2006; Staub et al., 2006; Raz et al., 2008). Therefore, it is of extreme importance to investigate the cellular alterations that take place in the EC during the development and progression of AD as well as normal ageing.

In the present thesis, I have focused on astroglia. Astroglia is critical for brain homeostasis (Wang and Bordey, 2008; Sofroniew and Vinters, 2010; Verkhratsky et al., 2011). In this thesis, the work has been mainly aimed at studying the morphological and functional alterations of astrocytes in EC during the progression of AD. In Chapter 3, I investigated the changes in astrocytic cytoskeleton. Later in Chapter 4, I analysed the stability of astrocytic function controlling glutamate homeostasis via the glutamate-glutamine shuttle. And finally, in Chapter 5, I studied the morphological changes in the astrocytes during ageing not only in EC, but in its major output area also deeply involved in mnesic functions and highly vulnerable in memory related pathologies including AD, the hippocampus. In addition, I shall discuss some possible potential future work, that in fact are already carried out in our Laboratory, to have a clearer scenario of the relevance of astroglial morphological and functional changes as well as their heterogeneity in response to the AD related pathology and impairments, therefore becoming a key player in the potential therapeutic strategies.
2. Astrocytic changes in AD

AD is an age-related and irreversible neurodegenerative disease with severe neuronal and synaptic loss. Abnormal accumulation of β-amyloid and aggregation of hyperphosphorylated tau, in the form of SPs and NFTs, are considered as the main pathological feature of AD (Braak and Braak, 1991). In addition to the alteration and misfolding of proteins, astroglial changes are also a prominent pathological phenomenon in AD (Rodriguez et al., 2009; Olabarria et al., 2010; Verkhratsky et al., 2010). Astrocytes are ubiquitous and the most numerous glial cells in the brain, covering neural network with their processes and exerting their function in numerous homeostatic tasks. A growing body of evidence indicates that astrocytes are also involved in information processing in the CNS (Araque et al., 1999; Fellin et al., 2004; Wang and Bordey, 2008; Sofroniew and Vinters, 2010). Dysfunction of astrocytes contributes to pathogenesis and progression of neurological diseases, including AD (Verkhratsky et al., 2010; Verkhratsky et al., 2011). Several studies described reactive astrogliaosis in response to AD pathology (Muramori et al., 1998; Porchet et al., 2003; Vanzani et al., 2005). However, the role of astrocyte and the consequence of astrocytic changes in AD are not fully understood.

In the present thesis, I have focused on the EC, the first and most affected region in AD, and the dysfunction of this area is associated with cognitive deficits and memory impairment in AD. For this purpose, I used 3xTg-AD mouse model, which is one of the most relevant animal model of AD and mimicking the spatiotemporal progression of Aβ and tau pathology in AD human patients (Oddo et al., 2003a; Oddo et al., 2003b).

2.1 Changes in the Astroglial Cytoskeleton in the EC of 3xTg-AD Mice

In chapter 3, I performed morphological analysis of GFAP-IR astrocytes in the EC of 3xTg-AD animals at different ages. The GFAP-IR astrocytes distribute widely through the entire EC with higher density in layers I and VI. Surprisingly, in contrast to the generally accepted reactive astrogliaosis with a massive increase in GFAP proliferation, which was first recognised by Alois Alzheimer and later by Ramon y Cajal, during AD progression (Alzheimer, 1907; Muramori et al., 1998; Porchet et al.,
2003; Nagele et al., 2004; Vanzani et al., 2005) we observed a general atrophy of astrocytes, shown by reduction in GFAP positive surface and volume, of 3xTg-AD animals when compared to age-matched non-Tg animals. To our best knowledge, this is the first evidence revealing an early and sustained atrophy of astrocytes in the EC of 3xTg-AD mice. These morphological changes occurred in both superficial and deep layers in young and fully matured animals (from 1 to 6 months) whereas it only took place in the deep layers at a later age (12 months). The observed astroglial atrophy in the EC corresponds to previous report in the hippocampus also presented by our Laboratory, which showed statistically significant atrophy in the DG at the age of 12 months and in the CA1 at the age of 18 months (Olabarria et al., 2010).

Human post-mortem studies have showed hypertrophic and reactive astrocytes in the EC of AD brains (Muramori et al., 1998; Porchet et al., 2003; Vanzani et al., 2005), however, there is also evidence indicating distinct responses of specific human cortical astrocytes to AD. The intralaminar astrocytes tend to be hypertrophic whereas the interlaminar astrocytes have loss or disruption in their processes and altered dynamic properties (Colombo et al., 2002). Although GFAP immunolabeling did not reveal the total volume and actual surface of astroglia (Bushong et al., 2002), the decreased GFAP-IR surface coincided with a reduction in astroglial processes by 26%-45%, suggesting a reduced astrogial profiles and arborisation. In addition, the smaller volume of GFAP-IR astrocytes may also reflect smaller or decreased astrocytic processes. Taken together, a general atrophy of astrocytes is a sign of potential decline in astrocytic coverage and domain. Moreover, Simpson et al. (2011) indicated reduced gene transcriptomes of cytoskeletal proteins (including myosin and actin) and junctional proteins in GFAP positive astrocytes isolated from AD temporal cortex, implying alterations in astrogial morphology, motility and communication (Buss et al., 2004; Potokar et al., 2007; Fellin, 2009). Therefore, it is time to re-consider the role of astrogia in neuropathology, and particularly in AD. Given the multiple functions that astrogia exert, the cytoskeletal atrophy may lead to decreased synaptic coverage by astrocytic processes, and thus to compromised astrocytic support and synaptic maintenance. Synaptic connectivity might be impaired in consequence. Indeed, several reports already revealed loss of synapses/dendritic spines in the neocortex and the hippocampus; specifically, loss of perforated synapses in the CA1 subfield of the hippocampus may results in impaired LTP and reduced field excitatory postsynaptic...
potential in several brain regions of 3xTg-AD mice (Oddo et al., 2003b; Bertoni-Freddari et al., 2008; Bittner et al., 2010; Noristani et al., 2011). Astroglial atrophy in the EC occurred very early when there is no apparent sign of AD pathology. This is somewhat similar to the loss of dendrites and synaptic disconnection and/or altered synaptic plasticity in the cortex in both human patients and 3xTg-AD mice, which all appear at the incipient and mild stage of AD (Heinonen et al., 1995; Ingelsson et al., 2004; Reddy et al., 2005; Baloyannis et al., 2007; Bittner et al., 2010). In 3xTg-AD animals, loss of dendritic spines coincides with dendritic dystrophy, with absence of SPs and NFTs but appearance of intracellular soluble Aβ (Bittner et al., 2010). Human post-mortem studies revealed pronounced synaptic loss shown by reduction in presynaptic and postsynaptic proteins as early events in the course of AD (Heinonen et al., 1995; Ingelsson et al., 2004; Reddy et al., 2005). This is often accompanied with prominent alterations in dendritic morphology (Baloyannis et al., 2007). It is known that astrocytes are responsible for synaptic stability, ion and neurotransmitter homeostasis, which are important for synaptic activity (Kofuji and Newman, 2004; Ullian et al., 2004). In addition, astrocytes can also eliminate the abnormal synapses by secreting proteolytic enzymes (VanSaun and Werle, 2000). Cytoskeletal atrophy of astrocytes may lead to reduced astrocytic synaptic coverage, and subsequently compromise astroglial maintenance of synaptic structures and functions as well as astroglial removal of dystrophic neurites. Taken together, it may imply potential association of astrocytes with synaptic degeneration in early AD. Astroglial atrophy in both superficial and deep layers at young ages is sustained into later age (12 months) only in deep layers. Nonetheless, it is of note that this phenomenon is not due to the recovery of atrophic astrocytes. In fact, the transgenic animals exhibited a constant lower expression of GFAP at all ages, but more importantly with age entorhinal astrocytes in non-Tg mice showed a tendency toward atrophy as ageing advances (which will be discussed later; Fig. 6.1).
Figure 6.1. Schematic Illustration of astroglial changes in the EC during ageing and AD. It is evident that entorhinal astrocytes undergo structural atrophy at the very early stage of AD and this atrophy sustain till the late stage of AD. During the ageing process, astrocytes exhibit normal morphology in young and matured brain, however, becoming atrophic at old ages. The degree of AD pathology (ex. Aβ plaques) is indicated by the size of red squares. Mild AD pathology may occur between middle and late ages whilst the presence of severe AD pathology is usually at late age.

Early atrophy of astrocytes in the EC is similar to temporal progression of AD pathology, which is characterised by abnormal accumulation of Aβ and hyperphosphorylated tau (Braak and Braak, 1991; Braak et al., 1999). The appearance of Aβ deposits begins in the temporal cortex, including the EC, and then advances to neighbouring cortical regions before finally reaching the hippocampus (Braak and Braak, 1997). The neurofibrillary changes also begin primarily in the transentorhinal/entorhinal regions, and then proceed to other limbic structures. NFTs can be found in all cortical structures at very late stage (Braak and Braak, 1997; Braak et al., 1999; Thal et al., 2000b). Shrinkage of the EC, which may be caused by loss of
neurones and/or synapses, occurs earlier than structural atrophy in other regions (Heinonen et al., 1995; Gomez-Isla et al., 1996; Du et al., 2001; Kordower et al., 2001; Scahill et al., 2002; deToledo-Morrell et al., 2004). Therefore, the early astrocytic atrophy again reflects the fact that the EC is the first affected region during AD progression.

Previous studies of our laboratory indicated concomitant existence of hypertrophic and atrophic astrocytes in the hippocampus. Astrocytes away from the plaques displayed atrophic morphology, while astrocytes in the vicinity of Aβ underwent reactive gliosis (Olabarria et al., 2010). Thus, it was important to investigate whether the reactive astrogliosis is also present around Aβ deposits in the EC. To our surprise, Aβ deposits and senile plaques did not induce reactive astrogliosis at least based on the morphological criteria. Furthermore, the presence of GFAP-IR astrocytes around Aβ deposits is rare in the EC. This distinct behaviour of entorhinal astrocytes is in line with the study in the prefrontal cortex, which also indicated the scant existence of reactive astrocytes in the vicinity of Aβ (Kulijewicz-Nawrot et al., 2012b), suggesting that cortical astrocytes respond to pathology differently when compared to the hippocampal astrocytes. These regional differences in astrocytic responses to AD pathology most likely reflect heterogeneity of astroglia. Relative indifference of entorhinal astrocytes to AD pathology might provide the explanation for high susceptibility of EC to AD pathology.

2.2 Glutamate Homeostasis in the 3xTg-AD Animals

Cytoskeletal changes of entorhinal astrocytes in the 3xTg-AD mice throughout the development and progression of AD pathology may result in compromised astrocytic functions in synaptic maintenance and modulation. It has been suggested that disturbed glutamate homeostasis and glutamatergic transmission is involved in neuronal/synaptic degeneration and synaptic dysfunction in AD (Walton and Dodd, 2007). Therefore, we investigated whether glutamate-glutamine cycle can be attributed to astrogliial atrophy. Astrocytes clear up the majority of glutamate in the synapses by glutamate transporters and subsequently convert glutamate into glutamine by the enzyme glutamine synthetase (GS), which is specifically expressed in astrocytes. Glutamine is then released by
astrocytes and is transported back to neurones, where it serves as substance for glutamate synthesis.

In chapter 4, I investigated whether astroglial contribution to glutamate balance is altered by measuring GS expression in the EC of 3xTg-AD animals. GS-IR astrocytes had a relatively stable expression, as indicated by constant Nv of GS-IR cells and GS expression during the progression of AD. However, with dual immunofluorescence staining of GS and GFAP, we observed different subpopulations of astrocytes in the EC: GS-positive, GFAP-positive and GS/GFAP-positive astrocytes, indicating functional diversity of astrocytes. In addition, we also found stable GS surface and volume in GS- and GS/GFAP-IR astrocytes.

Previous studies about glutamate changes in AD have brought controversial results. Some reports revealed decreased GS expression and activity as well as deficits in glutamate transporters at late stages of AD (Le Prince et al., 1995; Castegna et al., 2002; Hynd et al., 2004), whilst other studies indicated these alterations are caused by reduction in GFAP and withdrawal of astrocytic processes (Robinson, 2001; Hughes et al., 2004; Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2012a). On the contrary, Burbaeva and colleagues (2005) indicated increased expression of glutamate metabolising enzymes including GS and glutaminase in the prefrontal cortex of AD, suggesting increased glutamatergic transmission in AD. However, there is also evidence showing stable levels of glutamate and/or glutamate uptake in AD cortex (Mohanakrishnan et al., 1995; Beckstrom et al., 1999). Our results showing similar GS expression in the EC of 3xTg-AD and non-Tg mice are in agreement with the above studies, suggesting unaltered glutamate-glutamine cycle during the progression of AD. Nonetheless, there is a slight increase, although not statistically significant, in GS expression in 3xTg-AD animals at later age when compared to age-matched non-Tg group, implying a higher demand of GS, which might be caused by increased glutamatergic transmission.

Dual immunolabelling of GS and GFAP revealed the existence of astroglial subsets (Fig 4.3), being GS-IR, GFAP-IR and GS/GFAP-IR, indicating cellular differences. This is in agreement with the study by Robinson (2000), which suggested GS positive astrocytes could be a complementary population to GFAP-IR astrocytes despite that we
also observed astrocytes expressing both GS and GFAP. Moreover, this phenomenon reflects astrogial heterogeneity within the same area, corresponding to the studies demonstrating heterogeneity of astrocytic identity and functions (Walz and Lang, 1998; Holthoff and Witte, 2000; Emsley and Macklis, 2006; Simpson et al., 2010). Moreover, GS positive astrocytes do not exhibit morphological changes in AD progression, in accordance with the report indicating regular morphology of astrocytes in AD cortex (Robinson, 2000). There are no significant changes in GS profiles in GS/GFAP positive astrocytes in spite of the evident GFAP atrophy (Fig 4.4); suggesting cytoskeleton atrophy has no direct influence on GS-IR architecture.

In this respect, one may ask what the effects of GFAP atrophy can exert if GFAP only delineate part of astroglia population in the cortex (Walz and Lang, 1998; Robinson et al., 2000). It is known that each single astrocyte covers around 90,000 synapses in the rodent cortex (Oberheim et al., 2006), thus atrophy of GFAP-IR astrocytes may cause reduced astrocytic coverage over synapses as well as decreased endfeet contacts with blood vessels, resulting in compromised ability of astrocytes to maintain extracellular ion homeostasis and reduced support to local active synapses (Walz, 2000; Oberheim et al., 2006; Nedergaard and Verkhratsky, 2012). In line with this, studies using transgenic animal models of AD demonstrated decreased GFAP labelled endfeet along with decreased K⁺ channel expression on astrocytes (Oberheim et al., 2006; Wilcock et al., 2009). Additionally, decrease in astrocytic cytoskeleton protein may also be accompanied with decline in connexins, such as Cx43, suggesting a decrease in astrocytic gap junctional connectivity and compromised spatial ion buffering (Walz, 2000; Simpson et al., 2011). The diminished communication and buffering ability of GFAP-IR astrocytes may lead to neuronal hyperactivity in the EC, which was already observed in 3xTg-AD mice (Arsenault et al., 2011). Altered astrogial modulation and neuronal activity may result in a consequent network dysfunction, which is essential for cognitive processes (Fellin, 2009).

In addition to intra-regional heterogeneity of astrocytes, this highly diverse groups of cells react to neuropathology in distinct manner by displaying region- and time-dependent differences in morphology changes and gene expression (Hill et al., 1996; Zhang and Barres, 2010). Previous studies of GS-IR cells in the PFC and the hippocampus in 3xTg-AD showed distinct changes in GS-positive astrocytes in contrast
to astrocytes in the EC. GS expression is decreased in the PFC at early and middle ages whereas a manifest decline in the hippocampus occurs at later ages (Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2012a). The variation of astrocytic ability to maintain glutamate balance via glutamate-glutamine shuttle, measured by GS expression, in different brain regions of 3xTg-AD mice implicated regional heterogeneous responses of astrocytes to the disease.

2.3 Heterogeneous Alterations in Astroglial Morphology during Ageing

Changes in astroglia and consequent effects on synaptic network and neuronal survival during physiological ageing are not fully understood. In chapter 5, I performed morphological analysis of GFAP-positive astrocytes in both the hippocampus and EC in aged animal to study the how astrocytes react to ageing. I found distinct ageing effects on hippocampal and entorhinal astrocytes. Astrocytes in the hippocampal fields, DG and CA1, exhibited elevation in GFAP-IR surface and volume, indicative of hypertrophy; entorhinal astrocytes displayed an atrophy, revealed by a significant reduction in GFAP.

It is generally accepted that reactive astrogliosis, which is characterised by hypertrophy of astrocytic processes, up-regulation of intermediate filament (especially GFAP) and re-expression of nestin (Pekny and Nilsson, 2005), is a common alteration during ageing (Goss et al., 1991; Kohama et al., 1995; Nichols, 1999; Cotrina and Nedergaard, 2002). In chapter 5, I revealed astrogliosis in the DG and CA1 subfields of the hippocampus, corresponding to previous studies indicating that hippocampus is the most affected region by astrogliosis during ageing (David et al., 1997; Amenta et al., 1998). In this regard, it is important to know if this astrocytic modification leads to changes in functions and cause subsequent synaptic/neuronal alterations in the hippocampus that may underlie the basis of memory deficits during ageing. Indeed, a decline in GS-positive cell areas was also detected in the DG and CA1 of aged animals. This alteration is in agreement with the evidence revealing attenuated astroglial function of glutamate balance in aged animals (Gottfried et al., 2002; Gracia-Matas et al., 2008), which might be concomitant with the occurrence with GFAP hypertrophy (Weir and Thomas, 1984; Ortinski et al., 2010).
In contrast to hypertrophic astrocytes, however, there is also evidence indicating that
cortical astrocytes have a tendency toward atrophy during ageing and this alteration
become manifest in demented aged subjects (Senitz et al., 1995). In chapter 5, I
reported such astroglial atrophy, reflected by decreased GFAP expression, in the EC of
aged animals. Accordingly, a growing body of reports focusing on the potential role of
astrocytes in pathogenesis and disease progression have shown early involvement of
astroglial atrophy in a variety of neurodegenerative diseases including AD, amyotrophic
lateral sclerosis, and Wernicke encephalopathy (Rossi et al., 2008; Rossi and Volterra,
2009; Heneka et al., 2010; Verkhratsky et al., 2010; Rodriguez and Verkhratsky, 2011).
However, astroglial atrophy here did not result in increase or decrease of GS-positive
 cellular domain, corresponding to the studies showing unaltered GS expression in the
cortex of aged brain (Goss et al., 1991; Wu et al., 2005). As discussed in the previous
section, there are different subsets of astrocytes in the EC, and the GFAP atrophy may
affect other astroglial functions rather than glutamate balance via glutamate-glutamine
shuttle in this region. Astroglial control over ion and extracellular volume as well as
 glial responses to synaptic activity decrease in aged brain, suggesting reduced astroglial
 support and altered neuron-glial signalling (Sykova et al., 1998; Lalo et al., 2011).
There are also reports indicating altered astrocytic functions in metabolism as well as
 less protection for neurones against oxidative stress, which is intimately associated with
ageing, from aged astrocytes (Deng et al., 2006; Lin et al., 2007; Ritz et al., 2009). It
should be noted that accumulating evidence demonstrates reduced input and disrupted
connection from EC layer II to the hippocampus during ageing, suggesting that
disrupted synaptic connections underlie the cognitive decline during ageing (Geinisman
et al., 1992; Nicolle et al., 1999; Smith et al., 2000; Scheff et al., 2006; Stranahan et al.,
2011). This reduction in synaptic connectivity might be attributed to a decreased
support and modulation of astrocyte due to astroglial atrophy in the EC during ageing.

Furthermore, different responses of astroglia in the hippocampus and EC show
heterogeneous remodelling and functional alterations of astroglia in different brain
regions during ageing. This again corroborates astroglial heterogeneity, implicating
different function of astrocytes in different regions, which might be associated with the
local microenvironment and demands (Zhang and Barres, 2010). Moreover, changes in
the aged astrocytes are similar to those astrocytes in the EC of pathological brains

202
(3xTg-AD; Fig 6.1), implying that the alterations in the neuronal activity and synaptic connectivity might be comparable in ageing and AD.
3. General Conclusion

Alteration and dysfunction of the EC in ageing and age-related neurodegenerative disease, AD, have been well documented. Many studies revealed the role of the EC in cognitive and memory decline during ageing as well as AD progression (Du et al., 2001; Kordower et al., 2001; Raz et al., 2005; Scheff et al., 2006; Stoub et al., 2006). It is of note that the elderly population and AD incidence increase dramatically. In particular, the prevalence of AD, which was estimated to reach 106.23 million worldwide in 2050, will result in enormous social and economic pressure (Brookmeyer et al., 2007; Wilmo and Prince, 2010). Numerous experimental and clinical researches have focused on this disease, however, effective therapeutic strategy for AD is still lacking.

The conspicuous involvement of the EC in AD progression is shown by early presence of plaques and tangles as well as early structural atrophy (Braak and Braak, 1991, 1997; Thal et al., 2000b; Thal et al., 2000a; deToledo-Morrell et al., 2004). In addition, cells in the transentorhinal/EC areas are the first neurones undergoing changes in the distal dendrites, which precede the occurrence of AD pathology (Braak and Braak, 1995). Moreover, it is indicated that the disruption of the perforant path, which might result from neuronal or synaptic degeneration of EC layer II neurones, is one of the earliest event in AD (Hyman et al., 1984; Lippa et al., 1992; Gomez-Isla et al., 1996; Salat et al., 2009). This disrupted synaptic connectivity between the EC and the hippocampus is considered to account for memory deficit, which may occur prior to the onset of AD pathology (Hyman et al., 1984; Coleman et al., 2004; Stoub et al., 2006). Therefore, understanding changes of the EC in AD may provide a therapeutic target to mitigate and slacken off, if not stop, the early cognitive decline in AD.

So far, many studies have focused on neurones of the EC; however, more and more attention has transferred to astroglia, the other major cell component in the brain. Astroglia controls the CNS homeostasis at different levels, ranging from molecular homeostasis (e.g. ions and neurotransmitter homeostasis) to organ homeostasis (e.g., induction and maintenance of BBB; (Verkhratsky et al., 2010). Dysfunction of astrocytes causes homeostasis failure, in turn may initiate and mediate the pathological progression of neurological diseases (Rodriguez et al., 2009). Therefore, astrogial alterations in the EC may lead to deficient support to neurones and synapses and
subsequently compromised synaptic activity and connectivity, underlying the mechanism of cognitive deficits of incipient AD (Rodriguez et al., 2009; Verkhratsky et al., 2010; Verkhratsky et al., 2011).

Our data showed astrocytic atrophy, which is demonstrated by a significant decrease in the surface and volume of GFAP-IR cells, in the EC of 3xTg-AD mice. This cytoskeletal changes in astrocytes begin at very young age (1 month) and are sustained till later stages. The consistent decrement in the GFAP expression indicates general astrocytic atrophy in the EC throughout the progression in AD. This is in agreement with previous study revealing that astrocytes in the hippocampus had atrophic tendency at the age of 6 months and became significantly atrophic at a later ages (12 months and 18 months) in the same animal model (Olabarria et al., 2010). This temporal variation of changes in astroglial morphology not only indicates the fact that the EC is the first region affected by AD but also implicates that astroglial alterations in the hippocampus follow as a consequence of astrocytic atrophy in the EC. It is of note that GFAP labeling did not reveal actual morphology and size of astrocyte (Bushong et al., 2002); however, the observed reduction in surface and volume of GFAP-IR cells is concomitant with a decline in GFAP-IR profiles, suggesting atrophy of GFAP-IR astrocytes in the EC may represent a decreased astrocytic domain and synaptic coverage, which leads to decreased metabolic support, dysfunction of neurovascular unit as well as disturbance in ion/neurotransmitter balance. These changes can result in the loss of synapses or altered synaptic activity, which in turn causes the dysfunction of neuronal network and thus cognitive and mnesic impairment (Verkhratsky et al., 2010). In fact, there is evidence indicating decreased membrane surface area, which might be a result of loss of dendritic spines, in the EC (Arsenault et al. 2011). Other reports have revealed loss of synapses, rather than neuronal death, and compromised synaptic efficacy in different regions, suggesting synaptic disconnection in 3xTg-AD animals (Oddo et al., 2003b; Bertoni-Freddari et al., 2008; Bittner et al., 2010; Noristani et al., 2011). Therefore, astroglial atrophy in the EC could lead to disrupted synaptic connectivity between the EC and hippocampus as well as changes in the microenvironment and the subsequent astrocytic alteration in the hippocampus.

In addition, early changes of astrocytes in the EC are similar to the AD pathology. For example, Aβ deposits start in the neocortex as well as the EC, and then occur in the
hippocampus and finally appear in all subcortical regions (Braak and Braak, 1997; Thal et al., 2000b). This again corroborates EC as the initial lesion in AD. Furthermore, the rare appearance of astrocytes around the plaques and lack of astrogliosis triggered by Aβ deposits may account for the susceptibility of the EC, which is the first and most vulnerable during the progression of AD.

In order to understand whether the astroglial atrophy can compromise the local homeostasis, we analysed the expression of GS, which is a critical element in the glutamate balance. Our data showed constant density of GS-IR cells and comparable IOD of GS, suggesting unaltered GS expression in the EC. In addition, the morphology of GS-IR cells, measured by surface and volume, were not changed regardless of its co-localisation with GFAP, which was significantly reduced in both surface and volume. These data indicated that astroglial atrophy has no direct effect on GS level and the structure of GS positive cells. Thus, GFAP atrophy does not disturb glutamate-glutamine cycle in the EC, but may rather affect the extracellular environment and neural circuit via other mechanisms (e.g., potassium buffering and intercellular signalling).

Furthermore, dual labelling of GS and GFAP revealed distinct subset of astrocytes in the EC, being GS-, GFAP- and GS/GFAP-IR subsets, indicating heterogeneity in astroglial identity within the region, which is also demonstrated by other studies (Walz and Lang, 1998; Holthoff and Witte, 2000; Emsley and Macklis, 2006; Simpson et al., 2010). Moreover, when compared with the decrement of GS in other regions in 3xTg-AD animals, such as the PFC and the hippocampus, the constant GS expression in the EC suggested regional variation in astrocytic glutamate uptake, and thus spatiotemporal differences of astroglial responses to AD.

In addition to AD, I was also interested in the astroglial reaction to ageing. Although it is commonly regarded that astrocytes undergo reactive astrogliosis in the ageing brain (Cotrina and Nedergaard, 2002), our data showed a diverse astroglial alterations in the aged mouse brain. Aged astrocytes in the DG and CA1 were hypertrophic, as indicated by increased GFAP surface and volume. On the contrary, astrocytes in the EC exhibited significant reduction in GFAP level. The cellular area of GS-positive astrocytes decreased significantly in the DG and CA1 whereas it remained unaltered in the EC.
The astroglia atrophy in the EC may contribute to disruption of synaptic connectivity between the EC and the hippocampus via deregulation of extracellular ions, less metabolic support, compromised protections against oxidative stress and reduced neuron-glia interactions, and thus leading to cognitive and memory deficits during ageing (Geinisman et al., 1992; Nicolle et al., 1999; Smith et al., 2000; Deng et al., 2006; Scheff et al., 2006; Lin et al., 2007; Ritz et al., 2009; Stranahan and Mattson, 2010).

Differential changes of astrocytic morphology in the hippocampus and the EC confirm the regional heterogeneity in the astrocytic responses to ageing in different brain regions. The diversity of astroglial alteration implies differences in astrocytic physiology and functions (Zhang and Barres, 2010). Moreover, aged astrocytes in the EC show similar morphological changes in the 3xTg-AD mice, suggesting that general atrophy of astroglial may result in reduced astrocytic coverage and modulation of synapses and account for the synaptic dysfunction in ageing and AD.

It should not be overlooked that GFAP labeling did not reveal the total population and actual volume of astrocytes (Bushong et al., 2002; Robinson et al., 2000). Moreover, vimentin, the other cytoskeleton protein, is also expressed by mature astrocytes and essential for IF organisation whereas re-expression of nestin and synemin is found in reactive astrocyte, which is featured by a hypertrophic morphology (Frisen et al., 1995; Lin et al., 1995; Pekny et al., 1995; Eliasson et al., 1999; Jing et al., 2007). More evidence from other cytoskeleton proteins (such as vimentin) as well as astrocytic markers (ex. S100β) are needed for explicit understanding of overall changes in astrocytic morphology/structure in AD pathology and during ageing processes.
4. Future work

Further investigations are required to elucidate the full scenario of entorhinal astrocytic subsets and alterations in AD. It would be vital to study the changes in astrocytes labelled by S100β, which may be another subset of astrocyte in the EC. It would be also critical to investigate if those astrocytes have different responses to AD pathology, such as Aβ deposition. In addition, it would be interesting to study the effect of enriched mental exposure and exercise on the astroglia. In addition, it would be essential to know if astrogial cytoskeletal atrophy affects the synaptic activity and plasticity. This aspect is carried out by studying the changes in the cells or neuritis labelled by polysialylated neuronal cell adhesion molecule. This particular aspect will be covered in the same period mentioned above. It would be fundamental to study via which mechanism astrogial atrophy can affect local and global synaptic network. All the studies mentioned above are currently executed in our Laboratory and are expected to be completed in January 2013.
References


References


References


References


Duffy AM, Fitzgerald ML, Chan J, Robinson DC, Milner TA, Mackie K, Pickel VM (2011) Acetylcholine alpha7 nicotinic and dopamine D2 receptors are targeted to many of the same postsynaptic dendrites and astrocytes in the rodent prefrontal cortex. Synapse 65:1350-1367.


hyperphosphorylation through Wnt/beta-catenin pathway rescue in PC12 cells. J Mol Med (Berl) 84:253-258.


Ferbinteanu J, Holsinger RM, McDonald RJ (1999) Lesions of the medial or lateral perforant path have different effects on hippocampal contributions to place learning and on fear conditioning to context. Behav Brain Res 101:65-84.


Harkany T, Dijkstra IM, Oosterink BJ, Horvath KM, Abraham I, Keijser J, Van der Zee EA, Luiten PG (2000a) Increased amyloid precursor protein expression and
serotonergic sprouting following excitotoxic lesion of the rat magnocellular nucleus basalis: neuroprotection by Ca(2+) antagonist nimodipine. Neuroscience 101:101-114.


Kaut KP, Bunsey MD (2001) The effects of lesions to the rat hippocampus or rhinal cortex on olfactory and spatial memory: retrograde and anterograde findings. Cogn Affect Behav Neurosci 1:270-286.


Mesulam MM, Mufson EJ, Levey AI, Wainer BH (1983) Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the
References

septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. The Journal of comparative neurology 214:170-197.


Naber PA, Lopes da Silva FH, Witter MP (2001) Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. Hippocampus 11:99-104.


Parsons CG, Danysz W, Quack G (1999) Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist--a review of preclinical data. Neuropharmacology 38:735-767.


intermediate filaments but develop and reproduce normally. EMBO J 14:1590-1598.


Phillips ML, Ladouceur CD, Drevets WC (2008) A neural model of voluntary and automatic emotion regulation: implications for understanding the
References


Ramon y Cajal SRY (1918) la microfotografía estereoscopica y biplanar del tejido nervioso. madrid: rab. del Lab. de Inv. biol.


References


Saunders RC, Mishkin M, Aggleton JP (2005) Projections from the entorhinal cortex, perirhinal cortex, presubiculum, and parasubiculum to the medial thalamus in


References


References


Witter MP (2007) The perforant path: projections from the entorhinal cortex to the
Witter MP, Van Hoesen GW, Amaral DG (1989) Topographical organization of the
entorhinal projection to the dentate gyrus of the monkey. J Neurosci 9:216-228.
entorhinal cortex of the rat: distribution, morphology, ultrastructure of neurons,
and co-localization with gamma-aminobutyric acid and parvalbumin. J Comp
Neurol 425:177-192.
Xu T, Pandey SC (2000) Cellular localization of serotonin(2A) (5HT(2A)) receptors in
Yang W, Ang LC, Strong MJ (2005) Tau protein aggregation in the frontal and
Neuron 16:921-932.
Reduction of AMPA-selective glutamate receptor subunits in the entorhinal
cortex of patients with Alzheimer's disease pathology: a biochemical study.
Yeh CY, Vadhwana B, Verkhratsky A, Rodriguez JJ (2011) Early astrocytic atrophy in
the entorhinal cortex of a triple transgenic animal model of Alzheimer's disease.
Yeterian EH, Pandya DN (1991) Prefrontostriatal connections in relation to cortical


Appendix
Early astrocytic atrophy in the entorhinal cortex of a triple transgenic animal model of Alzheimer's disease

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ABSTRACT

The EC (entorhinal cortex) is fundamental for cognitive and mnesic functions. Thus damage to this area appears as a key element in the progression of AD (Alzheimer’s disease), resulting in memory deficits arising from neuronal and synaptic alterations as well as glial malfunction. In this paper, we have performed an in-depth analysis of astroglial morphology in the EC by measuring the surface and volume of the GFAP (glial fibrillary acidic protein) profiles in a triple transgenic mouse model of AD [3xTg-AD (triple transgenic mice of AD)]. We found significant reduction in both the surface and volume of GFAP-labelled profiles in 3xTg-AD animals from very early ages (1 month) when compared with non-Tg (non-transgenic) controls (48 and 54% reduction respectively), which was sustained for up to 12 months (33 and 45% reduction respectively). The appearance of Aβ (amyloid β-peptide) depositions at 12 months of age did not trigger astroglial hypertrophy; nor did it result in the close association of astrocytes with senile plaques. Our results suggest that the AD progressive cognitive deterioration can be associated with an early reduction of astrocytic arborization and shrinkage of the astroglial domain, which may affect synaptic connectivity within the EC and between the EC and other brain regions. In addition, the EC seems to be particularly vulnerable to AD pathology because of the absence of evident astrogliosis in response to Aβ accumulation. Thus we can consider that targeting astroglial atrophy may represent a therapeutic strategy which might slow down the progression of AD.

Key words: Alzheimer’s disease, astrocyte, dementia, entorhinal cortex, glial fibrillary acidic protein (GFAP), memory.

INTRODUCTION

The EC (entorhinal cortex), a part of the temporal cortex, is involved in mnesic processes by establishing the cortico-hippocampal circuits. The EC is divided into superficial (I–III) and deep layers (IV–VI) that show differential anatomical and functional organization (Suzuki and Amaral, 1994; Witter and Amaral, 2004). The superficial layers are the main recipient of intracortical information and the major output source to the HC (hippocampus), whereas the deep layers are mainly responsible for the projections to cortical regions (Figure 1; Suzuki and Amaral, 1994; Witter and Amaral, 2004). The neurons from the EC layer II terminate in the middle and outer molecular layer of the DG (dentate gyrus) and send collaterals to the hippocampal CA2 and CA3 fields (Witter et al., 1989; Tamamaki and Nojyo, 1993; Suzuki and Amaral, 1994). Layer III neurons project mainly to the CA1 and subiculum, which in turn feedback to layer V of the EC (Naber et al., 2001). Functionally, activation of the EC and persistent neuronal activity in the EC are involved in the...

Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid β-peptide; EC, entorhinal cortex; GFAP, glial fibrillary acidic protein; GFAP-IR, glial fibrillary acidic protein-immunoreactive; HC, hippocampus; LEC, lateral entorhinal cortex; NFT, neurofibrillary tangle; non-Tg, non-transgenic; Nv, numerical density; PB, phosphate buffer; PHF, paired helical filament; RT, room temperature; SP, senile plaque; 3xTG-AD, triple transgenic mice of AD; TS, Tizma-based saline.

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EC

Superficial Layers (I–III)

Hippocampus

Hippocampal Formation and Subiculum

Deep Layers (VI–IV)

Parahippocampal Areas

Figure 1 Schematic illustration of entorhinal inputs to the HC

In brief, the superficial layers (I–III) project to the hippocampal formation and subiculum, while the deep layers (IV/VI) receive reciprocal inputs from these areas. Meanwhile, deep layers also innervate to parahippocampal areas and the HC.

The AD-related neuronal loss and atrophy of the EC are well documented in human patients (Gomez-Isla et al., 1996; Calhoun et al., 1998; de Toledo-Morrell et al., 2000; Du et al., 2001; Kordower et al., 2001; Ribe et al., 2005). However, little is known about AD-associated changes in EC astroglia. Post-mortem studies have revealed an increase in GFAP (glial fibrillary acidic protein) protein levels as well as in the number of GFAP-positive astrocytes within the EC, which seems to be associated with the Aβ load (Muramori et al., 1998; Porchet et al., 2003). Recently, we reported the concomitant occurrence of astrogial atrophy and astrogliosis in the HC of the transgenic mouse model of AD. The atrophy appears as a generalized process, whereas astrogliosis was triggered by developing SPs and Aβ aggregates (Rodríguez et al., 2009a, 2009b; Heneka et al., 2010; Olabarria et al., 2010; Verkhratsky et al., 2010; Rodríguez and Verkhratsky, 2011). In the present paper, we extended our analysis of AD-associated changes in astroglia to the EC.

MATERIALS AND METHODS

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

Animal models

The generation of 3xTg-AD (triple transgenic mice of AD) mice has been well described (Oddo et al., 2003a, 2003b; Billings et al., 2005; Rodríguez et al., 2008; Rodríguez et al., 2009a, 2009b). The 3xTg-AD mice were derived from mixed 129/C57BL6 mice, which harbour APPswe, PS1M146V and TauP301L mutations, and the same spatiotemporal progression of amyloid and tau protein pathology in human AD. The non-Tg (non-transgenic) control mice were from the same strain and the same genetic background. All 3xTg-AD and non-Tg littermates were from homozygous breeders. Mice were grouped by gender and genotype, housed under controlled temperature and 12 h light/12 h dark cycles with ad libitum access to food and water.

Fixation and tissue processing

As previously described (Olabarria et al., 2010), male 3xTg-AD at 1, 3, 6, 9 and 12 months of age (n=5, 4, 4, 5 and 5 respectively) and their age-corresponding non-Tg controls (n=4, 4, 4, 5, 5 respectively) were anaesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. The brains were fixed by aortic arch perfusion of 3.75% acrolein (25 ml; TAAB, UK) in 2% (w/v) paraformaldehyde (Sigma, St. Louis, MO, U.S.A.) and 0.1 M PB (phosphate buffer) at pH 7.4, followed by 75 ml of 2% paraformaldehyde. Brains were removed, cut into 2–3 mm
coronal slabs containing the EC, post-fixed in 2% para-formaldehyde for 24 h and sectioned at 40–50 μm with a vibrating microtome (VT1000S; Leica, Milton Keynes, U.K.). Free-floating sections in 0.1 M PB were collected and stored in a cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal sections at levels −2.30 mm/−3.88 mm [LEC (lateral entorhinal cortex)] posterior to Bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos and Franklin (2004).

**Antibodies**

To detect and determine the changes in the astrocytic cytoskeleton in the EC, we used a monoclonal mouse antiserum against GFAP (anti-GFAP; Sigma–Aldrich; #G3893). For the identification of Aβ aggregation as well as the relation of astrocytic cytoskeleton alteration with Aβ, we used a polyclonal rabbit anti-GFAP serum (Sigma–Aldrich; #G9269) and a monoclonal mouse antiserum against amino acid residues 1–16 of Aβ, which reacts with abnormally processed isoforms and precursor form of Aβ [EFHDS: anti-Aβ 6E10 (SIG-39320), Signet Laboratories, Dedham, MA, U.S.A.]. For the detection of NFTs, we used a monoclonal mouse antiserum against PHF (paired helical filament) tau protein, which recognizes phosphorylated Ser-202/Thr-205 of tau protein (Innogenetics, Zwijndrecht, Belgium). The immunolabelling pattern we obtained with this antibody is equivalent to that obtained previously in different brain regions (Oddo et al., 2003a). Positive and negative control immunohistochemistry was used to test non-specific labelling and/or cross-reaction between antisera derived from different host species, showing no immunoreactivity (data not shown). The specificity of these antisera was confirmed by immunohistochemistry and Western blotting in previous papers (Goedert 1995; Halliday et al., 1996; Rodríguez et al, 2008).

**Immunocytochemistry**

The procedure for immunocytochemistry was as described previously (Olabarria et al., 2010; Noristani et al., 2010). All sections from both non-Tg and 3xTg–AD groups at different ages were processed at the same time using precisely the same experimental conditions to minimize methodological variability. Sections were pretreated with 30% (v/v) methanol and 3% H2O2 in 0.1 M PB for 30 min and subsequently a 1% sodium borohydride (Sigma–Aldrich) solution for 30 min. After washing with PB profusely, sections were then rinsed with 0.1 M TS (Tizma-based saline; Sigma–Aldrich), followed by incubation in 0.5% BSA (Sigma–Aldrich) and 0.25% Triton X-100 (Sigma) for 30 min. The sections were incubated in 0.1% BSA in 0.1 M TS at pH 7.6 and 0.25% Triton X-100 containing the following antibodies: monoclonal mouse anti-GFAP (1:5000; Sigma), monoclonal mouse anti-Aβ (1:1000; Covance, Emeryville, CA, U.S.A.) or monoclonal mouse anti-PHF tau protein (1:1000; Innogenetics) at room temperature (RT) (20–25˚C) for 48 h. The sections were rinsed with TS and incubated in biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Peterborough, U.K.) at RT for 1 h. After rinsing with TS for 30 min, sections were incubated with avidin–biotin peroxidase complex (Vector Laboratories). The peroxidase reaction was carried out by incubation with a solution containing 0.022% DAB (diaminobenzidine; Aldrich) or 0.003% H2O2, or the SG peroxidase substrate kit (Vector Laboratories) for 5 min. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and subsequently xylene, and then permanently coverslipped with the use of Entellan (Merck).

For single fluorescent labelling, the sections were then incubated for 48 h at RT in the primary antibody solution as mentioned above. Sections were then rinsed with 0.1 M TS and incubated in secondary solution containing FITC-conjugated goat anti-mouse IgG (1:100; Jackson Immunoresearch, Baltimore Pike, PA, U.S.A.) for 1 h at RT.

For dual labelling, the sections were incubated for 48 h at RT in a primary antibody cocktail containing (i) monoclonal mouse anti-Aβ antibody (1:1000; Covance) and (ii) rabbit anti-GFAP (1:5000; Sigma) simultaneously. Sections were washed with 0.1 M TS and incubated in rhodamine [TRITC (tetramethylrhodamine β-isothiocyanate)]-conjugated goat anti-rabbit for the detection of GFAP, followed by rinsing with TS. Subsequently, the sections were incubated in FITC-conjugated goat anti-mouse IgG (Invitrogen, Paisley, U.K.) for the detection of Aβ. All the sections were mounted in an aqueous medium (Vectashield; Vector Laboratories).

**Morphological analysis of the astrocytic cytoskeleton**

GFAP-positive astrocytes (n=30–35 in single fluorescent labelling experiments) throughout the layer of the LEC were imaged by using confocal microscopy (Leica SPS, Mannheim, Germany) with 0.2 μm z-step size. Parallel confocal planes were superimposed and Cell Analyst program (Chvatal et al., 2007) was used for analysis. Five digital filters (average 3 × 3, convolution, gauss 5 × 5, despeckle, simple objects removal) with a threshold of 50 were used to determine the surface and volume of the GFAP-positive astrocytic cytoskeleton. When analysing astroglial morphology in relation to Aβ plaques, all cells with somata within 50 μm from the plaque border were regarded as plaque associated, and cells with somata located more distantly (>50 μm) as cells not associated with plaques (Olabarria et al., 2010).

**GFAP-IR (glial fibrillary acidic protein–immunoreactive) cell count in the EC**

To determine the Nv (numerical density) of GFAP-IR astrocytes in the EC, we counted the number of GFAP-IR astrocytes throughout the layers of the whole LEC, an area that permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.
of 435000 µm² in coronal sections with 40 µm thickness in three or four representative non-consecutive sections. Confocal stack images were used for this purpose. GFAP-IR astrocytes were intensely labelled against dark background, which made them easy to identify with equal chances of being counted. The analysis was performed blindly with regard to mouse genotype.

Statistical analysis
Data are expressed as means ± S.E.M. The paired or unpaired Student’s t test was used to examine differences in the Nv, surface, volume and somata volume of GFAP-labelled cells between 3xTg-AD and non-Tg animals, using Graphpad Prism (GraphPad Software, La Jolla, CA, U.S.A.). P < 0.05 was considered statistically significant.

RESULTS
GFAP-IR cells were widely distributed throughout the EC of both non-Tg and 3xTg-AD mice (Figures 2A–2F), with layers I and VI being the ones showing more GFAP-IR cells and stronger immunoreactivity (Figures 2A and 2B). GFAP-IR showed multiple branched processes extending in different directions from elongated cell bodies (Figures 3D–3G).

Astrocytic cytoskeletal atrophy in 3xTg-AD mice
From 1 month of age, we observed morphological remodelling of EC astrocytes in 3xTg-AD mice compared with non-Tg animals. The astrocytes in 3xTg-AD animals had reduced primary branches (on average by 26%) originating from the soma and a markedly reduced presence of secondary processes extending from the primary branches (reduced by 30% on average), as well as distal ramifications (reduced by 45%; Figures 3D–3G). These changes indicate a morphological atrophy which was further confirmed by a significant decrease in both the surface and volume of GFAP profiles in the EC of 3xTg-AD animals when compared with non-Tg controls (Figures 3A and 3B). At younger ages (1 month), astrocytes of 3xTg-AD mice showed a significant decrease in GFAP surface when compared with the control. The surface area of GFAP-IR profiles was reduced by 48% (378.45 ± 64.56 µm² compared with 728.47 ± 96.67 µm²; P = 0.017; Figure 3A). The volume of GFAP-IR cells in 3xTg-AD was reduced by 54% (115.38 ± 25.82 µm³ compared with 252.94 ± 32.73 µm³; P = 0.012; Figure 3B). This atrophic glial appearance was sustained through all ages up to 12 months (Figures 3A and 3B). This reduction was significant at 3 and 6 months of age; the surface area was decreased by 44 and 39% (378.56 ± 64.53 µm² compared with 680.20 ± 53.70 µm², P = 0.036; 354.68 ± 67.74 µm² compared with 581.26 ± 70.13 µm², P = 0.025 respectively; Figure 3A). The volume of GFAP-IR cells decreased by 42 and 42% respectively (130.25 ± 25.82 µm³ compared with 224.32 ± 18.80 µm³, P = 0.0490; 116.15 ± 25.63 µm³ compared with 200.64 ± 31.07 µm³, P = 0.025 respectively; Figure 3B). However, from 9 months of age onwards the degree of atrophic changes became somewhat less pronounced because of parallel atrophic changes developing in healthy aged controls. At 9 months of age, the surface and volume of astrocytes in 3x-Tg-AD animals were reduced, compared with the control, by 26% (391.04 ± 30.75 µm² compared with 529.47 ± 48.21 µm²,
Layer-specific astrocytic atrophy during AD

We found a significant decrease in GFAP-IR cell surface areas in layers II, III and VI of 3xTg-AD animals when compared with controls from 1 month of age by 57% (323.57 ± 53.89 μm² compared with 769.28 ± 180.91 μm²; P = 0.035), 45% (305.36 ± 70.05 μm² compared with 564.42 ± 62.21 μm²; P = 0.031) and 56% (416.65 ± 87.62 μm² compared with 964.04 ± 118.84 μm²; P = 0.007; Figure 4A) respectively. This decrease in the GFAP surface was also paralleled by a reduction in the cell volume in all layers: a reduction in volume of 66% in layer II (84.78 ± 17.82 μm³ compared with 251.43 ± 65.01 μm³; P = 0.028), 68% in layer III (59.57 ± 16.16 μm³ compared with 187.70 ± 26.64 μm³; P = 0.004) and 71% in layer VI (132.08 ± 44.52 μm³ compared with 360.13 ± 38.71 μm³; P = 0.0072; Figure 4B). However, we did not find any significant difference in the other layers, layers I and V.

However, at middle age (6 months), these atrophic changes in surface and volume occurred only in layers II and V. The surface decreased by 38% (320.53 ± 71.52 μm² compared with 520.03 ± 79.62 μm²; P = 0.021) and 41% (386.35 ± 97.00 μm² compared with 650.50 ± 79.62 μm²; P = 0.004) in layers II and V respectively (Figure 4C); the volume decreased by 45% (93.74 ± 24.85 μm³ compared with 169.19 ± 38.39 μm³; P = 0.023) and 42% (130.43 ± 37.92 μm³ compared with 224.85 ± 34.16 μm³; P = 0.011) in layers II and V respectively (Figure 4D). However, no significant decline in either surface or volume was found in layers I, III and VI.

Finally, at later ages (12 months), the decrease in either surface or volume was restricted to layer VI but had a similar significance. 3xTg-AD animals showed a decrease in GFAP surface by 43% (418.81 ± 53.84 μm² compared with 739.91 ± 56.20 μm²; P = 0.007) and in volume by 49%
Astrocytic atrophy in the EC is not associated with loss of GFAP-IR astrocytes

The density of astrocytes in the EC and in different layers of EC of 3xTg-AD mice when compared with non-Tg mice was constant at all ages (Figure 2E). The distribution of GFAP-IR cells throughout the EC was also similar at all ages (Figure 2).

Rare association of astrocytes with Aβ plaques and absence of hypertrophic reaction

The presence of intraneuronal Aβ accumulation in EC is evident from 9 months of age, although plaques and extracellular aggregates are not present until 12 months of age (Figure 5). However, notwithstanding Aβ accumulation, GFAP-IR astrocytes rarely appear to be associated with Aβ-positive neurons, extracellular Aβ aggregates or SPs in the 3xTg-AD animals. All in all, less than 5% of astrocytes were located at a distance <$50 \mu m$ to the SP. Most importantly, however, we failed to observe any signs of hypertrophy in EC astrocytes in 3xTg-AD animals at all ages; astroglial cells always demonstrated atrophic morphology.

DISCUSSION

The EC is regarded as a funnel for cortico-hippocampal information transfer and integration, playing an essential role in cognition and memory (Kerr et al., 2007; Canto et al., 2008; Coutureau and Di Scala, 2009). In the present study, for the first time we present evidence indicating an early (from 1 month of age) atrophy of astrocytes in the EC of 3xTg-AD mice; this atrophy being sustained through more advanced ages (up to 12 months). This atrophic remodelling occurred in the superficial and deep layers at early and middle age (1–6 months), although at later ages (12 months) it remained only in the deeper layers. The morphological atrophy of astroglia is not accompanied by an astrocytic loss at all ages. The generalized atrophy characterized by a major reduction in astrocytic primary branches and massive reduction in secondary and distal processes in the EC appears in very young 3xTg-AD animals (1 months old), which did not show yet any signs of AD pathology. This atrophic appearance remains in later ages (12 months), distinguished by developed Aβ plaques and NFTs, which are confirmed by the immunoreactivity of Aβ and phosphorylated tau protein (Supplementary Figure S1 at http://www.asnneuro.org/an/003/an003e071add.htm; Oddo et al., 2003b; Rodrı´guez et al., 2009b). Consistent reduction in the GFAP expression is in agreement with our previous observations of generalized atrophy of astrocytes in the HC, which similarly appear before the neuropathological marks (from 9 months) and are sustained in later ages (up to 18 months; Rodrı´guez et al., 2009b; Olabarria et al., 2010). Although some post-mortem studies have revealed hypertrophic astrocytes, characteristic of astrogliosis in the EC of AD human patients (Muramori et al., 1998; Porchet et al., 2003, Vanzani et al., 2005), this is only

(149.03 ± 23.85 µm³ compared with 290.03 ± 29.20 µm³; P=0.004; Figures 4E and 4F).

Figure 4 Comparison of astrocytic GFAP EC surface and volume at different ages

The histograms show decreased GFAP surface (A, C, E) and volume (B, D, F) within specific layers between 3xTg-AD animals at the ages of 1, 6 and 12 months respectively. Results are means ± S.E.M. (*P<0.05; **P<0.01 compared with the age-matched non-Tg control).

Figure 5 Distribution and relationship of GFAP immunoreactive astrocytes and β-amyloid presence

Confocal images of dual labelling of GFAP (red) and Aβ (green) show that GFAP-IR astrocytes are distant from intracellular Aβ deposits (with a distance of 86 µm) at 12 months and distant from the Aβ plaques (with a distance of 125 µm) at 18 months. Arrowheads indicate astrocytes.
related to intralaminar astrocytes, since interlaminar astrocytes also showed disrupted processes and dynamic properties (Colombo et al., 2002). Furthermore, microarray analysis also showed a decrease in gene transcription of astrocytic cytoskeleton proteins in AD, implying a down-regulation of the astrocytic cytoskeleton (Simpson et al., 2011). At the same time, the density of GFAP-IR cells remains constant at all ages in both the control and 3Xtg-AD. This indicates that neither aging nor AD pathology triggers cell loss. These data again correspond to our previous study and argue against the prominent astroglial proliferative response in the 3Xtg-AD brain (Olabarria et al., 2010).

Astrocytic atrophy in the EC occurred very early, at 1 month of age. This is somewhat similar to the general observation that the AD pathology is manifested by Aβ accumulation, which begins in neocortex and rhinal cortices, including the EC, subsequently progressing to the HC and eventually appearing in all subcortical areas (Braak and Braak, 1997; Thal et al., 2000). The formation of neurofibrillary tangles interacts in the transentorhinal as well as entorhinal cortices, advancing to the HC through the perforant path and neighbouring cortical regions (Braak et al., 1999). The spatiotemporal occurrence of astroglial atrophy is therefore consistent with pathological hallmarks of AD, indicating that the EC is the region first affected by AD pathology. The evident changes in entorhinal astrocytes appear within layers II, III and VI at early age (1 month); layers II and V are affected at middle age (6 months), while they are restricted in layer VI at later ages (12 months). Nevertheless, it should be noted that there is no recovery of atrophic astrocytes in transgenic mice in the superficial layers at later ages but a decrease in GFAP in non-Tg controls, implying an aging effect on control animals (Figure 3).

Astroglia sustain multiple functions, including balance of neurotransmitters, release of trophic factors, metabolic support and extracellular ion buffering, all of which maintain brain physiology and support neuronal connectivity (Danbolt, 2001; Nedergaard et al., 2003; Wang and Bordey, 2008; Verkhratsky et al., 2010, 2011). In addition, astrocytes are able to sense synaptic activity, regulate synaptic plasticity and synchronize neuronal networks, thus being involved in conscience and cognitive processes (Araque et al., 1999; Fellin et al., 2004; Henneberger et al., 2010; Lalo et al., 2006, 2011). Finally, by virtue of astrogliosis, astrocytes form the innate brain defence system, localizing the lesions and assisting in pathological remodelling of the affected circuitry (Sofroniew, 2009). Early astroglial atrophy observed in EC may result in reduced astroglial coverage of synapses and thus may appear to be a key factor in altered synaptic connectivity (Rodríguez et al., 2009b; Verkhratsky et al., 2010). In consequence, the synaptic condition and/or activity may be affected, causing synaptic remodelling, altered synaptic connections and network activity. It is likely that this early astrocytic atrophy leads to compromised EC output to other areas, especially the HC that receives innervation from both the superficial and deep layers of the EC (Figure 1). In fact, there is no significant neuronal loss in memory-associated areas, such as the EC and HC, in the 3Xtg-AD animal model (Oddo et al., 2003a, 2003b; Rohn et al., 2008; Bittner et al., 2010). Nonetheless, loss of synapses and/or dendritic spines as well as compromised long-term potentiation have been detected in several brain regions, suggesting impaired synaptic connectivity and functions in this animal model (Oddo et al., 2003a; Bertoni-Freddari et al., 2008; Bittner et al., 2010; Noristani et al., 2011). Therefore the astrocytic atrophy in the EC may underlie synaptic pathology being the basis of cognitive and memory impairment at the early stages of the AD.

In contrast with the HC (Olabarria et al., 2010), arrival of Aβ depositions and SPs in the EC does not trigger astroglial reaction at least from the morphological criteria. We failed to observe a close association of astrocytes with SPs; nor did we find any signs of astroglial hypertrophy. This peculiar indifference of EC astrocytes to the AD-specific lesion may underlie the particular vulnerability of the EC to the AD-like pathology and provide an explanation of why EC is the first brain region to be affected in the course of AD. Obviously, further studies are required to extend the findings from the AD animal model to human pathology.

REFERENCES

Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Rabinovici GD, Alafuzoff I, Cesani L, C exported_150768 278 2011 The Author(s) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/). Canto CB, Wouterlood FG, Witter MP (2008) What does the anatomical organization of the entorhinal cortex tell us? Neural Plast 2008: 381243.

Astroglial entorhinal cortex atrophy in Alzheimer's disease


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