Investigating Therapeutic Strategies in a Preclinical Model for Alzheimer’s Disease

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2017

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Final Word Count: 29,364
Abstract

Alzheimer’s disease (AD) is a worldwide, incurable disease, and the most common form of dementia. Numbers of cases are rising, and since its discovery the only approved medications have treated only the symptoms, not the pathological cause.

With the cost to society rising, the debilitating nature of the disease and the pressure put on the family members and support network of patients, disease modifying therapies are in dire need. Current models have proven an invaluable tool with which to study certain aspects of the disease and the genetics behind it, however the lack of clinically approved medications in the last 20 years suggests new models are needed.

Based on the amyloid cascade hypothesis, this thesis initially characterises two models of β-Amyloid oligomer (Aβo) induced cognitive deficits. Both models are created by ICV injection of soluble Aβo into the brain of rat. The models differ only by the molecular weight of the Aβo 1-42, one, referred to as low molecular weight (LMW) Aβo, with stable dimers, trimers and tetramers, the other, referred to as high molecular weight (HMW) Aβo, consisting of assemblies ranging from ~50 to ~150 kDa. It was found that behavioural deficits were similar between the two, with a robust object recognition deficit, but no working memory deficit. Both models also showed a deficit in the synaptic marker PSD-95; however the LMW Aβo caused a deficit in the frontal cortex, whereas the HMW Aβo caused a hippocampal deficit.

The role of the cellular prion protein (PrP<sup>C</sup>) was explored, by blocking its binding to Aβo with the antibody 6D11. Interestingly the two models showed different results. The HMW Aβo deficits were completely blocked by the 6D11 application, however the LMW Aβo deficits were only partially prevented.

Finally, Fasudil, a vasodilator approved in parts of Asia, was used to inhibit Rho-kinase, showing a prevention of the cognitive deficits in the HMW Aβo model.

The results of this thesis show the ICV administration of Aβo to be a useful model for investigating the effects of Aβo, provides a platform with which to study the differing effects of Aβo with different oligomeric assemblies, and a model to test therapeutic strategies with relevance to AD.
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Acknowledgements

I would like to thank Dr Mike Harte for his persistent help and guidance throughout the past 4 years. Thank you to the members of the lab group and office past and present for keeping me sane throughout the most testing of times, and for sharing their laughs and tears. Thank you Andy, Antonio, Bushra, Chlœ, Daniela, Giovanni, Harry, James, Jo, Jojo, Kathryn, Katie, Marianne, Matt, Maurizio, Meg, Michelle, Naz, Sara, and Will.

I would also like to thank the team members who have helped with data collection at some points. Many thanks to William Watremez, Heledd Jarosz-Griffith, Andrew Hayward, Bushra Almari, Giovanni Podda, Chlœ Piercy, Victoria Fasolino and Claire White. Thank you to Professor Nigel Hooper, Dr Richard Killick, and the team at King’s for their input.

Thanks to Alzheimer’s Research UK, the Alzheimer’s Drug Discovery Foundation, the Experimental Psychology Society, and the Faculty of Biology, Medicine and Health for giving me the opportunity to present my work around the world.

Thank you to all my friends, too numerous to mention, for the encouragement you gave me, especially in the last months whilst writing up.

Finally, thank you to my family, who have supported me so that I could reach this point. I wouldn’t have got here without you.
Chapter 1: General Introduction
1.1 Disease and theories of mechanism

1.1.1 Alzheimer’s disease

It is estimated that there are over 24 million people with dementia worldwide (Ferri et al. 2005; Reitz & Mayeux 2014), the majority of which being caused by Alzheimer’s disease (AD) (Rizzi et al. 2014). With this number expected to rise and the debilitating and incurable nature of the disease, AD is a worldwide health concern, both from a humanitarian and an economic point of view.

AD is a progressive neurodegenerative disease first characterised by Alois Alzheimer in 1906, who was presented with a 51 year old female patient with a progressive mental illness. He described her deteriorating memory and behaviours, noting that there were no severe abnormalities of her motor skills and reflexes until close to her death, four and a half years after she was first diagnosed with an illness. Post-mortem many abnormalities of the brain tissue were noted, including neuroinflammation, and what we now know to be the hallmarks of AD: dense, insoluble extracellular amyloid plaques, and intracellular insoluble twisted fibres, neurofibrillary tangles (NFTs) (Tiraboschi et al. 2004), formed from paired helical filaments (PHFs) of tau, a microtubule-associated protein. Although this patient was suffering at the age of 51, most frequently AD is diagnosed in people over the age of 65 (Brookmeyer et al. 1998). The patient described appears to have a rare form known as early onset Alzheimer’s which may occur at an earlier age than the majority of AD cases. Early symptoms
often appear to be those of normal ageing or stress, and are commonly mistaken as such (Waldemar et al. 2007).

In a review of cognitive markers Bäckman et al. (2004) suggest that many at risk individuals have impairments in multiple cognitive domains several years prior to a clinical diagnosis of AD, during what is referred to as the “pre-clinical stage”, or Mild Cognitive Impairment (MCI). The most informative measures to identify individuals who are at high risk appear to be executive functioning, episodic memory and perceptual speed. The severity of measured impairments is important, as these areas are known to decline in normal ageing. Diagnosis of the pre-clinical stage is limited by the overlap of cognitive scores between individuals who will, and individuals who will not, go on to be clinically diagnosed with AD. Nygård (2003) suggests that complex daily activities are affected, and that these changes should be incorporated into the diagnosis of MCI. It should be noted that although individuals with MCI are at a higher risk to develop AD (10-15%/year compared to 1-2% for general population) not all MCI individuals go on to develop full AD symptoms (Petersen 2007). The ability to reliably diagnose and predict early AD is therefore limited, and if the goal to treat AD at an early stage is to be achieved, a more reliable diagnostic tool needs to be developed. There have been many attempts at finding early biomarkers of AD, or characterising either blood or cerebrospinal fluid (CSF) markers so that diagnoses can be made on living patients before the onset of moderate to severe cognitive symptoms. Whilst CSF markers are currently used to diagnose AD when dementia is present, more work is required to assess their ability to predict AD in the prodromal stage (Lovestone 2014). Recently, a predictive blood profile has
been characterised with the ability to predict progression to AD from the prodromal stage with an accuracy of 87%, sensitivity of 85%, and a specificity of 88% (Hye et al. 2014), however, more work is required to investigate whether this profile fits with accuracy in other cohorts.

With increasing cognitive impairment, a patient may be diagnosed with AD. In the early stages, patients show memory impairment and may suffer loss of language skills, as was the case with Alzheimer’s original patient. The individual may still be capable of living independently for most of the time, but will likely require aid with some organisational matters (Förstl & Kurz 1999). Once the disease progresses from mild AD to a more moderate stage, patients’ memory formation will increasingly deteriorate, however they may more easily recall older memories, causing them to “live in the past” (Beatty et al. 1988). There are increasing issues with language, reading and writing skills and patients begin to lose the ability to comprehend their condition and eventually the ability to perform normal routine tasks, such as dressing and eating. Mood changes may occur, causing strain on family relations, as well as visual hallucinations in around a fifth of cases (Förstl & Kurz 1999). The disease will eventually progress to a severe state, where language is almost entirely lost and patients are often unable to carry out the most basic of tasks, including chewing and swallowing unaided, and motor function is significantly impaired (Förstl & Kurz 1999). In 1991, Braak & Braak devised a way of categorising the stages of AD progression in relation to the underlying neuropathology, based not on amyloid deposits, which they found had high variability between patients, but on NFTs, which showed clear patterns, allowing the
differen
tiation of stages. Six stages were determined. Stages I and II relate to NFTs
confinement to the transentorhinal region, III and IV are used when the limbic region is
affected, and stages V and VI are when the neocortex is extensively involved. These stages
appear to correlate with the cognitive domains affected by AD, for example the
transentorhinal region thought to be involved in memory formation and which is the first
area to be pathologically affected, and a loss of memory formation is one of the first
symptoms with which patients present. Additionally the later dysfunction of executive
function and speech is mirrored in the NFT formation in the cortical areas responsible for
these processes (Nelson et al. 2009). Although these stages can show a tight correlation with
neuropathology at autopsy, and cognitive function prior to death, it is not uncommon to see
both plaques and tangles without dementia. Additionally there are dementias that present
without the presence of either plaques or tangles (Nelson et al. 2009), therefore the
mechanisms by which these histological hallmarks may cause disease progression need to be
investigated further.
1.1.2 Theories of mechanism

1.1.2.1 The amyloid hypothesis

In 1984, β-amyloid (Aβ) was identified as the main component of the plaques seen in AD (Glenner & Wong 1984). Additionally, the main protein in the plaques seen in Down’s syndrome (DS) patients, who often exhibit AD like behaviour by middle age, was also shown to be Aβ (Masters et al. 1985). DS patients have an extra copy of chromosome 21 (Lott & Head 2005; Nistor et al. 2007), which has been shown to contain the gene encoding for Amyloid Precursor Protein (APP) (Hardy 1992). APP is an integral membrane protein, found in a variety of tissues, but concentrated in synapses (Priller et al. 2006). Although its function is unknown, it is thought to be involved in synaptic contact (Schubert et al. 1991) and formation (Priller et al. 2006) and neuronal plasticity (Turner et al. 2003). It undergoes extensive post-translational modification, including cleavage extracellularly by β-secretase and α-secretase, and in the membrane domain by γ-secretase (Figure 1). α and γ-secretase cleavage results in soluble APPα (sAPPα) and a fragment of Aβ known as p3 peptide, which does not assemble into oligomers (Dulin et al. 2008). It is the cleavage of APP by β and γ-secretase that initiates the amyloidogenic pathway, and generates soluble APPβ (sAPPβ) and a fragment of Aβ, 36-42 amino acids in length (Hardy & Selkoe 2002). BACE1 has been shown to encode for β-secretase activity, and in knock-out studies, deletion of this gene abolishes Aβ generation and deposition, prevents neuronal loss, and reverses memory deficits in 5XFAD APP/PS1 transgenic mice (Ohno et al. 2007). The γ-secretase complex consists of four proteins: presenilin, nicastrin, presenilin enhancer 2 (PEN-2) and anterior pharynx-defective 1
Investigating therapeutic strategies in a preclinical model for Alzheimer’s Disease

(APH-1). In 1992 Hardy & Higgins presented the Amyloid Hypothesis, which suggests that the accumulation of Aβ generated in this way is the fundamental neuropathological catalyst in AD pathogenesis. In support of this, transgenic mouse models expressing mutations in APP and Presenilin 1 have been shown to increase the levels of amyloid plaques, total Aβ, and cognitive defects (Hsiao et al. 1996). As mentioned, DS patients exhibit AD like pathology, suggesting that an extra copy of the APP gene may be sufficient to cause AD. Subsequent genetic work has also shown that most known mutations that cause early-onset AD result in an increase of Aβ (Tanzi & Bertram 2005).

Despite an initial focus on amyloid plaques, little translational benefit has been seen as a result of this research, and more recently the focus has been on the soluble forms of Aβ. There is increasing evidence to suggest that these soluble oligomers are the more toxic form of Aβ (Zhao et al. 2012), as they have been shown to inhibit synaptic transmission (Moreno et al. 2009) and disrupt axonal transport (Pigino et al. 2009). β-amyloid oligomers (Aβo) have been show to correlate with cognitive decline (Jongbloed et al. 2015), a correlation much stronger than between Aβ plaques and cognition (Nelson et al. 2012; Morris et al. 2014). The different oligomers and their mechanisms will be discussed and explored in subsequent results chapters.
1.1.2.2 Tau as the main causative factor

The other main hallmark of AD is the NFTs composed of tau filaments that form intracellularly. As these tangles appear to correlate with cognitive decline and neuronal loss better than amyloid plaques, tau has attracted considerable attention, and several mechanisms have been suggested. This hypothesis suggests that tau molecules are somehow hyperphosphorylated at specific sites, which causes them to bind to other tau molecules, forming intracellular neurofibrillary tangles (Goedert et al. 1991). As tau is an important

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*Figure 1 APP Processing. APP is cleaved at the γ-site, and either the α-site, resulting in sAPPα and p3, or β-site, resulting in sAPPβ and Aβ production.*
microtubule protein, its aggregation and dysfunction causes the collapse of the neuronal transport system (Iqbal et al. 2005). This results in the inability of the cell body to communicate and transport molecules to and from the periphery of the cell, inhibit cell communication, and results in cell death (Chun & Johnson 2006). As neurons are more elongated than regular cells, they are the most affected by the inhibited tau function. There are six isoforms of tau in human brains as a result of alternative splicing at exons 2, 3 and 10. They can have either 0, 1 or 2 inserts at the N-terminal, and 3 or 4 repeats at the C-terminal (Zhong et al. 2012). The longest isoform is known as 2N4R, and the shortest as 0N3R (Figure 2). The location and conformation of tau is modulated by these different isoforms, and therefore the function between them can differ, and the availability and affinity for ligands and microtubules depends on these changes (Andreadis 2012). These isoforms may, therefore, play different roles in AD and other tauopathies. Although it is generally agreed that the dysfunction of tau is important in the progression of AD, most researchers believe that tau lies downstream of Aβ in the cascade (Mudher & Lovestone 2002).
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1.1.2.3 The role of Inflammation

In AD there are molecular changes which suggest that there is immune and inflammatory activation (Wyss-Coray & Rogers 2012). Whilst this is supported by some evidence that use of non-steroidal anti-inflammatory drugs (NSAIDs) has a benefit to patients with the disease, most long term, placebo controlled clinical studies have not shown benefit from the use of these drugs, possibly as they are administered too late in the disease progression and pathological process to have a substantive effect (Imbimbo et al. 2010). These drugs may be useful as a prevention tool, but have so far shown no effect on slowing disease progression. Whether the inflammation is a response or a causative factor in AD is unknown, but there is some evidence to suggest that it may play a role in the pathology (Figure 3), in a positive feedback loop with Aβ production and interacting with APOE (Imbimbo et al. 2010). Recent evidence has suggested that only a certain class of NSAIDs, the fenamates, are of benefit to

---

Figure 2 Tau isoforms. (Taken from Wang & Mandelkow 2015). Showing the alternative splicing which results in the 6 isoforms found in the brains of man.
the disease, acting via inhibition of the NLRP3 inflammasome (Daniels et al. 2016). It is believed that Aβ in the brain can activate microglia (Prinz et al. 2011). In 2013, two independent groups conducted Genome-wide Association Studies (GWAS) highlighting TREM2 mutations as a risk factor for late-stage AD (Guerreiro et al. 2013; Jonsson et al. 2012). This mutation carries a risk factor similar in size to that of APOE-e4. The TREM2 gene codes for the protein Triggering receptor expressed on myeloid cells 2 (TREM2). As its name suggests, this protein is expressed in brain microglia and, on the assumption that the mutations causes a loss of function, it is suggested that it normally aids in the clearance of Aβ. Its loss of function could cause the microglia to activate in a pro-inflammatory phase, and may be part of the increased inflammation we see in AD (Niemitz 2013). Whether the Aβ directly affects TREM2 is unknown, but with earlier evidence that Aβ does in fact activate the microglia (Prinz et al. 2011), it is conceivable that a build-up of Aβ may inhibit TREM2, causing less Aβ clearance and inflammation, and starting a self-activating cycle.

More evidence suggesting the role of inflammation in AD comes in the form of knock-out experiments. NLRP3 is an inflammasome which activates Caspase-1, which in turn is required for the activation of interleukin-1β (IL-1β). IL-1β is produced by activated microglia, and plays an important role in the pro-inflammatory response (Entrez Gene, 2013). Heneka et al. (2013) showed that both NLRP3 and Caspase-1 knock-out APP/PS1 positive mice were protected against cognitive deficits, and had increased amyloid phagocytosis.
Figure 3 Possible interplay between Aβ and inflammation (taken from Imbimbo et al. 2010) Showing a feedback loop for Aβ production via inflammation. C1q, C3b, C3a and MAC (membrane attack complex) are complement factors, cytokines include interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and TGF-β, ApoE is apolipoprotein E and α1-antichymotrypsin (α1-ACT) is an acute phase reactive protein.

1.1.2.4 ApoE ε4

In 1993, analysis of apoliprotein E (ApoE) in AD patients and controls revealed that the type 4 variant (ApoE ε4) was highly associated with development of AD (Strittmatter et al. 1993), and consequently a lot of research has since been focused on its function and allele frequency in differing populations. Meta-analysis has shown that Caucasian and Japanese individuals homozygous for the ε4 allele were up to 30 times more likely to develop the disease than non-carriers. Although this allele causes an increased risk for AD, there is
evidence that one allele, ε2, has a protective role. Heterozygous ε2/ε3 carriers have a low risk, ε3/ε4 have a slight increase in risk, and interestingly ε2/ε4 carriers have the same risk as ε3/ε3, considered normal, suggesting that the protective effects of ε2 negate the damaging effects of ε4 (Table 1) (Corder et al. 1994). The exact function of ApoE is unknown, but it is thought that it aids in the proteolytic breakdown of Aβ, and that the ε4 allele is not as effective at this as the other alleles (Jiang et al. 2008), adding to the Amyloid cascade hypothesis, and the evidence that Aβ is a major cause of AD pathology.

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<td>1.1</td>
<td>1</td>
<td>4.4</td>
<td>19.3</td>
</tr>
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</table>

*Table 1 ApoE allele risk in unrelated subjects (Data taken from Corder et al. 1994).*

### 1.1.2.5 The Cholinergic theory

The oldest theory of AD suggests a deregulation of acetylcholine (Ach) (Francis et al. 1999), and most drugs licensed today aim to compensate for the deficit of Ach. These drugs have shown some benefits in treating the cognitive deficits in AD, but do little to target the underlying mechanisms or slow the progression of the disease (Birks & Harvey 2006). As a result this theory is no longer widely accepted.
1.1.2.6 Wnt signalling

One potential link between Aβ and tau is the Wnt family and its signalling pathways (Figure 4). The members of the Wnt family of proteins are secreted, lipid-modified, cysteine-rich glycoproteins, 350-400 amino acids in length, and are evolutionarily conserved (Cadigan & Nusse 1997; Wodarz & Nusse 1998). They play an integral role in cell adhesion and determination, as well as in the development of both vertebrates and invertebrates. More relevant to AD, they also have a key role in synaptic assembly, function and remodelling mediated by activity (Wodarz & Nusse 1998; Gogolla et al. 2009; Inestrosa & Arenas 2010). This is especially interesting, as the loss of synapses is seen prior to neuronal cell death caused by Aβ (Hsia et al. 1999). Wnt can signal in several recognised pathways, although the three major ones are the Planar Cell Polarity (PCP) pathway, the Wnt/Ca2+ pathway and the most studied Canonical pathway (Moon et al. 2002; Semenov et al. 2007). In the Canonical pathway, Wnt binds to Frizzled (Fz) and LRP5/6 via dishevelled (Dvl) to form the Fz-LRP5/6 complex, and promotes LRP6 phosphorylation via GSK3 (a protein known to phosphorylate tau) and CK1. This results in the inhibition of β-catenin degradation. β-catenin has several functions, but one of them is to control the expression of Dickkopf-1 (Dkk-1) (MacDonald et al. 2007), a known canonical Wnt signalling antagonist (Kawano & Kypta 2003). Additionally, canonical pathway activation leads to the inhibition of GSK3β (Alvarez et al. 2004). Dkk-1 has been shown to inhibit the canonical pathway by binding to LRPs (Caricasole 2004) and preventing the formation of the Fz-LRP5/6 complex. Interestingly Dkk-1 has been shown to be upregulated in the AD brain as a result of Aβ exposure, it promotes the phosphorylation
of tau (Caricasole 2004) via p53 (Wang et al. 2000). Work by Purro et al. (2012) have shown that Dkk-1 is required for Aβ mediated synaptic loss, highlighting Dkk-1 as an interesting target for therapy. The CLU gene has been suggested as a risk gene for sporadic AD, and recently its product, clusterin, has been shown to regulate the toxicity of Aβ via Dkk-1 (Killick et al. 2014). It is suggested that Dkk-1 induces the PCP pathway, which is activated by Wnt activation of Fz only, and results in the activation of several pathways regulating the actin cytoskeleton and cell adhesion (Semenov et al. 2007). As a result of this, Early growth response protein 1 (EGR-1) could be activated by c-jun (Hoffmann et al. 2008), activating CDK5 and promoting tau phosphorylation (Lu et al. 2011). The mechanisms of the different Wnt pathways could be of extreme importance to AD, and a better understanding of the protein interactions involved could help identify therapeutic targets, and this will be explored further in Chapter 6.

Figure 4 Potential links between Aβ and Tau via Wnt signalling. Aβ causes an increase in DKK-1, causing the canonical pathway to be inhibited, and the PCP pathway to be activated.
1.1.2.7 PrP\textsubscript{c}

Cellular prion protein (PrP\textsubscript{c}) has long been of interest in the field of neurodegeneration, not only for its role in Creutzfeldt–Jakob disease, but it has also been reported to co-localise with A\textsubscript{β} plaques in AD brains (Voigtländer \textit{et al.} 2001) and in transgenic mice (Schwarze-Eicker \textit{et al.} 2005) promoting the theory that PrP\textsubscript{c} increases A\textsubscript{β} aggregation (Schwarze-Eicker \textit{et al.} 2005). However there is evidence that it may also be involved in APP processing, perhaps via inhibition of β-secretase 1 (BACE1), thereby decreasing A\textsubscript{β} production (Kellett & Hooper 2009). It has been observed that in the early stages of AD, PrP\textsubscript{c} levels are increased. This might suggest a pathological role for PrP\textsubscript{c} in AD, however, within AD high PrP\textsubscript{c} levels correlate with lower phosphorylated tau levels (Vergara \textit{et al.} 2014), perhaps due to a decrease in A\textsubscript{β} induced fyn activation (Larson \textit{et al.} 2012). PrP\textsubscript{c} is thought to require metabotropic glutamate receptor 5 (mGluR5) coupling for the activation of fyn (Um \textit{et al.} 2013), which can alter N-methyl-d-aspartate receptor (NMDA-R) function, the phosphorylation of tau and links extracellular glutamate to protein translation in dendrites (Figure 5) (Nygaard \textit{et al.} 2014).

It appears that PrP\textsubscript{c} plays a protective role in AD, reducing pathological processing of APP and tau misphosphorylation, but the disruption of PrP\textsubscript{c} via interaction with A\textsubscript{β} is thought to contribute to the cognitive decline seen in the disease (Laurén 2014). It is therefore considered a therapeutic target in AD, and this will be explored further in Chapter 5.
Figure 5 Aβ signals via PrP<sup>C</sup>-mGluR5 coupling, (taken from Nygaard et al. 2014). Aβ binding to PrP<sup>C</sup> causes mGluR5 to alter NMDA-R function, tau phosphorylation and protein translation via fyn.

1.1.3 Current Treatments.

As mentioned earlier, four out of the five pharmacological treatments used for AD are acetylcholinesterase inhibitors aimed at boosting the amount of acetylcholine in the brain, with the fifth being an NMDA-R antagonist (Pohanka 2011). None of these five treatment options have a significant effect on the delay of onset, nor prevent disease progression, and a significant proportion of patients suffer from side effects (Birks & Harvey 2006).
Due to the lack of effective pharmaceutical intervention and the debilitating nature of the disease, there is an increasing cost to society in the form of psychosocial therapy and care giving. It is estimated that in the UK alone, AD costs the economy £23 billion a year (ARUK, 2012) and with an aging population, this figure is only going to increase without effective management or prevention. There are a number of models with which to test new pharmacological agents, however most promising compounds do not appear to translate from these models to humans. New models may provide a better system with which to test new drugs.

1.2 Current models

In order to better understand the mechanisms of AD, and to test possible drugs and molecular targets with cognitive output, a number of animal models have been created for aspects of the disease. By far the most common method is transgenic mouse models.

1.2.1 Transgenics

Mouse lines containing mutant copies of human genes are generated in order to study the effects of the genes in living organisms.

1.2.1.1 APP mutations

As current theories of AD mechanisms predict that amyloid is an upstream molecule of AD pathology, and is a product of APP processing, many transgenic lines have been produced
with APP mutations. Indeed the first successful transgenic mouse model was created to overexpress mutant human APP, which had a single amino acid change (phenylalanine for valine at 717), which caused the mouse to develop multiple AD-like pathology, including plaque formation, microgliosis and synaptic loss (Games et al. 1995). This line has also demonstrated memory loss similar to that seen in AD (Kobayashi & Chen 2005). The creation of this model was a significant boost to the Amyloid Hypothesis.

Additional APP mutant mice have been created, such as the Tg2576 which overexpress the Swedish double mutant form of APP (Bryan et al. 2009). Although a number of these animals suffered from motor problems, elimination of these animals in the analysis shows that there are memory, and other cognitive deficits, in this strain compared to controls (Kobayashi & Chen 2005). Both these strains of mice show an inability to distinguish between contextual cues in fear conditioning (Corcoran et al. 2002). Fear conditioning is thought to involve the amygdala (LeDoux 2003) and hippocampus, both of which are affected in AD (Poulin et al. 2011; Mu & Gage 2011).

There is now a wide variety of mice with APP mutations. Mice with different genetic backgrounds can respond differently with identical mutations, so it is important to consider not only the gene being manipulated, but also the strain of mouse, when using these models for research.
1.2.1.2 Presenilin Mutations

In addition to mutations in APP, there are also mouse models with mutated Presenilin1 (PS1) and Presenilin2 (PS2). Presenilins are a family of proteins, and comprise part of the γ-secretase complex which cleaves APP within the membrane-spanning domain (Tabaton et al. 2010). Mutations in these proteins can lead to abnormal APP cleavage and accumulation of Aβ in humans (Brunkan & Goate 2005). Initial attempts to create a knock-out PS1 mouse resulted in all the mice dying shortly after birth, with enormous neuronal loss and impaired neurogenesis, suggesting PS1 plays a vital role in neuronal survival and neurogenesis (Shen et al. 1997). There are now several PS1 and PS2 knock-out models that survive, and all show cognitive deficits, but not to the extent of Tg2576 animals (Bryan et al. 2009). Over expressing mutant Presenilins results in an increase of Aβ production, but does not produce plaques or changes in behaviour (Spires & Hyman 2005).

1.2.1.3 Tau Mutations

The other hallmark of AD, NFTs, has been modelled using transgenic mice with tau mutations. Initially overexpression of 4R human tau was used, resulting in the hyperphosphorylation of tau and somatodendritic localisation, but no NFT formation was seen (Götz et al. 1995). This suggests that an increase in tau is not enough to induce AD-like pathology. However overexpression of 3R human tau can develop small amounts of NFTs in extremely old mice (Ishihara et al. 2001). Conversely, no evidence of hyperphosphorylation
or NFTs was found in mice expressing all six isoforms of Human tau (Duff et al. 2000), however when crossed with tau knock-out mice, cell loss and lesions were seen.

As these results did not model human AD well, pathogenic mutations of tau were introduced to mice. A Frontotemporal dementia with parkinsonism-17 (FTDP-17) associated gene caused NFT formation in several areas of the CNS and PNS, along with cell loss and motor deficits (Lewis et al. 2000). Mice with the same mutation have also been shown to correlate NFT numbers with decline in memory tasks and cognitive decline (Arendash et al. 2004; Ramsden et al. 2005).

These models have allowed researchers to inject Aβ into the hippocampus and observe the effects on NFT formation, which is shown to be increased by Aβ 1-42 (Gotz 2001).

1.2.1.4 Combining Transgenics

By combining both APP (Tg2576) and PS1 (PS1M146L) mutations, Holcomb et al. (1998) were able to produce animals that better mimicked the effects and timescale of AD. They showed increased Aβ deposition earlier than single transgenics, and an increase in soluble Aβ. The increase in Aβ seen was greater than the combined amounts seen in single transgenic APP or PS1 mice, suggesting that there is an interaction which greatly enhances AD-like pathology. Unlike single transgenic Tg2576 mice, younger double mutants perform as well as controls with the deficits appearing only in older mice on most cognitive tests, with the exception of the Y-maze where both old and young animals present with deficits when compared to controls (Bryan et al. 2009). This model also showed a cognitive decline that preceded Aβ
deposition, which supports the now accepted theory that soluble Aβ is a toxic species, and synaptic deficits and cognitive decline precede plaque formation in AD.

Triple transgenics, combining tau, presenilin and APP mutations show the most complete transgenic model for AD, developing plaques and subsequently NFTs in the hippocampus following plaque formation in the neocortex. These mice also have Long-Term Potentiation (LTP) deficits prior to plaque and NFT formation, (Oddo et al. 2003), and Morris Water Maze (MWM) and Novel Object Recognition (NOR) tasks deficits (Cantarella et al. 2015)

1.2.1.5 The relevance and usefulness of transgenic animals.

A number of promising therapeutic targets have arisen from transgenic research, with a number progressing to clinical trials. Main approaches include removal of plaques and NFTs, and a reduction in their formation to begin with via, for example, inhibition of γ-secretase. While there have been some promising results in the inhibition of γ-secretase, as previously mentioned total knock-out results in unviable offspring (Shen et al. 1997), so great caution must be taken. Despite a number of promising targets, there is as of yet, no viable treatment for AD. Although no direct benefit in the form of treatment has come from these models, their study has still contributed to the overall knowledge of potentially relevant genes and their interactions and the basic biology of AD related mechanisms and testing of novel molecules.
In recent years there has been the development of a double transgenic AD model in rat (Cohen et al. 2013). This model, with mutant APP (APPsw) and presenilin 1 (PS1ΔE9) genes, presents with age dependent amyloidosis, tauopathy, gliosis and neuronal loss in a manner similar to AD. This new generation of model presents an exciting avenue with which to explore the roles of these genes, as the rat physiology is more relevant to that of man in comparison to mouse (Do Carmo & Cuello 2013), there is a chance that discoveries will be made with this model that cannot be found in the mouse.

Current models do not accurately model the entirety of the disease, focussing mainly on amyloid deposition and NFT expression. Until a greater understanding of the disease is gained, we will only be able to model aspects of AD. It is not known that the plaques and NFT formation are not a compensatory response to an upstream event, and therefore focussing on their removal could be irrelevant to disease progression, or worse, contribute to the cognitive decline.

1.2.2 Oligomer models

Due to the increasingly accepted view that Aβ oligomers are the toxic species in AD, attempts to model their mechanisms and pathology synthetically have been made. Synthetic Aβ oligomers can come in a variety of assemblies (summarised in Table 2)
<table>
<thead>
<tr>
<th>Oligomeric assembly</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protofibril (PF)</td>
<td>Intermediates of synthetic Aβ fibrillization; up to 150 nm in length and ~5 nm in width; β-sheet structure: bind Congo red and Thioflavin T</td>
</tr>
<tr>
<td>Annular assemblies</td>
<td>Doughnut-like structures of synthetic Aβ; outer diameter of ~8–12 nm; inner diameter of ~2.0–2.5 nm</td>
</tr>
<tr>
<td>Aβ-derived diffusible ligands (ADDLs)</td>
<td>Synthetic Aβ oligomers smaller than annuli; may affect neural signal transduction pathways</td>
</tr>
<tr>
<td>Aβ*56</td>
<td>Apparent dodecamer of endogenous brain Aβ; detected in the brains of an APP transgenic mouse line and may correlate with memory loss</td>
</tr>
<tr>
<td>Secreted soluble Aβ dimers and trimers</td>
<td>Produced by cultured cells; resistant to SDS; resistant to the Aβ-degrading protease IDE; alter synaptic structure and function</td>
</tr>
</tbody>
</table>

Table 2 Oligomeric assemblies of Aβ, (adapted from Haass & Selkoe 2007)

There have been a number of proposed models of the toxicity and cognitive effects of Aβ in both rat and mouse models, as well as observations of the direct effect of Aβ on cell cultures, for example primary cultured neurons from mice and rats (Killick et al. 2014), however for the purposes of reviewing animal behaviour effects, these have not been considered. As mentioned above, there are different assemblies of synthetic Aβ oligomers, and on top of this, there are also different forms of Aβ used. Most commonly used are Aβ 1-42, 1-40 and 25-35. Whilst 1-40 and 1-42 are recognised to be commonly found in the brain, 25-35 has been suggested to contain the "active" portion of Aβ (Kubo et al. 2002) and it is known to polymerise into amyloid-like fibrils and cause a reduction of cell survival, most likely by apoptosis (Forloni et al. 1993). It has been suggested that Aβ 1-42 is more toxic than 1-40, and that both forms are broken down into Aβ 25-35 (Kubo et al. 2002).
Because of this inconsistency and lack of understanding in what form of Aβ is responsible for the changes seen in AD (if any) a number of models use different forms of Aβ in different animals. Even in studies which use the same animal and Aβo species, often the method of delivery differs, and so it is hard to find results which entirely support each other. An attempt to catalogue a number of different models, with their species, peptide, method of administration and cognitive and neuropathological effects can be seen in Table 3. Most of these models fail to induce the aggregation of Aβ and NFTs (Lawlor & Young 2011), so the models’ use on the reduction of these aggregates is limited, however, as a tool to study the interactions of soluble Aβ, at known concentrations and time points, and its effects on a cognitive level, it is unparalleled.

Whilst controversial, the findings on oligomer models do agree in a few areas (Table 3). The majority do find memory impairments of some kind, usually hippocampal related (Nabeshima & Nitta 1994; Yamada et al. 1999; Frautschy et al. 2001; Nakamura et al. 2001; Nag et al. 2001; Lecanu et al. 2006; Malm et al. 2006; Liu et al. 2008; Brouillette et al. 2012; Frozza et al. 2013; Zussy et al. 2013) and synaptic deficits (Frautschy et al. 2001; Frozza et al. 2013) are commonly seen. Several studies also saw an inflammatory response (Frautschy et al. 2001; Malm et al. 2006; Frozza et al. 2013; Zussy et al. 2013), and several saw changes in tau phosphorylation (Lecanu et al. 2006; Brouillette et al. 2012; Zussy et al. 2013) and neuronal loss (Malin et al. 2001; Lecanu et al. 2006; Zussy et al. 2013). Conversely others saw only minimal neuronal loss in response to Aβ0 injection (Morimoto et al. 1998). It is not
always clear if the studies did not see controversial findings, or if they were simply not reported.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Species</th>
<th>Peptide</th>
<th>Method of delivery</th>
<th>Study length</th>
<th>Effects of Aβo</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zussy et al.</td>
<td>Rat, Sprague-Dawley, Male</td>
<td>25-35</td>
<td>Acute 10 mg ICV</td>
<td>6 weeks</td>
<td>• ↑ APP expression tau phosphorylation, Aβ1–42 generation.</td>
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<td></td>
<td>• Short- and long-term memory impairments.</td>
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<td>• ↑ corticosterone plasma levels.</td>
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<td>• ↑ brain oxidative (lipid peroxidation), mitochondrial (caspase-9 levels) and reticulum stress (caspase-12 levels).</td>
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<td></td>
<td></td>
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<td></td>
<td>• Astroglia &amp; microglial activation.</td>
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<tr>
<td>Morimoto et al.</td>
<td>Rat, Sprague-Dawley, Male</td>
<td>25-35</td>
<td>Acute HC injection</td>
<td>14 days</td>
<td>• Each Aβ species caused neuronal loss.</td>
<td>Dizocilpine maleate reversed neuronal loss Animals also injected with ibotenic acid</td>
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<td></td>
<td></td>
<td>1-40</td>
<td></td>
<td></td>
<td>• Astroglial &amp; microglial activation.</td>
<td></td>
</tr>
<tr>
<td>Brouillette et al. 2012</td>
<td>Mouse, C57BL/6</td>
<td>1-42</td>
<td>Injected HC via cannula 2ul over 5 mins for 6 days</td>
<td>7 days from 1st injection</td>
<td>• Neuronal loss.</td>
<td>TTR reduced neuronal loss and memory deficits</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Daily injection to HC via Cannula</td>
<td>1 month</td>
<td>• Tau hyperphosphorylation.</td>
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<td></td>
<td>• Deficits in hippocampus-dependent memory.</td>
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<tr>
<td>Frautschy et al.</td>
<td>Rat, Sprague-Dawley, Female</td>
<td>1-42</td>
<td>Acute I CV</td>
<td>15 days</td>
<td>• Oxidative damage.</td>
<td>Curcumin reduced most effects of Aβ</td>
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<td>• Synaptophysin loss.</td>
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<td></td>
<td>• Microglial response.</td>
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<td></td>
<td>• Aβ deposits.</td>
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<td>• MWM deficits.</td>
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<td>• ↓ PSD-95.</td>
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<tr>
<td>Frozza et al.</td>
<td>Rat, Wistar, Male</td>
<td>1-42</td>
<td>Cannula in right ventricle, Continuous infusion (300 pmol/day)</td>
<td>14 days</td>
<td>• ↓ Y-maze performance</td>
<td>Resveratrol reversed most effects</td>
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<td></td>
<td>• Synaptic loss.</td>
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<td>• Astroglia &amp; microglial activation.</td>
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<td></td>
<td>• ↑ JNK &amp; GSK-3β activation</td>
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<tr>
<td>Yamada et al.</td>
<td>Rat, Wistar, Male</td>
<td>1-42</td>
<td>Cannula in right ventricle, Continuous infusion (300 pmol/day)</td>
<td>14 days</td>
<td>• Y-maze and MWM impairments.</td>
<td>Idebenone and α-tocopherol prevented the behavioural deficits</td>
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<td></td>
<td>• Passive avoidance impairments.</td>
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<td>Study</td>
<td>Species, Strain</td>
<td>Treatment Details</td>
<td>Duration</td>
<td>Behavioral Effects</td>
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</table>
| Nakamura et al. 2001       | Rat, Fischer, Male                       | Continuous infusion for 3 days in right ventricle 10 or 20 µg/rat | 20 days and 80 days | • Dose and time dependent impairments in Y-maze, MWM and passive avoidance retention  
  • ↓ in HC-3 binding in HC  
  • Ventricular enlargement and neuronal damage in the CA1  
  Learning impairments worse at 80 days than 20 |
| Liu et al. 2008            | Rat, Wistar                             | 5µg ICV injection  | 21 days  | • Astrocyte activation, ↓ levels of the a7 and a4 protein subunits of nAChR, and ↑ expression of a7 mRNA, Impaired learning and spatial memory (MWM)  
  High-Cholesterol diet potentiated effects on L&M |
| Malm et al. 2006           | Mouse, C57BL & Rat, SHR                 | 14 day release of 40µg via miniosmotic pumps connected to catheter | 6 months | • Learning deficits in 9 month old mice but not 2.5 month old  
  • Learning deficits in rats after 6 months (MWM)  
  • Dysfunctional astrocytes |
| Lecanu et al. 2006         | Long-Evans Rat                          | Osmotic micropump in left ventricle 2.5 µl/h. | 4 weeks  | • Memory impairment  
  • Hyperphosphorylated tau,  
  • Thioflavin-S-positive amyloid deposits  
  • Neuronal loss and gliosis  
  Impairments only with Fe 2+ and buthionine-sulfoximine |
| Nabeshima & Nitta 1994     | Rat, Kbl Wistar, Male                   | Continuous via catheter, 2 weeks, 3-300pmol/day ICV | 15 days  | • Impairments on MWM and PA  
  • ChAT ↓ in FC and HC |
| Nag et al. 2001            | Rat, Sprague-Dawley, Male               | ICV over 14 days  | 14 days  | • NOR deficit,  
  • No sensorimotor gating effect  
  Phystostigmine had no effect |
| Olariu et al. 2002         | Rat, Wistar, Male                       | Osmotic micropump ICV 12µl/day 14 days | 14 days  | • Decreased PKC activity  
  • Affinity for PDBu binding in HC, |
| Malin et al. 2001          | Rat, Sprague-Dawley, Male               | Injection into 7 HC sites | 17 days  | • Neuronal loss & Gliosis  
  • Plexiglas sunburst maze impaired on retention trial speed & accuracy |

Table 3 A summary of Aβ oligomer models in rodents (Abbreviations: APP = Amyloid Precursor Protein; FC= Frontal Cortex; HC= Hippocampus; L&M= Learning & Memory; MYM= Morris Water Maze; PA= Passive Avoidance; PDBu= Phorbol 12,13-dibutyrate; SHR= Spontaneously hypertensive rat; TTR= Transthyretin)
1.3 Aims of the project

The history of AD animal models is not one of great success when measured by the output of therapeutic drugs. Early AD models were based on the cholinergic hypothesis (Smith 1988) leading to 4 out of the 5 treatments currently approved for AD. All of these drugs provide palliative effects on the disease (Casey et al. 2010), but no effect on the mechanisms of disease (Birks & Harvey 2006). Following these models, transgenic models of AD have been developed. Whilst these models have proven huge benefit to the knowledge of disease mechanisms (LaFerla & Green 2012), the vast majority of AD cases are sporadic (Bird 1993; Reitz & Mayeux 2014) with no known underlying cause (LaFerla & Green 2012). Additionally, these transgenic models are focused on the aspects of AD which appear later on in disease progression such as Aβ-plaque and NFT formation (Kitazawa et al. 2012), rather than early changes which precede cognitive decline. According to the revised Amyloid Cascade hypothesis, soluble Aβos cause synaptic disruption, downstream changes in glial cells and eventually neuronal loss resulting in dementia (Hardy & Selkoe 2002). It is therefore important to investigate these oligomers and their molecular functions. Where-as in the field of transgenics, differing groups often share animals of the same lineage, currently the field of Aβo animal models is chaotic and disorganised, with groups using models which differ by animal species and genetic background, Aβo dosage, Aβo peptide lengths, Aβo aggregation state, Aβo assembly and method of Aβo delivery.

It is the aim of this project to investigate two different types Aβo 1-42; one of low molecular weight assemblies (LMW) consisting of dimers trimers and tetramers, and one of high
molecular weight (HMW) consisting of assemblies ranging from ~50 to ~150 kDa. By keeping as much the same between the two models as possible, it will be possible to identify more clearly the different effects of these AβOs, both on cognition and the neuropathological deficits caused by AβO, and thereby providing a platform for drug screening with relevance to AD.

1.4 Model and rationale

Rats have been chosen as the organism species for these models. As AD is a disease involving cognitive and memory decline, an organism with extensive historical testing is desirable. Rats are thought to be more capable in the learning of a wider variety of cognitive tasks than mice and other small animal models (Iannaccone & Jacob 2009). Tau is thought to be a major protein implicated in AD, and rats have a tau proteome more similar to humans than mice (Hanes et al. 2009). Although this model is based on amyloid, downstream tau changes are hypothesised and it makes sense to use a model organism more closely relating to the human disease. The size of the rat is also preferable over the mouse. As the model will involve surgery, the relative proportion of brain tissue damaged in surgery will be smaller than that in the mouse. Two types of AβO will be administered via Intracerebroventricular injection. The low molecular weight (LMW) oligomers will be provided by Synaging, who have characterised these oligomers in vivo in mice and in vitro (Youssef et al. 2008; Florent et al. 2006; Malaplate-Armand et al. 2006). High molecular weight (HMW) oligomers will be
obtained by purchasing Aβ monomers and oligomerising and characterising them as
described in the method section. Multiple cognitive tasks will be performed by the rats in
order to detect any cognitive changes caused by the administration of these oligomers, and
synaptic markers will be investigated.

Novel object recognition (Ennaceur & Delacour 1988) will be used to assess the animals’
visual recognition memory. Reliant on the inquisitive nature of rats and their preference for
novelty, this test is thought to involve the perirhinal cortex at short (minutes) intertrial
intervals, and the hippocampus in the longer (hours) intervals (Antunes & Biala 2012). A
variation of this test, novel object location, will also be used, with this spatial memory
thought to be hippocampal and dependent (Williams et al. 2007). The 16-holeboard maze
(Oades & Isaacson 1978) will be used to assess working memory (Kuc et al. 2006), which is
thought to involve the prefrontal cortex and hippocampus (Yoon et al. 2008). Additionally the
Y-maze will also be used to assess working memory (Wolf et al. 2016).

PSD-95 and synaptophysin have been chosen as markers of synaptic integrity. PSD-95 is the
major scaffolding postsynaptic protein and a regulator of synaptic strength (Chen et al. 2011)
and a reduction should indicate post synaptic impairment. Synaptophysin is an integral
presynaptic protein (Wiedenmann & Franke 1985), and again, changes in synaptophysin
levels should indicate alterations in presynaptic integrity. Bcl2 will be investigated as a
general marker of apoptosis to investigate whether neuronal death is the cause of any
synaptic loss seen, and GluN2b will also be looked at, to investigate whether Aβ application is
sufficient to cause an increase in this NMDA subunit thought to be responsible for the
excitotoxicity seen in AD (Paoletti et al. 2013; Zhou & Sheng 2013; Zhou 2014). The hippocampus will be investigated due to its implication in the literature summarised in table 3, and the frontal cortex will also be investigated, as previous work on the LMW oligomers has shown an inflammatory response and synaptic deficit in this region.

1.5 Project outline

Chapter 2 will outline the general methods and procedures used in characterising and testing these models. Chapters 3 and 4 will involve characterising the cognitive effects of the administration of LMW and HMW Aβo into rats respectively. Chapter 5 will then investigate the effects of blocking Aβ binding to PrPc in both models, and in Chapter 6 the therapeutic effects of ROCK inhibition will be investigated.
Chapter 2: General Methods
2.1 Study outline

In each study adult rats received a single Intracerebroventricular (ICV) injection of either vehicle or Aβ oligomers, referred to as Day 0. They then underwent one or more behavioural test, before being sacrificed and their brains harvested for post-mortem analysis. Details of any treatment are specified within each individual study.

2.2 Experimental Animals

All animals, except where specified otherwise, were female Lister-hooded rats (Charles River) weighing approximately 220 ±20g at the start of experimentation, housed in single sex groups of five in individually ventilated double decker GR1800 cages (Tecniplast, Italy), on a 12hr light cycle (Lights on from 07:00 to 19:00). Temperature (21 ± 2°C) and Humidity (55 ± 5%) were both controlled. Food (Special Diet Services, UK) and water were provided ad-libitum. All behavioural experiments were undertaken between the hours of 0800 and 1200, and conducted in accordance with UK Animals (Scientific Procedures) 1986 Act and University of Manchester ethical guidelines.

2.3 Surgical procedure

Rats were anaesthetized using 4% isoflurane in O₂ in an induction chamber. A clipper was then used to shave the head in preparation for surgery. The rat was mounted in a stereotaxic frame and anaesthesia was maintained at 3% isoflurane. Blunt ear bars coated with local anaesthetic (EMLA cream: 2.5% lidocaine and 2.5% w/w prilocaine) were used to position the
head. To protect the eyes, an ocular lubricant (Laci-lube: Paraffin and wool alcohol) was applied. The exposed skin of the head was swabbed with an alcoholic iodine solution (iodine 2.5% w/v, potassium iodine 2.5% w/v) and an analgesic (Vetergesic: Buprenorphine) was applied subcutaneously at 0.1mg/kg. The pedal reflex was checked to ensure the animal was fully unconscious, and a midline sagittal incision was made on the skin over the skull, clamp scissors were used to hold the skin away. The anaesthesia was then lowered to 2% and the coordinates of bregma were recorded and used to calculate the position of the left ventricle of the brain (ML -1.5; AP -0.8) (Paxinos & Watson 1997) (Figure 6). At the recorded position a hand held drill was used to gently drill a hole in the skull only. The vertical location of the ventricle was calculated (DV 4.5) (Paxinos & Watson 1997) and a Hamilton syringe containing 10µL of Aβ-oligomers or vehicle was lowered to this position. The solution was injected at a rate of 2.5µL/min, and the syringe removed. The skin was then sutured using Coated Vicryl, and an antibiotic (Cloxacillin 500mg) was applied to prevent infection. 1.5mL of saline was administered to aid rehydration, the anaesthesia removed and the animal was allowed 45 minutes single-housed in an incubator (30°C) to recover before returning to its home cage.

Initial experiments were based using low molecular weight (LMW) oligomers from Synaging, and the surgery protocol was designed based on their ICV surgery in mice (Youssef et al. 2008). The volume of Aβo was increased in line with the larger rat brain, although the concentration was kept the same, and injected unilaterally as in their studies. For the high
molecular weight (HMW) studies, experimental parameters were kept the same to reduce variability.

Figure 6 Rat brain after injection of a purple dye. Showing the dye in the left lateral ventricle (A,B) surrounding the hippocampus (C,D). The brain was cut freehand along the coronal plane. Unevenness in the cutting has given the impression of a larger left hippocampus, though it is just a more posterior view than the right. No such distortion was seen post-mortem at the end of experiments.

2.4 β-amyloid Oligomers

2.4.1 Low molecular weight oligomers (stable dimers, trimers and tetramers): β-amyloid 1-42 was supplied by Synaging (Nancy, France) at a concentration of 5nMol. The toxicity of these
oligomers have been reported previously by Synaging (Florent et al. 2006; Malaplate-Armand et al. 2006; Youssef et al. 2008)

2.4.2 High molecular weight oligomers: HMW oligomers were prepared as described by Rushworth et al. (2013). Briefly, biotin-Aβ1-42 (Anaspec) was dissolved in Hexafluoropropanol (HFIP) to disaggregate for 1 hr, aliquoted and then HFIP was evaporated off using N₂. The peptide was resuspended in dimethyl sulfoxide (DMSO) to bring it to a 1mM concentration, and then diluted in Ham’s F12 to 100μM. It was then allowed 16hr to aggregate at room temperature.

2.5 Novel Object Recognition

2.5.1 Apparatus

The test box was a 52x52x51cm PVC arena, with black walls and white flooring with a 3x3 grid pattern. The objects were two from a can of Coca-Cola, a white plastic bottle filled with water, an inverted black flowerpot filled with cement and a brown glass bottle filled with water. The novel object was counterbalanced for left/right position to account for spatial bias. All objects had previously been validated for bias by other members of the lab group.
2.5.2 Procedure

2.5.2.1 Habituation

Animals were placed in the NOR boxes with their cage mates on two consecutive days for 30 and 15 minutes respectively prior to the first experimentation. This was carried out in order to reduce the stress during the testing phase.

2.5.2.2 Acquisition phase

Two identical objects (A1 & A2) were positioned in the box, equidistant from the walls (6cm) and corners. Camera recording was initiated, and rats were placed individually into the box (Figure 7A). The animal was then left for 3 minutes, before being removed to its home cage.

2.5.2.3 Inter trial interval (ITI)

The rat was left in its home cage for 1 minute.

2.5.2.4 Retention Phase

The two familiar objects were removed, and replaced with two new objects (A3 and B1, where A3 is identical to A1 & A2, and B1 is completely different). The recording equipment was switched back on, and the rat was placed back into box, and left for 3 minutes to explore (Figure 7B). At the end of the retention phase, the rat was placed back in its home cage, and the text box and objects were cleaned with 70% ethanol.
2.5.2.5 Data Analysis

The time spent investigating (sniffing, chewing, licking, touching) each object was recorded blind to treatment and object novelty. The exclusion criterion for the NOR test was defined; if an animal failed to explore one or both of the objects for less than 2s in either the acquisition or retention trial, or if the animal knocked over an object, or escaped the testing chamber, it was excluded from the final data analysis. The discrimination index (DI) was used to measure the ratio of discrimination between the novel and familiar object. The DI was calculated by dividing the difference in exploration time by total exploration time. The mean and standard deviation were calculated, and a one way ANOVA followed by planned comparisons to determine significance between groups, or if appropriate, a Student T-test was used to determine significance between novel and familiar object exploration. Locomotor activity was recorded by counting the total number of times an animal entered a

Figure 7 Novel Object Recognition. A rat investigated two identical objects in the acquisition phase (A) and two different objects in the retention phase (B).
new sector. One line crossing was counted as each time the base of the rat’s tail crossed over a line of the 3x3 grid on the base of the testing chamber, described earlier.

2.6 Post-mortem

2.6.1 Brain perfusion

Rats were anaesthetized with an overdose of isoflurane (5% in O2 2L/min) until deep anaesthesia was achieved, and checked with paw and blink reflex. The rat was maintained on anaesthetic throughout the procedure. The diaphragm and ribcage were cut open and the heart exposed. A butterfly needle was inserted into the left ventricle, and phosphate-buffered saline (PBS) pumped in at ~20mL/min for approximately 7 minutes until the flow coming out of the heart was clear.

2.6.2 Dissection

Brains were removed and dissected immediately to limit the freeze/thaw cycles. Immediately after perfusion, death was confirmed and the head was removed. The skin was cut back, and using scissors and Hull forceps the skull was removed exposing the brain, which was gently scooped out and placed in ice cold PBS on a petri dish, on ice. The olfactory bulbs and cerebellum were removed with a scalpel, and the brain separated into right and left hemispheres. Using visual markers the frontal cortex (defined as posterior and dorsal to the olfactory bulbs and anterior to the most anterior point of the corpus callosum) and hippocampus of each hemisphere was removed (Palkovits et al. 2013), placed in a
microcentrifuge tube and immediately snap frozen in -80°C isopentane. The samples were then stored at -80°C for later use.

### 2.6.3 Tissue preparation

Tissue was homogenised on ice in 10-fold (mg/μL) volume of homogenisation buffer [10 mM Trizma base (Sigma-Aldrich, UK), 320μM sucrose, 2 mM EDTA, pH 7.4, and protease (Roche, UK) and phosphatase inhibitor cocktails (Sigma-Aldrich, UK)]. Homogenates were spun down at 800g at 4°C for 15 minutes. The pellet (P1) was discarded, and the supernatant (S1) was spun again at 12000g at 4°C for 20 minutes. The supernatant (S2) was stored at -20°C and the pellet (P2) was resuspended in PBS buffer (10 mM Phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) and stored at -20°C (Figure 8)

![Flow Diagram](image.png)

*Figure 8. A flow diagram demonstrating the tissue preparation process*
2.6.4 Protein assay

A Bradford assay was used to determine protein concentration in homogenised brain samples. Samples were diluted 1:10 in ultrapure H$_2$O to reduce sample waste. In a 96-well plate, BSA standard (Bio-Rad) was added at concentrations of 1, 0.75, 0.5, 0.25, 0.125 and 0 in triplicate. 100µL of diluted samples were added in triplicate, and 200µL of dye reagent (Bio-Rad) was added to every well. The plate was incubated for 5 minutes and read on a plate reader at 595nm. The standards were used to construct a standard curve, and this was used to determine protein concentration of the diluted sample, which was multiplied by 10 to determine protein concentration of sample.

2.6.5 Western Blot

Prepared samples were diluted in Laemmli sample buffer (5% β-mercaptoethanol) (Bio-Rad) and distilled water so as to attain 30µg of protein per well with each well having an equal volume of diluted sample. Samples were boiled for 5 minutes at 90°C and briefly centrifuged at 5000rpm. Pre-cast Criterion TGX Stain-Free gels (Bio-Rad) were placed into a Criterion Cell tank (Bio-Rad) filled with running buffer (Bio-Rad). The gels were loaded with 30µL of sample in each well and 10 µL of ladder in one of the wells. Gels were then run at 120v for 30-60 minutes until the bands reached the foot of the gel. Once run the gels were scanned and activated on the ChemiDoc™ MP System and then transferred to a nitrocellulose membrane. The membrane was incubated in 3% milk for 1hr at RT, and incubated with primary antibody
overnight at 4°C, then incubated with the secondary antibody for 1hr at RT. The membrane was then incubated with Clarity Western ECL solution (Bio-Rad) for 5 minutes then imaged on the ChemiDoc™ MP System. With the exception of the Clarity Western ECL solution, the membrane was washed with PBST in-between each step.

2.6.6 Elisa

100µL of capture antibody (R&D systems) diluted in PBS to manufacturer’s instructions was incubated on 96-well Nunc-immuno plate (ThermoFisher Scientific) over night at RT. Following this the plate was incubated with 300µL of BSA (1% in PBS) (Bio-Rad) for 1hr at RT, then 100µL of prepared sample, diluted as needed in BSA, or 100µL of standards (R&D systems), diluted according with manufacturer’s instruction, was added to each well in triplicate and incubated at RT for 2hr. 100µL of detection antibody (R&D systems) diluted in PBS to manufacturer’s instructions was then added and incubated for 2hr at RT. 100µL streptavidin (R&D systems) (0.5% in BSA) was incubated in the plate in a dark room for 20 minutes, followed by 100µL of freshly mixed substrate reagent (50:50 Colour reagents A&B) (R&D systems) for a further 20 minutes. To this 100µL of H₂SO₄ was added to cease the reaction. The plate was then read at 450nm on a plate reader. With the exception of the substrate solution and H₂SO₄, the plate was washed with PBST 3x in-between each step. The standards were used to construct a standard curve, and this was used to determine the target protein concentration of the sample. The results of the protein assay were then used to determine the target protein/total protein concentration.
2.6.7 Meso Scale Assay

Pre-coated plates were purchased (Meso Scale Discovery) and blocked with 150μL of blocking solution (Meso Scale Discovery) at RT for 1hr. 25μL of prepared samples or standards (Meso Scale Discovery) were then incubated at RT for 3hrs followed by 25μL of detection antibody solution (Meso Scale Discovery) incubated for 1hr at RT. 150μL of Read Buffer T (Meso Scale Discovery) was added and read with a SECTOR Imager immediately. With the exception of the read buffer, plate was washed with Tris Wash Buffer (Meso Scale Discovery) 3x in-between each step.

2.7 Power calculations

Power calculations were made using the most restrictive tests, with estimates of the effect size based on previous studies performed by the team on separate projects. A type 1 error alpha of 5% and power of 80% was set, and it was calculated that 10 animals were required to obtain significant results in the behavioural tests.
Chapter 3: Investigating the effects of LMW Aβo on behavioural and pathological markers in the rat
3.1 Introduction

Within the Alzheimer’s disease (AD) brain, several species of soluble β-amyloid (Aβ) can be found, along with insoluble fibrils and plaques, and several attempts at identifying the toxic species of soluble oligomers have been made. In mice, intracerebroventricular (ICV) injection of high molecular weight (HMW; ranging from ~50 to ~150 kDa), and low molecular weight (LMW; dimers-tetramers) oligomers have been shown to act differently, with LMW oligomers causing long lasting, synaptic alterations, and HMW oligomers causing short term NMDA receptor associated cognitive disruption (Figueiredo et al. 2013). The prion protein (PrPSc) has been identified as an Aβ receptor (Kellett & Hooper 2009), and HMW oligomers are known to interact with PrPSc in AD brains (Dohler et al. 2014), supporting the Figueiredo et al., (2013) finding that HMW oligomers affect NMDA function, as PrPSc bound Aβ has been shown to decrease surface NMDA receptors (Um et al. 2012). Interestingly Velasco et al., (2012) found that in rat hippocampal cells, it was the HMW oligomers, not the LMW oligomers, as shown by Figueiredo et al., (2013) which showed synaptic binding. Conversely other studies have all shown a role for LMW oligomers in synaptic loss or dysfunction (Shankar et al. 2007; Chen & Glabe 2006; Townsend et al. 2006). LMW oligomers have also been shown to induce the collapse of the endoplasmic reticulum (ER) and destabilise microtubules in rat hippocampal cells (Lai et al. 2009). ER dysfunction has also been shown in the APPswe mutant mice and human AD brain (Lai et al. 2009; Taguchi et al. 2000) suggesting that these LMW oligomers are relevant to AD pathology.
Breaking the LMW oligomer category up, Aβ dimers have been shown to be elevated in AD brains late on in the disease, when compared with Aβ*56 (Lesné et al., 2013). These dimers correlated with plaque load, and Aβ*56 correlated with pathological tau, and negatively correlated with post-synaptic proteins, suggesting that Aβ*56 precedes Aβ dimers in AD. This is interesting as others have found that Aβ dimers have the ability to form higher oligomerisations of Aβ (O’Nuallain et al. 2010; Tsigelny et al. 2014).

Aβ trimers have been shown to appear in mice before cognitive deficits, and have been proposed as the building blocks of higher aggregations of Aβ (Lesné et al., 2006; Matsumura et al., 2011). Whilst Hung et al., (2008) have shown that trimers are neurotoxic in vitro, they have been shown to have no effect on LTP ex vivo (O’Malley et al. 2014) or memory formation in vivo (Reed et al. 2011).

The literature surrounding the different oligomerisations is controversial. This may be due to the large variety of oligomer preparation methods, differences in using either synthetic or purified Aβ, or the tendency of Aβ to aggregate spontaneously. Whilst elucidating the role of each type of Aβ oligomer is important, it is also crucial to understand how Aβ oligomers act when in a more physiological mixed form.

As one of the key symptoms of AD is memory loss, and this occurs before neuronal loss, the mechanism of memory loss is important to understand. It has been shown that electroencephalogram (EEG) gamma band activity is disrupted in the AD brain (van Deursen et al. 2008), and this has been replicated in several mouse models of AD (Goutagny et al. 2013; Verret et al. 2012). The integrity of these oscillations is thought to be crucial to
memory consolidation (Goutagny & Krantic 2013), and so understanding the mechanisms behind such alterations is important. Some evidence suggests that over-excitability of the networks could be caused by reduced inhibitory signalling (Verret et al. 2012). This signalling is thought to be controlled in part by parvalbumin expressing interneurons (Verret et al. 2012; Sohal et al. 2009). These interneurons have been shown to be decreased in AD brains (Brady & Mufson 1997; Satoh et al. 1991).

This study aims to characterise the behaviour of a preclinical model relevant to AD. Rats will receive ICV injection of stabilised LMW Aβ oligomers composed of dimers, trimers and tetramers. Novel object recognition (NOR), novel object location (NOL) and 16-holeboard maze tasks will be performed to access any cognitive changes caused by the oligomers. It is conceivable that parvalbumin interneuron deficits may be caused by Aβ in AD, so levels of these neurons will be investigated post mortem.
3.2 Material and Methods

3.2.1 Animals

A total of 20 female lister-hooded rats weighing approximately 200±20g at the start of the experiment were used in these studies. See section 2.2 for housing conditions. Animals initially had free access to food (Special Diet Services, UK) One week prior to hole-board training, the animals were food restricted to 90% of their free-feeding body weight (Approximately 10g chow/rat/day). Food restriction was maintained throughout training and experimentation. Access to water was not restricted. All behavioural experiments were conducted in accordance with UK Animals (Scientific Procedures) 1986 Act and University of Manchester ethical guidelines.

3.2.2 Aβ oligomers

Aβ 1-42 was supplied by Synaging (Nancy, France) at a concentration of 5nMol. The control solution was an equal volume of the vehicle solution, PBS (10 mM Phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4).

3.2.3 Experimental design

Animals were injected with 10μL of either vehicle or 5nmol Aβ oligomers. Novel object recognition (NOR) was then performed on days 4, 35 and 70. The 16-holeboard maze task was performed from day 49 to day 61 and the novel object location (NOL) task was
performed on day 71 (Figure 9). The animals were then sacrificed and their brains extracted for immunohistochemistry.

Figure 9 An outline of the behavioural experiments in the characterisation of LMW Aβo study

3.2.4 Surgery

Aβ oligomers were injected as in section 2.3.

3.2.5 Novel Object Recognition

The apparatus was as described in section 2.5.1. The rats were habituated prior to NOR testing (see section 2.5.2.1) and tested 4, 35 and 70 days after surgery. For details on the testing conditions, see section 2.5.2. Behavioural assessment details can be found in section 2.5.2.5.
3.2.6 Novel Object Location

The apparatus for the Novel Object Location task was identical to that for NOR, described in section 2.5.1. Animals were tested 71 days after surgery. Rats were not formally habituated as they had had previous exposure to the testing environment in the NOR tasks. In the acquisition phase two identical objects (A1 & A2) were positioned in the corners of the box, equidistant from the walls (6cm) and corners. Camera recording was initiated, and rats were placed individually into the box. The animal was then left for 3 minutes, before being removed to its home cage. Animals were left in the home cage for an ITI of 1 minute, then returned to the enclosure with two new objects (A3 and A4, identical to A1 & A2). A3 was placed in the same position as A1, however A4 was moved to a different corner to A2. The recording equipment was switched back on, and the rat was placed back into box, and left for 3 minutes to explore (Figure 10). Behavioural assessment details can be found in section 2.3.2.5.
Figure 10 NOL diagram. The animal is free to explore two identical objects in the acquisition phase. It is then removed for the inter-trial interval, and the two objects are replaced with two identical objects, one in the same location, one in a different location. The animal is then placed back into the test chamber and allowed to explore in the retention phase.

3.2.7 16-Holeboard Maze

The animals were tested for working memory using the 16-holeboard Maze test 50 days after surgery. 1 week before the start of the experiment rats were food restricted to 90% of their free feeding body weight by daily administration of ~10g of standard rat chow (Special Diet Services Ltd., UK). Rats were weighed regularly and food quantity altered to ensure a healthy and stable bodyweight.

3.2.7.1 Apparatus

The apparatus consisted of an open box made of Plexiglas (70x70x50cm) positioned 30 cm above the floor on a small table inside a designated behavioural test room, environmental conditions were identical to the animal holding room (21 ±2°C and Humidity 55 ±5%). The walls of the box were clear and the floor was grey. There were 16 individual equally spaced
bait holes on the floor of the box which were 4cm in depth (Figure 11). A camera was positioned above the 16-holeboard maze and connected to a portable TV positioned outside the behavioural test room to allow observation and scoring of animal behaviour without any disturbance from the experimenter. Background radio music was constantly played within the behavioural test room.

### 3.2.7.2 Handling

As the 16-holeboard maze paradigm requires a great deal of handling of the rats by the experimenter during the training phase, the rats were handled daily for 1 week prior to training on the 16-holeboard maze; handling includes scruffing and firmly holding the rats until they appeared relaxed. This habituation process ensured that the rats were not stressed during the training protocol, allowing them to rapidly learn the task without fear of the experimenter.

### 3.2.7.3 Habituation

The 16-holeboard maze was cleaned with 70% ethanol into which 2g per 100 ml of TestDiet AIN-76A Rodent Tablet was dissolved, in order to mask lingering olfactory trails prior to habituation and also to prevent the rats using olfactory stimuli to find the rewards. During habituation, all 16 holes were baited, and regularly re-filled with food pellets (TestDiet AIN-76A Rodent Tablet). Rats were habituated in cage groups (n=5) and placed in the 16-holeboard maze for 30 minutes each day for 5 days.
3.2.7.4 Training

Training sessions were performed in the morning (09:00h) and again in the afternoon (14:00h) for 5 days. During training, individual rats underwent sessions of 10-trials with an ITI of 20 seconds, whereby the rats were gently held by the experimenter whilst the 16-holeboard maze was thoroughly cleaned with 70% ethanol/TestDiet AIN-76A Rodent Tablet solution.

Each trial required 6 food pellets to be placed in the same 6 holes throughout the entire training procedure. The trial ended when all of the 6 food pellets had been found and eaten or after a period of 2 minutes whereby the rats had not been successful in finding the 6 food pellets.

3.2.7.5 Testing

10 days after the last training session, the rats were tested in an identical manner to a training session.

3.2.7.6 Data analysis

The number of visits to each hole and the time (latency) taken to complete the task (find and eat all the food pellets) were recorded manually by an experimenter blind to group type. The number of visits was used to calculate the Working Memory Score (WMS). This was calculated by dividing the number of visits to a hole that contained food (i.e. 6) by the number of total visits to all holes, with the maximum score being 1 (i.e. 6/6). The number of
revisits to both rewarded and non-rewarded holes was also recorded. Repeated measures ANOVAs were used to determine if the rats learned the task, as well as to determine differences in WMS and latency between the groups. A two-tailed two-sampled Student T-test was used to determine significance in the difference in revisits to both rewarded and non-rewarded holes.

![16-holeboard maze](image)

*Figure 11 16-holeboard maze (not to scale). A diagram showing the location of the rewarded and non-rewarded holes. Rewards were placed in 6 holes, the location of which remained the same throughout the experiment.*
3.2.8 Parvalbumin Immunohistochemistry

Rats were culled by overdose of anaesthetic (5% isoflurane in O₂), and brains were perfused with phosphate buffered saline (PBS) 0.1 M, and perfusion-fixed with 4% paraformaldehyde in PBS. Brains were rapidly extracted and incubated in 4% paraformaldehyde for 24hr followed by 30% glucose for 48hrs at 4°C, before being stored at -80°C. Coronal slices containing the frontal cortex [roughly AP +3.5 - +2.7] and prefrontal cortex [roughly from the anterior of the brain up to AP +3.5, (Paxinos & Watson 1997)] were cut and free floating sections were stained for parvalbumin with a mouse monoclonal anti-parvalbumin antibody (Swant, PV235) and revealed by ABC and DAB kits (Vector Laboratories, PK-6100 and SK4100) as described by McKibben et al. (2010). Sections were mounted on slides, coded and analysed blind to treatment. Stained sections were scanned at 4x magnification using an Olympus BX51 microscope interfaced to an Image ProPlus (version 6.3) analysis system (Media Cybernetics, USA) via a JVC 3-CCD video camera. Estimations of neuronal density (cells/mm²) were carried out in every 6th section with a minimum of 6 sections per animal counted. The region of interest was highlighted and parvalbumin-positive neurons were counted live at a higher magnification (20x) using randomly generated points and a 2D counting frame. There was no staining in sections where the primary antibody was omitted.
3.3 Results

3.3.1 ICV injection of stabilised LMW Aβo induced a long lasting NOR deficit

Memory deficits were tested using NOR 4, 35 and 70 days after injection of vehicle or Aβ oligomers (Aβo).

3.3.1.1 Acquisition

Figure 12 (A-C) shows that on days 4, 35 and 70 after injection of vehicle or soluble Aβ oligomers, total exploration time is unchanged between groups; with no preference for either left (L) or right (R) objects in the acquisition trial in either vehicle (p>0.05) or Aβo (p>0.05) animals. There was no difference between groups on total exploration (F(1,36)=0.01, p>0.05), however object exploration was significantly affected by day of testing (F(2,36)=44.42, p<0.001).

3.3.1.2 Retention

After a 1 minute inter-trial interval (ITI), vehicle animals spend significantly more time investigating the novel (N) object than the familiar (F) on day 4 (p<0.001), whereas Aβo animals explored both familiar and novel objects equally (p>0.05). Similar results were seen for day 35 with vehicle animals again showing a preference for the novel object over the familiar (p<0.001), and the Aβo animals continued to investigate both familiar and novel objects equally (p>0.05). 70 days after surgery the vehicle animals again investigated the novel object more than the familiar (p<0.001), and the Aβo animals investigated both equally.
There was no difference between groups on total exploration ($F_{(1,36)}=0.00$, $p>0.05$), however object exploration was significantly affected by day of testing ($F_{(2,36)}=5.79$, $p<0.01$).

### 3.3.1.3 Discrimination index

There was a significant effect of treatment group on the DI ($F_{(5,53)}=26.85$, $p<0.001$) (Figure 12D). There was a significant difference between groups on days 4 ($t_{(5)}= 6.418$, $p<0.001$), 35 ($t_{(5)}= 7.570$, $p<0.001$), and 50 ($t_{(5)}= 5.955$, $p<0.001$).

In summary, the vehicle group spent a significant amount of time investigating the novel, over the familiar object on each day of testing, an effect that was abolished in Aβo treated animals.
3.3.2 ICV injection of stabilised LMW Aβo had no effect on performance in the 16-holeboard maze.

3.3.2.1 Effect of twice-daily training on Working Memory Score in the 16-holeboard maze 6 weeks after ICV injection with vehicle or Aβ oligomers.

A repeated measure ANOVA revealed that 10 training sessions had a significant effect on WMS in the 16-hole board maze for vehicle animals ($F_{(9,81)}=12.358$, $p<0.001$) and those injected with Aβ oligomers ($F_{(9,81)}=8.723$, $p<0.001$). Post-hoc analysis revealed significant
differences on training sessions 5-10 compared to training session 1 in the vehicle group ($p<0.01$). In the Aβ oligomer group, significance was found on training sessions 4, 6-10 in comparison to training session 1 ($p<0.01$) (Figure 13). However the repeated measures ANOVA showed no difference between the groups ($p>0.05$), and planned post-hoc comparisons confirmed that they did not differ in any session.

![Figure 13](image.png)

**Figure 13:** Working memory scores over 10 training sessions showing working memory scores over 10 training sessions for animals 6 weeks after ICV injection with either Aβ oligomers or vehicle. The results are expressed as means ±SEM (n=10 per group).
3.3.2.2 Effect of twice-daily training on Latency to complete the 16-holeboard maze 6 weeks after ICV injection with Aβ oligomers or vehicle.

A repeated measures ANOVA showed that over the 10 training sessions, latency was significantly reduced in the vehicle (F(3,29)=8.63, p<0.001) and Aβ (F(3,31)=15.73, p<0.001) groups (Figure 14). Post-hoc analysis revealed significant differences on training sessions 7-10 compared to training session 1 in the vehicle group (p<0.01), in the Aβ oligomer group, significance was found on each training session compared to training session 1 (p<0.01). There was no difference between groups (p>0.05)

![Graph showing decrease in latency over 10 training sessions for animals 6 weeks after ICV injection with either Aβ oligomers or vehicle. The results are expressed as means ±SEM (n=10 per group)](image)

*Figure 14 showing a decrease in latency over 10 training sessions for animals 6 weeks after ICV injection with either Aβ oligomers or vehicle. The results are expressed as means ±SEM (n=10 per group)*
3.3.2.3 Effect of twice-daily training on revisits to baited holes

A repeated measures ANOVA revealed that over the 10 training sessions, revisits to baited holes reduced significantly in vehicle (F\(_{(9,81)}\)=2.07, \(p<0.05\)) and Aβ oligomer (F\(_{(4,37)}\)=3.22, \(p<0.05\)) groups (Figure 15). Planned post-hoc comparisons showed no significant changes on any particular session from training session 1 in the vehicle group, however a significant difference on training sessions 3, 6-10 from training session 1 (\(p<0.05\)) was seen in the Aβo group. The repeated measures ANOVA showed that there was no difference between the groups (\(p>0.05\))

Figure 15 showing the total number of revisits to baited holes in each training session for both Aβo and vehicle injected rats 6 weeks after surgery. The results are expressed as means ±SEM (n=10 per group)
### 3.3.2.4 Effect of twice-daily training on revisits to non-baited holes

A repeated measures ANOVA showed that the vehicle group made significantly less revisits to non-rewarded holes over the training sessions ($F_{(3,31)}=5.01$, $p<0.005$), as did the Aβ oligomer group ($F_{(9,81)}=7.17$, $p<0.001$). Planned post-hoc comparisons showed significant differences on training sessions 5,6 ($p<0.05$) 7-9 ($p<0.01$) and 10 ($p<0.05$) compared to training session 1 in the vehicle group, and differences on training sessions 3 ($p<0.01$) 4 ($p<0.05$) 5-7 ($p<0.01$), 8 ($p<0.05$) 9 & 10 ($p<0.01$) compared to training session 1 in the Aβo group (Figure 16). A repeated measures ANOVA found no significant difference between the groups ($p>0.05$).

![Figure 16 showing the total number of revisits to non-rewarded holes in each training session for both Aβo and vehicle injected rats 6 weeks after surgery. The results are expressed as means ±SEM (n=10 per group)](image-url)
3.3.2.5 Effect of a 10 day interval on performance

Following the 5 days of twice-daily training, rats were given a 10 day break from the task before performing it one more time. Repeated measures ANOVAs revealed that there was no difference between the groups on WMS ($p>0.05$) latency ($p>0.05$), and paired two-sample T-tests showed no differences in revisits to either baited ($p>0.05$) or non-baited ($p>0.05$) holes (Figure 17)

Figure 17 showing results 10 days after initial training over 10 trial runs for: (A) the working memory scores; (B) latency; (C) revisits to baited holes; (D) revisits to non-baited holes. The results are expressed as means ±SEM. n=10 per group
3.3.3 ICV injection of stabilised LMW Aβo induced deficits in NOL

3.3.3.1 Acquisition

Figure 18A shows that 71 days after injection soluble Aβ oligomers or vehicle, total exploration time is unchanged between groups; with no preference for either left (L) or right (R) objects in the acquisition trial in either vehicle ($p>0.05$) or Aβ oligomer ($p>0.05$) animals.

3.3.3.2 Retention

After a 1 minute inter-trial interval (ITI), vehicle animals spend significantly more time investigating the novel (N) object location over the familiar (F) object location ($p<0.001$), whereas Aβ oligomer animals explored both familiar and novel object locations equally ($p>0.05$). Total exploration time is decreased in the Aβ oligomer group for the retention trial vs acquisition ($p<0.001$).

3.3.3.3 Discrimination index

Figure 18B shows that following Aβ oligomer administration rats spend significantly less time investigating the novel location in comparison to their exploration time of the familiar location, when compared to the vehicle group ($p<0.01$) after a 1 minute ITI.

In summary, the vehicle group spent a significant amount of time investigating the object in the novel, over the object in the familiar location, an effect that was abolished in Aβo treated animals.
3.3.4 ICV injection of stabilised LMW Aβo has no effect on weight gain or locomotor activity

A repeated measures ANOVA revealed that there was no significant difference in weight change between the Aβo and vehicle groups (Figure 19). Both groups lost weight shortly after surgery, but this was not sustained and the animals continued to gain weight after this initial loss. Both groups of animals lost ~10% of their free feeding weight when food restricted, and regained this within a week of restriction being lifted.
Figure 19 Weight changes of the animal groups over time. 0 denotes week of surgery. Food was restricted from week 5 until week 9. The results are expressed as means ±SEM. No differences were seen between the groups. (n=10 per group)

The locomotor activity of the animals was measured by counting the number of times the animals crossed the lines of the grid in the test chamber. Animals moved less during the retention phase in comparison to the acquisition phase, but there were no significant differences between the groups on any day (Figure 20A). This was also seen in the NOL task (Figure 20B).
3.3.5 ICV injection of stabilised LMW Aβo induced a deficit in Parvalbumin immunoreactive interneurons.

The brains of the rats were removed post mortem and fixed with formaldehyde 71 days after the administration of Aβo. Parvalbumin-positive staining was found throughout the frontal and prefrontal regions with no staining in sections where the primary antibody was omitted.
Parvalbumin-positive cell density was significantly reduced in both the frontal cortex ($p<0.01$) and prefrontal cortex ($p<0.001$) (Figure 21).

Figure 21 Parvalbumin staining (A) Density of parvalbumin expressing neurons is significantly reduced in Aβ treated animals in both the frontal cortex and the prefrontal cortex. The results are expressed as means ±SEM. (***$p<0.001$, **$p<0.01$, n=10 per group) (B) Showing a typical section from the frontal cortex of a vehicle injected animal, (C) Showing a typical section from the frontal cortex of an Aβ oligomer injected animal
3.4 Discussion

This study aimed to investigate the effects of administration of LMW Aβ oligomers on cognitive performance in young adult rats. A NOR deficit was seen on every day of testing, along with a deficit in NOL after 71 days following Aβo injection. This long lasting deficit is in accordance with other studies, that have shown LMW Aβ oligomers cause long lasting deficits (Figueiredo et al. 2013). Strong deficits were seen in NOR and NOL, along with a reduction in parvalbumin immunoreactive interneurons in the frontal and prefrontal cortex. However no deficit could be found in working memory with the 16-Holeboard maze task.

3.4.1 Novel Object Recognition

In this study, a single ICV injection of soluble Aβ was sufficient to induce deficits in NOR after just four days. This deficit was sustained and evident after thirty-five and seventy days. This is in support of Nag, Tang, & Yee (2001) who found that ICV injection over 14 days also resulted in NOR deficits and Balducci et al., (2010), who found an impairment after an acute ICV injection. The NOR test is based on the rats tendency to investigate novelty, and a deficit in performance suggests a decrease in recognition memory performance, and has been suggested as a stress free alternative to the Morris Water maze for evaluating the presence of cognitive deficits in AD models (Zhang et al. 2012), although these two tasks are thought to be dependent on different memory types (Antunes & Biala 2012) with different brain regions implicated (D’Hooge & De Deyn 2001; Warburton & Brown 2015). The deficit in the present study could be explained by a lack of locomotor activity from the Aβo rats, however
analysis of line crossings revealed no difference in the groups. This suggests that the ICV injection of soluble Aβo did not have a significant effect on movement levels or general motivation, but rather the deficit is due to a decrease in cognitive ability. The general health of the animals was also unaffected, and there was no difference in weight between the two groups of animals.

The deficit seen in the Aβo group is convincing, however it is important to address the question of whether this is due to the animals forgetting the objects, or if they are unable to encode the information in the acquisition phase to begin with. Previous work by members of this lab has shown that when the ITI is removed entirely, i.e. the animal remains in the box when the objects are swapped, the Aβ-injected animals are able to discriminate between the novel and familiar objects, suggesting that the removal of the animals from the box is causing them to be distracted, and the memory of the objects is not being consolidated, rather than not being processed entirely.

### 3.4.2 16-Holeboard Maze Test

This study showed that a single ICV injection of soluble Aβ was not sufficient to induce a deficit in the 16-holeboard maze test 6 weeks post-surgery. The rats were able to learn the task, with significant increases in working memory score (WMS) for the vehicle and Aβ oligomer group after 5 and 4 training sessions respectively. The similarities in latency suggests that the locomotor activity is unchanged between the groups. Additionally, both groups made fewer revisits to both rewarded and non-rewarded holes over time, again in a similar manner to one-another. The 16-holeboard maze is thought to be a test of both spatial
and reference working memory (WM) (Kuc et al. 2006), and previous transgenic mouse models of Alzheimer’s disease have shown a deficit in this test (Kuc et al. 2006; Havas et al. 2011). Weiss, Shroff, & Disterhoft, (1998) have shown that older animals perform more poorly on this test than younger animals. It is unclear as to why no difference was found in this study. One reason may be that this model is of a very early stage of the disease. Evidence of WM decline in AD is inconsistent, but meta-analysis suggests that it is present in mild-AD, but not conclusive that it is in preclinical AD (Huntley & Howard 2010). It is therefore possible that this model is not at an advanced enough stage to see a WM deficit. Another possibility is that the test is not sensitive enough to pick up any differences between the groups. The test was modified from the original maze to include 6 baited holes rather than 4. This was done with the intention of making the test more difficult, as the rat would have more holes to remember, however it also increased the likelihood that the rat would come across the pellet by chance, and therefore decrease the difficulty. Additionally the animals in this study may have been too young to see a deficit, and compensatory mechanisms could have been in place, such as more competent Aβ clearance than seen in older animals, or over excitability in the activity of the animals in the search for rewards.

3.4.3 Novel Object Location

This study showed that a single ICV injection of soluble Aβ was sufficient to induce deficits in spatial memory 71 days after surgery. In a similar test, Goh & Manahan-Vaughan, (2013) showed spatial memory to involve LTP and LTD in the hippocampus, one of the earliest regions to be disrupted in AD (Hampel et al. 2008). LTD is thought to be dependent on
glycogen synthase kinase 3 β (GSK3β) (Collingridge et al. 2010) which is thought to play an essential role in AD which regulates many cellular processes (Takashima 2006; Hooper et al. 2008; Kremer et al. 2011). It is possible that GSK3β is disrupted in our model, potentially via Wnt signalling alterations, inhibiting LTD in the hippocampus, resulting in a spatial memory deficit. With further investigation to confirm this, it is possible that this test could be used in this model to examine the effectiveness of pharmacological agents on this pathway. The exploration times in this test were significantly lower than in the first NOR tests in the same box. It is likely that as these rats were so familiar with the test boxes, having being placed in them for tests 5 times previously, including a test the day before, that they did not feel as inclined to explore as they would normally in a novel surrounding. This was considered before the test, and so objects that had never been seen before by the rats were used to try and stimulate exploration. To some extent this appears to have been successful, as they investigated these objects more than in the final day of NOR testing the previous day.

3.4.4 Parvalbumin expression

The density of parvalbumin interneurons was significantly reduced in both the prefrontal and frontal cortices 71 days after a single ICV injection of LMW Aβ oligomers. Previous unpublished work by this group has shown that 14 days after injection with these oligomers, no reduction in total neurons were seen. One conclusion could be that these oligomers are toxic to only parvalbumin interneurons, or at least only specific neurons, in this model, and that the overall neuronal population is not decreased. However, the difference in time points means any conclusions must be rather tentative. Previous work within the lab in a rat model
of schizophrenia, the parvalbumin deficit takes 6 weeks to fully develop. It could be that similarly, in this model the parvalbumin interneurons take time to decline, or it could be that the single injection of Aβ oligomers initiates a cascade of Aβ production, and that a certain threshold must be met for Aβ to become neurotoxic in this model.

The reduction of these interneurons is in accordance with studies of human AD brains, and mouse models of AD, where parvalbumin neurons are known to be reduced (Satoh et al. 1991; Brady & Mufson 1997; Verret et al. 2012). This suggests that these parvalbumin reductions could be caused by the LMW Aβ oligomers in AD. This would support data showing that the altered oscillations in AD, thought to be caused by disrupted inhibitory signalling via parvalbumin inter neurons (Verret et al. 2012; Sohal et al. 2009), occur early in AD (Goutagny & Krantic 2013) and at a similar time that LMW oligomers, notably trimers, are found to be elevated (Lesné et al., 2006; Matsumura et al., 2011). This model may therefore be of potential utility in studying the Aβo mechanisms of early AD.

This study has shown a valid protocol for establishing a preclinical model of LMW Aβo, relevant to AD with an early memory deficit at 4 days lasting until at least 70 days. An alternative working memory test will be used to confirm or deny the lack of deficit seen in the 16-holeboard maze task. One such test in consideration is the Y-maze, and this will be used in future studies. This model will be used in future studies to investigate the effect of novel therapies on LMW oligomer induced deficits, as well as to study the effects of LMW Aβ oligomers on synapses.
Chapter 4: Investigating the effects of HMW Aβo on behavioural and pathological markers in the rat
4.1 Introduction

When the amyloid theory was first proposed, it was thought that the insoluble plaques composed primarily of β-Amyloid (Aβ) (Glenner & Wong 1984) were the toxic species in Alzheimer’s disease (AD) (Hardy & Higgins 1992). Since that time, the evidence for this theory has not been persuasive, and it is now suspected that soluble Aβ oligomers (Aβo) are the toxic species in AD (Walsh & Selkoe 2007; Kayed & Lasagna-Reeves 2012; Verma et al. 2015; Haass & Selkoe 2007).

Several animal models have been established in an attempt to confirm this theory and elucidate the mechanisms behind Aβo toxicity. Intracerebroventricular (ICV) injection of Aβ₁₋₄₂ oligomers into rats has been shown to cause neuronal loss, tau hyperphosphorylation, deficits in hippocampus-dependent memory, passive avoidance impairments and astrocyte activation (Lecanu et al. 2006; Liu et al. 2008; Nakamura et al. 2001; Yamada et al. 1999). Similarly injection of Aβ₁₋₄₂ oligomers into rat hippocampus caused neuronal loss, tau hyperphosphorylation and deficits in hippocampus-dependent memory (Brouillette et al. 2012). ICV injection of Aβ₁₋₄₀ oligomers has been shown to cause similar effects in rat (Olariu et al. 2002; Nag et al. 2001; Nabeshima & Nitta 1994) and hippocampus injection causes neuronal loss and gliosis (Malin et al. 2001). Models such as these allow us a different approach to the more conventional transgenic models, and it is hoped that they may provide a platform to gain valuable information on the mechanisms of Aβo induced toxicity and potential screening of novel therapeutic agents, however, different laboratory groups use
different models, usually of only one type, and with differing methods of describing the application of Aβo, the dosage is not always clear and consistent between groups.

Within this laboratory we have access to high molecular weight (HMW; ranging from ~50 to ~150 kDa), and low molecular weight (LMW; dimers-tetramers) Aβo. In this chapter, the aim is to characterise the behavioural effects of ICV injection of HMW Aβ_{1-42} oligomers. These synthetic Aβos, prepared as in section 2.4.2, have been used in several *in vitro* studies by the group led by Nigel Hooper at the University of Manchester. Atomic force microscopy (AFM) revealed that this preparation produced a homogeneous, fibril-free population of globular particles, the majority of which are spheres 5-6nm in diameter. Western blot revealed a broad band of high molecular mass (~55–170 kDa). Bands corresponding to dimer, trimer, and tetramer Aβ species were seen, but it was suggested these may be an artefact of SDS-PAGE. Dot blot revealed the Aβ oligomers displayed a fibrillar conformation, and not a pre-fibrillar conformation (Figure 22) (Rushworth *et al.* 2013). These oligomers bind to human SH-SY5Y neuroblastoma cells, cause cytotoxicity, activate the tau kinase fyn and impair the inactivation of β-secretase 1 (BACE-1) (Rushworth *et al.* 2013).
This chapter focuses on moving these oligomers from *in vitro* to *in vivo*, and characterising the behavioural and pathological effects caused by ICV injection of these Aβos, in lister hooded rats. Studies to determine an effective dose, and investigate the onset and duration of the effects will be carried out, along with a sex-difference study to determine if gender plays a role in the presentation of any deficits caused by these oligomers. Additionally, the general health and behaviour of these animals will be closely monitored to ensure there are no unexpected effects of the Aβo injection.
4.2 Methods

4.2.1 Animals

With the exception of the sex-difference study, all animals were female lister-hooded rats weighing approximately 200 ±20g at the start of experimentation. See section 2.2 for housing conditions. Animals had free access to food (Special Diet Services, UK) and water throughout experimentation. All experiments were undertaken between the hours of 0800 and 1200, and conducted in accordance with UK Animals (Scientific Procedures) 1986 Act and University of Manchester ethical guidelines.

4.2.2 Aβ oligomers

Aβ 1-42 was prepared as described in section 2.4.2 and 10nmol was administered unless otherwise stated. The control solution was an equal volume of the vehicle (10% DMSO in Ham’s F12 medium).
4.2.3 Experimental design

4.2.3.1 Dose-response study

To elucidate an effective dose of Aβo, animals were injected with either vehicle, 5nMol Aβo or 10nMol Aβo (n=10 per group). Novel object recognition (NOR) was then performed on day 14 and 35 post-surgery.

4.2.3.2 Time-course study:

Animals were injected with 10μL of either vehicle or 10nMol Aβ oligomers (n=10 per group). NOR was then performed on day 5, 7, 14, 35 and 50 post-surgery.
4.2.3.3 Sex-difference study:

Twenty male and twenty female lister hooded rats were used in this study. At the beginning of the experiment males weighed approximately 260g±25g and females weighed approximately 225g±20g. Animals were injected with 10μL of either vehicle or 10nMol Aβ oligomers (n=10 per group). NOR was then performed on day 7 and 14 post-surgery.
4.2.4 Additional characterisation

During the time course study, animals were also tested on Y-maze for working memory, and their body weight was monitored to ensure the Aβ0 administration was having no unexpected effects on general health. Y-maze was performed 8 days after surgery, and body weight was measured a week before surgery, the day of surgery, and on days 7, 14, 35 and 50 post-surgery. The brains were collected at the end of several studies, and synaptic and neuronal markers were investigated on brains from animals sacrificed on days 7, 14 and 50.

4.2.5 Behavioural testing

4.2.5.1 NOR

The apparatus was as described in section 2.5.1. The rats were habituated prior to NOR testing (see section 2.5.2.1) and at the indicated days after surgery. For details on the testing conditions, see section 2.5.2. Behavioural assessment details can be found in section 2.5.2.5.

4.2.5.1 Y-maze

4.2.5.1.1 Apparatus

The testing area was a radial 8-arms maze with three arms open with roughly 60° degrees between each open arm. The arms were 60cm in length and 10cm wide. The walls of the
arms were 29.5cm high made from black PVC. The testing area floor was white PVC. Outside the area external cues were visible to the animal in order for it to orientate itself. The entire chamber was enclosed by a black curtain to remove distraction and reduce anxiety. A camera was placed above the area with a live feed to a nearby computer for the experimenter to watch and score the animals (Figure 23).

![Figure 23 Radial 8-arm maze in y-maze set-up. The outline shows the orientation of open arms which the animal was free to explore.](image)

**4.2.5.1.3 Procedure**

Animals were placed at the end of one arm, and allowed to freely explore the arena for 8 minutes. The starting arm was randomised and balanced between groups. The curtains were
closed throughout the 8 minutes following which the animal removed and the chamber cleaned with 70% ethanol.

4.2.5.1.3 Data analysis

Each arm entry was recorded in order, and the percentage of spontaneous alternations was calculated. A spontaneous alternation was counted as when an animal entered three unique arms in three arm visits. The percentage of spontaneous calculations was derived for the first minute, the first 5 minutes and the total 8 minutes. A two-way ANOVA followed by planned comparisons was used to compare alternations between the two groups.

4.2.6 Post-mortem analysis

Animals were sacrificed after the last NOR test, and the brains were removed and homogenised as described in section 2.6. PSD-95 was then investigated with a Meso Scale assay as described in section 2.6.7, and synaptophysin, Bcl2 and GluN2b were investigated by western blot as described in section 2.6.5.
4.3 Results

4.3.1 Dose-response study:

4.3.1.1 Acquisition phase

During the acquisition phase all three groups explored the two objects equally on both Day 14 and Day 35 (Figure 24 A & B) with no difference in left/right object exploration on Day 14 ($F_{(5,52)}=0.75$, $p>0.05$) or Day 35 ($F_{(5,49)}=0.30$, $p>0.05$) independent of treatment. Total exploration was not significantly different between the groups ($F_{(2,27)}=1.047$, $p>0.05$).

4.3.1.2 Retention phase

4.3.1.2.1 Day 14

After a 1 minute ITI, there was a significant difference in the exploration of the novel and the familiar object ($F_{(5,52)}=2.51$, $p<0.05$). The vehicle group explored the novel object significantly more than the familiar ($p<0.01$), as did the 5nMol Aβo group ($p<0.01$). The 10nMol Aβo group however explored both objects equally ($p>0.05$). Total exploration time was not significantly different between groups ($F_{(2,26)}=0.092$, $p>0.05$).

4.3.1.2.2 Day 35

In the retention phase on Day 35, there was again a significant difference in the exploration of the novel and familiar objects ($F_{(5,48)}=2.384$, $p<0.05$). The vehicle explored the novel object significantly more than the familiar ($p<0.01$), however at this time point the 5nMol Aβo
group explored both objects equally \((p>0.05)\) as did the 10nMol Aβo group \((p>0.05)\). Total exploration time was not significantly different between groups \((F_{(2,24)}=0.42, p>0.05)\).

### 4.3.1.3 Discrimination index

During the retention phase there was a significant difference between the discrimination indices (DI) (Figure 24C) of the groups on Day 14 \((F_{(2,26)}=8.33, p<0.01)\), and Day 35 \((F_{(2,24)}=5.94, p<0.01)\). A planned contrast test showed that on Day 14 the vehicle group differed from the 10nMol Aβo group \((p<0.01)\) but not the 5nMol Aβo group \((p>0.05)\). On Day 35, the vehicle group significantly differed from the 5nMol Aβo group \((p<0.05)\) and again differed from the 10nMol Aβo group \((p<0.01)\).

In summary, both doses of Aβo elicited an NOR deficit, however the lower dose of 5nMol Aβo was only apparent on Day 35, whereas the higher 10nMol Aβo dose showed a deficit on Day 14. For future experiments, the higher dose of 10nMol Aβo will be used in order to produce a reliable deficit within a reasonable time.
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Figure 24 Dose response study showing HMW Aβo administration causes NOR deficit 14 (A) and 35 (B) days at 10nMol, and on day 35 but not on day 14 at 5nMol. Exploration times on day 14 (A), 35 (B) after ICV administration of Vehicle or Aβo. (C) DI on each day of test. n=10 *p<0.05, **p<0.01, (A,B) novel vs. familiar (C) vehicle vs Aβo; two-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group).
4.3.2 Time course study

4.3.2.1 Acquisition phase

During the acquisition phase both the vehicle and Aβo group explored the two objects equally on each day of testing (Figure 25 A-E). There was no difference of left/right exploration at any time point in either group ($F_{(3,120)}=0.70$, $p>0.05$). There was no difference in the total exploration of the two objects between the vehicle and Aβo group on each day of the test ($F_{(1,60)}=0.64$, $p>0.05$), however object exploration was significantly affected by the day of testing ($F_{(4,60)}=42.39$, $p<0.001$).

4.3.2.2 Retention phase

During the retention phase (Figure 25) there was a significant difference in the exploration of the novel and familiar objects ($F_{(3,117)}=18.23$, $p<0.001$). The vehicle treated group spent significantly more time investigating the novel object on days 7 ($p<0.01$), 14 ($p<0.05$), 35 ($p<0.01$) and 50 ($p<0.05$). However on day 5 there was no significant difference in object exploration in the vehicle group ($p>0.05$). There was no significant difference between the novel and familiar objects in the Aβo group at any time point ($p>0.05$). Like in the acquisition phase, there was no significant difference between the total exploration times of the vehicle and Aβo treated groups ($F_{(4,56)}=0.47$, $p>0.05$). Again, the total exploration times were significantly affected by the day of testing ($F_{(4,56)}=22.18$, $p<0.001$).
4.3.2.3 Discrimination Index

There was a significant effect of treatment group on the DI ($F_{(1,56)}=35.83$, $p<0.001$) (Figure 25F). There was a significant difference between groups on days 7 ($t_{(7)}= 2.184$, $p<0.05$), 14 ($t_{(7)}= 2.695$, $p<0.05$), 35 ($t_{(7)}= -3.915$, $p<0.01$) and 50 ($t_{(7)}= 2.904$, $p<0.05$). However on day 5 there was no significant difference between the DIs of each group ($t_{(7)}= 0.3950$, $p>0.05$).

In summary, the vehicle group spent a significant amount of time investigating the novel, over the familiar object. Aβo treated groups did not show this preference on any day after day 5. This suggests that the Aβo injected animals are expressing an NOR deficit, but it is not apparent due to the lack of a strong vehicle NOR performance.
**Figure 25** Time course study showing HMW Aβ0 administration causes NOR deficit from day 7-50, but not on day 5.

Exploration times on day 5 (A), 7 (B), 14 (C), 35 (D) and 50 (E) after ICV administration of Vehicle or Aβ0. (F) DI on each day of test. n=10 *p<0.05, **p<0.01). (A-E) novel vs. familiar; two-way ANOVA followed by Bonferroni planned comparisons. (F) Vehicle vs Aβ0; two-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group).
4.3.3 Sex difference study

4.3.3.1 Acquisition phase

During the acquisition phase the vehicle and Aβo group of both male and female animals explored the two objects equally on each day of testing (Figure 26 A-D). There was no difference in left/right exploration at any time point in any group ($F_{(7,68)}=1.71, p>0.05$). There was no difference in the total exploration of the two objects between the Aβo groups on each day of the test ($F_{(3,32)}=1.14, p>0.05$), however object exploration was significantly affected by the day of testing ($F_{(1,32)}=57.89, p<0.001$) in the Aβo groups of both male and females, and the vehicle group of the male animals ($p<0.001$) but not the vehicle group of the females ($p>0.05$).

4.3.3.2 Retention phase

During the retention phase (Figure 26 A-D) there was a significant difference in the exploration of the novel and familiar objects ($F_{(7,68)}=3.96, p<0.01$). The male vehicle treated group spent significantly more time investigating the novel object on day 14 ($p<0.01$) but not day 7 ($p<0.05$). The female vehicle treated group spent significantly more time investigating the novel object both on day 7 ($p<0.01$) and day 14 ($p<0.05$). There was no significant difference between the novel and familiar objects in the Aβo group in either males or females any time point ($p>0.05$). Like in the acquisition phase, there was no significant difference between the total exploration times of the vehicle and Aβo treated groups ($F_{(3,33)}=0.96, p>0.05$). Again, the total exploration times were significantly affected by the day
of testing ($F_{(1,33)}=33.41, \ p<0.001$), in the Aβo groups of both male and females, and the vehicle group of the female animals ($p<0.05$) but not the vehicle group of the males ($p>0.05$).

### 4.3.3.3 Discrimination Index

There was a significant effect of treatment group on the DI ($F_{(3,35)}=7.25, \ p<0.001$) (Figure 26E). There was a significant difference between vehicle and Aβo groups on days 7 in the females ($p<0.01$), but not males ($p>0.05$). On day 14, there was a significant difference in DI between the vehicle and Aβo groups in both males ($p<0.001$) and females ($p<0.01$). There was no gender*treatment effect on DI at day 7 ($p<0.05$) or day 14 ($p<0.05$).

In summary, the exploration times did not differ between male and female groups. The male vehicle animals did not spend significantly more time exploring the novel object on day 7; however they did on day 14. The female vehicle animals spent significantly more time exploring the novel object on both day 7 and day 14. The Aβo group did not spend significantly more time exploring the novel object at either time point in both males and females.
Figure 26 Sex difference study showing that Aβo administration induces an NOR deficit in both males (A,B) and females (C,D) 14 days after administration, and 7 days after administration in females, but not in males. There was no significant difference between the DIs of the males and females (E). n=10 *p<0.05, **p<0.01, ***p<0.001, (A-D) novel vs. familiar; two-way ANOVA followed by Bonferroni planned comparisons. (E) vehicle vs Aβo; one-way ANOVA followed by a student’s t-test. The results are expressed as means ±SEM. (n=10 per group).
4.3.4 Additional characterisation

4.3.4.1 Y-maze

Spontaneous alternations were calculated for 1, 5 and 8 minutes of maze exploration 8 days after surgery (Figure 27). There was no difference between groups at any time point of maze exploration ($F_{(1,34)}=0.00, p>0.05$).

![Spontaneous alternations graph](image)

*Figure 27 Y-maze showing no effect of Aβ₀ administration on Y-maze performance 8 days after surgery. Vehicle vs Aβ₀; two-way ANOVA. The results are expressed as means ±SEM. (n=10 per group).*

4.3.4.2 Post mortem data

PSD-95 was investigated as a post synaptic marker on days 7, 14 and 50 (Figure 28) in the left hippocampus. There was a significant difference between groups at each time point ($F_{(1,16)}=43.11, p<0.001$).
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Figure 28 PSD-95 levels showing Aβo administration caused a decrease in PSD-95 on day 7, 14 and 50 in the left hippocampus. Vehicle vs Aβo; two-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=5 per group). *p<0.05, ***p<0.001

There was no difference in PSD-95 in the right hemisphere on day 14 (Figure 29) and there was not a significant difference in either left or right frontal cortex on day 14 (Figure 29).

Figure 29 PSD-95 levels do not differ after Aβo administration in the right hippocampus (A), nor left or right frontal cortex (B). Vehicle vs Aβo; Student’s t-test. The results are expressed as means ±SEM. (n=5 per group).
On day 14 there was a significant decrease in synaptophysin (Figure 30) in the Aβo treated group when compared to control in the left hippocampus ($p<0.05$), but not the frontal cortex ($p>0.05$). There was no difference in Bcl2 in the hippocampus or frontal cortex ($p>0.05$). GluN2B expression was increased in the Aβo group compared with the vehicle group in the hippocampus ($p<0.01$) but not in the frontal cortex ($p>0.05$).

*Figure 30* Post-mortem markers showing Aβo administration caused a decrease in synaptophysin in the left hippocampus 14 days post-surgery (A). There was no change in synaptophysin in the frontal cortex. (B) Bcl2 expression was not changed in the Aβo group, however (C) GluN2B expression was increased. Vehicle vs Aβo; Student’s t-test. The results are expressed as means ±SEM. (n=5 per group). *$p<0.05$, *$p<0.01$. 
Synaptophysin levels in the left hemisphere were not significantly different between the vehicle and Aβo treated groups (Figure 31) on day 50 in both the hippocampus and the frontal cortex ($p>0.05$).

![Graph showing Synaptophysin levels in the left hemisphere between Vehicle and Aβo treated groups](image)

*Figure 31* Synaptophysin levels 50 days after surgery were not significantly different in the Aβo group when compared to the vehicle group in either the hippocampus or the frontal cortex. Vehicle vs Aβo; Student’s t-test. The results are expressed as means ±SEM. (n=5 per group)

### 4.3.4.3 Animal weight

Animals gained weight significantly throughout the study ($F_{(5,85)}=63.75$, $p<0.001$) (*Figure 32*), but there was no difference in weight between groups at any time point ($F_{(3,85)}=0.00$, $p>0.05$).
Figure 32: Animal weight showing the weight changes of the animal groups over time. 0 denotes week of surgery. The results are expressed as means ±SEM. No differences were seen between the groups Vehicle vs Aβo; two-way ANOVA. (n=10 per group).
4.4 Discussion

This study aimed to characterise the cognitive effects of HMW Aβo administration in rats, and effects on synaptic markers, and found that ICV administration of HMW Aβo caused:

- Long lasting NOR deficit from 7-50 days post-surgery at a 10nMol concentration;
- A deficit that was not apparent until day 35 at a 5nMol concentration.
- A similar NOR deficit in both males and female rats.
- No effect on performance in the Y-maze.
- Decreased synaptic markers.
- Increased NMDA receptor subunit GluN2B.
- No significant effects on markers of apoptosis.
- No significant effect on the general health and bodyweight of the animals.

4.4.1 Dose response study

In this study, animals were given either vehicle, 5nMol Aβo or 10nMol Aβo ICV injection, and assessed for visual recognition memory, using NOR, 14 and 35 days after surgery. On day 14, the vehicle group and the 5nMol Aβo group could both perform the NOR task. This was abolished in the 10nMol Aβo group. On day 35, the vehicle group could perform the NOR task, but both the 5nMol Aβo and 10nMol Aβo group could not, suggesting that the lower dose required more time to cause the NOR deficit in this model. As discussed in previous chapters, NOR has been shown to be affected by ICV injection of Aβ by other groups (Nag et
al. 2001; Balducci et al. 2010). Previous studies have suggested that the NOR task is as effective as the Morris water maze (MWM) task at evaluating the presence of cognitive deficit in models of AD and in the use of therapeutic compounds (Zhang et al. 2012), though they do not evaluate the same brain regions (D’Hooge & De Deyn 2001; Warburton & Brown 2015). Lecanu et al. (2006) found that a 4 week perfusion of Aβ1-42 was not sufficient to cause a memory deficit in the MWM task, in contrast to the findings of our study. The memory deficit found in the HMW Aβo model in this study presented with only an acute ICV injection of Aβ1-42, the concentration of the Lecanu et al study was 15μMol/L at 2.5μL/hr, compared to our acute administration of 100μMol/L at 2.5μL/min. It is possible that, although over the 4 weeks the Lecanu et al. study delivered more total Aβ1-42 (25.2 nMol vs 10nMol), the animals were able to process and clear the Aβ, preventing a pathological cascade being initiated before it built up to a toxic level. Aβ is thought to be cleared by the brain across the blood brain barrier (BBB) predominantly by Low density lipoprotein receptor-related protein 1 (LRP1) (Bates et al. 2009; Deane et al. 2009), and there is also evidence that Aβ is cleared via the lymphatic system (Iliff et al. 2012). It could be that with the slower application of the Aβ, the brain is able to clear the Aβ before significant damage occurs. Zussy et al. (2013) showed that with a single Aβ25-35 ICV injection at 10µg/rat, APP processing levels were significantly increased. It is possible that this injection causes a feedback loop whereby Aβ causes increased APP processing, and therefore more Aβ production. This could initiate a cycle whereby Aβ at a high enough level alters APP processing, leading to an increase in Aβ production. By using a slower application of Aβ, it is possible that Lecanu et al. did not trigger this altered APP processing before the Aβ was cleared. This could also explain why the lower
dose in this experiment caused a delayed deficit in NOR. At 5nMol, altered APP processing may be triggered, and Aβ may start to accumulate in the brain. However the effective level at which it causes enough damage would take longer to reach than when the starting injection was 10nMol.

The 10nMol injection of Aβo was successful at causing a significant NOR deficit on the first day of testing in this study, whereas the 5nMol took longer than 14 days to show a deficit. For this reason, it was decided that for remaining studies 10nMol of Aβo will be administered. Whilst the lower dose might be closer to physiological levels seen in the AD brain, the aim of this model is not to recreate AD, but rather to provide a platform with which to explore the mechanisms of Aβo action, and to identify novel drug targets and therapies. There were also no significant changes in apoptosis, suggesting the higher dose of 10nMol is not causing wide-spread toxicity, but is acting via specific mechanisms.

4.4.2 Time Course Study

This study aimed to validate the onset and persistence of the NOR deficit seen with injection of Aβo. Animals were given 10nmol Aβo and NOR was performed on day 5, 7, 14, 35 and 50. With the exception of day 5, there was a significant deficit in the Aβo animals when compared to controls. The performance of the vehicle group at this time point was decreased when compared to other time points. It could be that rather that the Aβo not causing a deficit, that 5 days is not enough time for the vehicle animals to recover sufficiently from surgery to perform well at the task. Whilst the vehicle animals could not perform well at
the NOR task at this time point, their overall activity levels appeared to be unaffected, as exploration during the acquisition phase was similar to or higher than that at other time points. There was no difference of object exploration during the acquisition phase in either vehicle or Aβo treated group at any time point, suggesting that there was no inherent left/right bias. In line with previous reports (Akkerman et al. 2012) both groups did show a decrease in overall exploration of the objects over time, suggesting that the animals were less motivated to explore as they became more used to the testing apparatus. As this change was seen in both groups, it can be concluded that this decrease in exploration did not bias the results.

The NOR deficit was persistent, and due to the decreased interest in exploration from the rats, it was decided to end the study after day 50, as any recovery from the deficit did not seem imminent, suggesting that the acute Aβo administration resulted in a persistent NOR deficit.

4.4.3 Sex difference study

This study aimed to identify any differences in response to ICV Aβo injection between male and female rats. Both male and female Aβo treated groups showed an NOR deficit on day 14, however only the female Aβo treated group showed a deficit on day 7. This could be due to the male animals taking longer to recover from surgery than the females, or it may be that due to the increased size of the males, the dose of Aβo was not high enough to induce an NOR deficit at this time point. On day 14 both male and female animals performed similarly,
with a similar Aβo induced deficit. It was decided that, due to the delayed NOR deficit in the males, only female rats would be used for the remainder of studies. Whilst there was no difference between the males and females on NOR performance at day 14, any conclusions drawn from drug studies would have to be validated in male animals, as the drugs themselves may act differently dependent upon sexual characteristics.

In the males there was a significant reduction in total exploration time during the acquisition phase in both vehicle and Aβo treated groups, whereas the female vehicle group did not reduce activity significantly. Fernandes et al. (1999) showed that male rat behaviour is driven by anxiety, suggesting that as the males became more habituated to the testing environment, their anxiety levels decreased, and they were less motivated to explore.

### 4.4.4 Y-maze

To assess working memory, the Y-maze task was performed on day 8. No difference between the vehicle treated and Aβo treated groups could be found after 1, 5 or 8 minutes of exploration, suggesting that at this time point, there was no working memory deficit in this model. This is in contrast to a previous study (Yamada et al. 1999) that showed a y-maze deficit caused by chronic ICV administration of Aβo after 8 days. It could be that the working memory deficit is caused by a chronic administration and not acute. Alternatively, it may be that the working memory deficit caused by Aβo administration in this model takes longer to manifest than the 8 days after which it was tested in this study. It could be useful to do a
similar time course study with y-maze as the NOR, to determine if a working memory deficit becomes apparent. Alternatively more advanced working memory tasks, such as the radial 8-arm maze could be used as a more sensitive test to detect a deficit.

4.4.5 Post mortem data

To investigate post synaptic integrity, PSD-95 was investigated as a marker. PSD-95 levels were significantly reduced at each time point investigated - day 7, 14 and 50 in the left hippocampus, but not the right hippocampus or either side of the frontal cortex. Synaptophysin was used to investigate presynaptic integrity. This was significantly decreased in Aβo treated animals on day 14 in the left hippocampus, but not frontal cortex. There was no difference in synaptophysin levels on day 50 in either the left hippocampus or frontal cortex. As a marker of apoptosis, Bcl2 was measured. There was no significant difference in levels of Bcl2 between the vehicle and Aβo groups in either the left hippocampus or frontal cortex. GluN2B is a subunit of NMDA receptors, and was seen to be increased in the hippocampus of the Aβo treated group compared to the controls.

The loss of PSD-95 is in accordance with previous studies showing the association and binding of Aβo to post synaptic proteins (Koffie et al. 2009; Um et al. 2012). This binding has also been shown to phosphorylate GluN2b (Um et al. 2012). NMDA receptors are already a target of AD medication, with Memantine (Kotermanski & Johnson 2009) being approved in 2003. Evidence suggests that GluN2b containing NMDA receptors are the receptors that
mediate excitotoxicity (Zhou 2014), and inhibition of GluN2b reverses some Aβ induced synaptic deficits (Paoletti et al. 2013; Zhou & Sheng 2013).

It is interesting that the deficit in synaptophysin is not persistent. It may be that after initial synaptic loss there is an attempt to regrow synapses, and this may explain the non-significant increase in synaptophysin seen on day 50. Aβ oligomers are thought to bind specifically to PSD-95 positive sites, and it may be that the initial loss of synaptophysin is not due to toxicity of the pre-synaptic sites, but rather that the loss of post synaptic sites results in a decrease of pre-synaptic sites due to disuse. This may explain why the synaptophysin levels were able to recover in this acute administration model.

Bcl2 levels were unaffected in the Aβo treated group when compared to controls, suggesting that at the time tested (day 14) there was no significant increase in apoptosis. This is in accordance with AD disease progression, whereby synapses are lost before neuronal loss occurs (Serrano-Pozo et al. 2011; Tu et al. 2014) suggesting that the dose of Aβo used in this study is targeting synapses, rather than causing non-specific cell death. Further testing at later time points would be required to confirm whether this model does induce neuronal death later on.

It is interesting that no markers were affected in the right side of the brain or in the frontal cortex. All animals received injection into the left lateral ventricle; close in proximity to the hippocampus. It could be that the Aβ cannot propagate further from the site of injection to the other regions. Alternatively it could be that as the Aβ spreads through the brain, it is at a lower concentration, and therefore insufficient to cause the damage seen in the
hippocampus. There is also the possibility that the receptors for the Aβo injected are only present in a high enough amount in the hippocampus, although this seems unlikely to be the main cause, as the right side of the hippocampus is also unaffected.

4.4.6 Animal weight

There was no significant difference in the general health or weight of the animals between the vehicle or Aβo treated groups at any time point, suggesting that the Aβo used is specific in its mechanism, and not causing system-wide harm to the animal.

This study has successfully characterised the cognitive effects of HMW Aβo administration in rats, and effects on synaptic markers. The dose response study revealed that the higher dose of 10nMol had a faster onset, and it was decided to use this dose for future studies. NOR deficit was present from 7-50 days in females, but not until day 14 in males. Female rats were chosen for the future studies. There was no difference in Y-maze performance between groups on day 8, however testing at later time points or using a radial 8-arm maze may reveal a working deficit. The post synaptic marker PSD-95 were decreased in the Aβo treated group at every time point tested, however synaptophysin, a marker of presynaptic integrity, was only reduced on day 14. Apoptosis does not appear to be changed in the Aβo treated groups, and the levels of NMDA receptor GluN2 are increased.

This chapter has demonstrated the ICV administration of HMW Aβo in rat to be a reliable and repeatable model for investigating the effects of HMW Aβo on cognitive and pathological
markers. Moving forward, the role of Aβ receptors and APP will be investigated, using female rats and a dose of 10nMol. NOR will be performed from 7-14 days, and PSD-95 will be investigated.
Chapter 5: Blocking the Binding of LMW and HMW Aβo to the Cellular Prion Protein *in vivo*
5.1 Introduction

Despite widespread support of the amyloid hypothesis (Musiek & Holtzman 2015), little is known about the neurotoxic mechanisms. Elucidating the process by which β-amyloid (Aβ) interacts with the cells in the brain could be crucial to understanding the pathology of Alzheimer’s disease (AD). Attempts have been made to understand the normal physiological role of Aβ, but it is not clear. One study (Luo et al. 2003) has shown that β-site amyloid precursor protein cleaving enzyme 1 (BACE-1) knock-out mice, in which Aβ is not generated, show no compensatory gene expression, nor do they exhibit any structural changes in any organ, suggesting Aβ production may have no vital role and could be a target for safe inhibition. There is some evidence that at low levels, Aβ actually has a neuroprotective effect, but that accumulation and oligomerisation blocks this neuroprotective role (Sadigh-Eteghad et al. 2014). Conversely a recent finding suggested that the aggregates may trap bacterial pathogens, and act as a natural antibiotic (Kumar et al. 2016), potentially leading to inflammatory pathways as possible drug targets in AD. Lacor et al. (2007) used a combination of microscopy and biochemical fractionation on hippocampal neuronal cultures showing that soluble Aβ oligomers (Aβo) bind with specificity to pyramidal neurons which are presumed to be excitatory (Lacor et al. 2004), causing decreased expression of N-methyl-d-aspartate receptor (NMDAR) and ephrin type B receptors (EphB2), resulting in abnormal spine morphology, although a receptor for Aβ was not proposed.

Identifying the receptors on which Aβ binds could provide a therapeutic target in AD.
Several receptors have been proposed, such as the receptor for advanced glycation end products (RAGE), NMDAR, α7-nicotinic acetylcholine receptor (α7 nAChR), EphB2, immunoglobulin G Fc gamma receptor IIb (FcyRIIb), and paired immunoglobulin-like receptor B (PirB) Clusterin and cellular prion protein (PrP^c) (Kam et al. 2014; Jarosz-Griffiths et al. 2016). As Aβ can exist in many different forms and aggregation states (Di Carlo 2010; Rushworth & Hooper 2010) it is important to take this into account when considering receptors. One receptor which is thought to distinguish between high molecular weight (HMW) and low molecular weight (LMW) Aβo is PrP^c (Jarosz-Griffiths et al. 2016).

PrP^c is thought to promote Aβ plaque formation (Schwarze-Eicker et al. 2005) and hippocampal slices from PrP^c knock-out mice have been shown to lack Aβo induced deficits in LTP, and in addition application of the anti-PrP^c antibody 6D11 on wild type slices also prevented the LTP deficits, suggesting that binding of Aβo to PrP^c is essential for Aβ impairment of synaptic plasticity (Laurén et al. 2009). Additionally, in vivo models have shown that memory impairments seen in transgenic models of AD require PrP^c by crossing transgenic AD models with PrP^c null mice. These APPswe/PSen1ΔE9, Prnp−/− mice presented without the axonal depletion, loss of the synaptic markers PSD-95 and synaptophysin, and memory deficits seen in the APPswe/PSen1ΔE9 mice with PrP^c (Gimbel et al. 2010) and the memory impairments in transgenic mouse models correlate tightly with the levels of HMW, PrP^c interacting, Aβos (Kostylev et al. 2015). ICV pre-treatment with the 6D11 antibody also successfully blocked the long-term depression (LTD) induced by Aβ in vivo in a rat model of AD (Hu et al. 2014).
This study will investigate the role of PrP\(^{C}\) using both the HMW and LMW A\(\beta\)o models previously discussed in earlier chapters. Animals will be pre-treated with 6D11 at the site of A\(\beta\)o or vehicle administration, the left lateral ventricle. NOR will then be performed after 7 and 14 days, and synaptic markers will be investigated.
5.2 Methods

5.2.1 Animals

All animals were female lister-hooded rats weighing approximately 200 ±20g at the start of experimentation. See section 2.2 for housing conditions. Animals had free access to food (Special Diet Services, UK) and water throughout experimentation and all studies were compliant with the Animal Scientific Procedures act (1986) and University of Manchester ethical guidelines.

5.2.2 Aβ oligomers

Both HMW and LMW oligomers previously described were used in this study.

Aβ 1-42 was prepared as described in section 2.4. The control solution was an equal volume of the vehicle. Each preparation of Aβ had its own vehicle as a control.

5.2.3 Antibodies

Anti-PrP\(^\text{C}\) antibody 6D11 was purchased from Biolegend and diluted in phosphate buffered saline (PBS) to 20μg/ml immediately before surgery. The control solution was an equal volume of PBS.

Rat IgG (Sigma-Aldrich) was diluted to 20μg/ml in PBS immediately before surgery.
5.2.4 Experimental design

During surgery prior to the injection of vehicle or Aβos, animals received ICV injection of 5μL of either PBS or 125ng 6D11 antibody in 5μL of PBS at a rate of 1μL/min (n=40 per group). Within the two groups, animals were then received ICV injection (10μL) of either vehicle, 10nMol HMW Aβ oligomers or 5nMol LMW Aβ oligomers (n=10 per group). An additional group (n=10) was injected with 5μL of rat IgG in 5μL of PBS at a rate of 1μL/min followed by 10nMol HMW Aβ oligomers. NOR was then performed on day 7 and 14 after surgery. On day 14 after after the NOR procedure, animals were sacrificed and their brains snap frozen in isopentane, and stored at -80°C.
5.2.5 Behavioural testing

5.2.5.1 NOR

The apparatus was as described in section 2.5.1. The rats were habituated prior to NOR testing (see section 2.5.2.1) and tested 7 and 14 days after surgery. For details on the testing conditions, see section 2.5.2. Behavioural assessment details can be found in section 2.5.2.5.

5.2.6 Post-mortem analysis

Animals were sacrificed after the last NOR test, and the brains were removed and homogenised as described in section 2.6. PSD-95 was then investigated using a Meso Scale as described in section 2.6.7.
5.3 Results

5.3.1 6D11 prevents HMW Aβo induced cognitive deficits

5.3.1.1 Acquisition phase

During the acquisition phase there was no difference in the exploration of the left/right object on day 7 ($F_{(9,84)}=1.172, p>0.05$) (Figure 33A) or day 14 ($F_{(9,82)}=0.2271, p>0.05$) (Figure 33B). There was no difference in the total exploration of the two objects between the groups on either day of the test ($F_{(4,40)}=0.71, p>0.05$), however object exploration was significantly affected by the day of testing ($F_{(1,40)}=18.88, p<0.001$) in the Aβo only group ($p<0.01$) but not in any of the other groups ($p>0.05$).

5.3.1.2 Retention phase

During the retention phase there was a significant difference in the exploration of novel and familiar objects on day 7 ($F_{(9,84)}=6.203, p<0.001$). The vehicle treated group spent significantly more time investigating the novel object on day 7 ($p<0.01$) as did the vehicle+6D11 treated ($p<0.001$) and the Aβo+6D11 ($p<0.001$) groups (Figure 33). However there was no significant difference in the object exploration in either the Aβo ($p>0.05$) or Aβo+IgG ($p>0.05$) treated groups. Similarly on day 14 there was a significant difference in the exploration of the novel and familiar objects ($F_{(9,80)}=3.327, p<0.01$), in the vehicle ($p<0.05$), vehicle+6D11 treated ($p<0.05$) and the Aβo+6D11 ($p<0.05$) groups. Again there was no significant difference in the object exploration in either the Aβo ($p>0.05$) or Aβo+IgG ($p>0.05$) treated groups. As with the acquisition phase, there was no difference in the total exploration of the two objects.
(F(4,40)=0.87, p>0.05), but the day of testing did effect the object exploration (F(1,40)=44.25, p<0.001), in the Vehicle, 6D11, Aβ+6D11, Aβ+IgG (p<0.05) and Aβ (p<0.01) groups.

5.3.1.3 Discrimination Index

There was a significant effect of treatment group on the DI (F(7,70)=3.545, p<0.001). On day 7 there was a significant difference between the vehicle treated group, and the Aβo (p<0.05) and Aβo+IgG (p<0.01) treated groups, but no difference was seen between the vehicle and 6D11 (p>0.05) or Aβo+6D11 (p>0.05) treated groups (Figure 33C). The DI of the Aβo group differed significantly from the 6D11 (p<0.05) and Aβo+6D11 (p<0.05) groups, but not from the Aβo+IgG group (p>0.05).

Similar results were seen on day 14, with a significant difference between the vehicle treated group, and the Aβo (p<0.05) and Aβo+IgG (p<0.05) treated groups, but no difference was seen between the vehicle and 6D11 (p>0.05) or Aβo+6D11 (p>0.05) treated groups. Again, the DI of the Aβo group differed significantly from the 6D11 (p<0.05) and Aβo+6D11 (p<0.05) groups, but not from the Aβo+IgG group (p>0.05) (Figure 33D).

In summary, unlike vehicle treated animals, animals treated with Aβo did not spend significantly more time exploring the novel object than exploring the familiar object; treatment with 6D11 prevents this deficit but IgG treatment did not. 6D11 treatment alone had no effect on the exploration times.
Figure 33 showing HMW Aβo induced NOR deficit is blocked by 6D11 on day 7 (A) and 14 (B). There was a significant difference in NOR performance in the Aβo and Aβo+IgG treated animals when compared to the vehicle, 6D11 and Aβo+6D11 treated groups on both day 7(C) and 14(D). (A,B) novel vs. familiar; two-way ANOVA followed by Bonferroni planned comparisons. *p<0.05, **p<0.01, ***p<0.001. (C,D) *p<0.05, **p<0.01 vs vehicle group, #p<0.05 vs Aβo group; one-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group).
5.3.2 The effect of 6D11 on LMW Aβo induced cognitive deficits:

5.3.2.1 Acquisition phase

During the acquisition phase there was no difference in the exploration of the left/right object on day 7 \((F(7,73)=0.323, p>0.05)\) (Figure 34A) or day 14 \((F(7,73)=0.515, p>0.05)\) (Figure 34B). There was no difference in the total exploration of the two objects between the groups on either day of the test \((F(3,33)=0.70, p>0.05)\), however object exploration was significantly affected by the day of testing \((F(1,33)=32.67, p<0.001)\) in the Vehicle group \((p<0.01)\) and Aβ+6D11 group, but not in the Aβ or 6D11 groups \((p>0.05)\).

5.3.2.2 Retention phase

During the retention phase there was a significant difference in the exploration of novel and familiar objects on day 7 \((F(7,73)=11.41, p<0.001)\). The vehicle treated group spend significantly more time investigating the novel object on day 7 \((p<0.001)\) as did the vehicle+6D11 treated \((p<0.001)\) and the Aβo+6D11 \((p<0.001)\) groups (Figure 34). However there was no significant difference in the object exploration in the Aβo \((p>0.05)\) treated group. Similarly on day 14 there was a significant increase in the exploration of the novel object \((F(7,67)=5.936, p<0.001)\), in the vehicle \((p<0.01)\) and vehicle+6D11 treated \((p<0.001)\). Again there was no significant difference in the object exploration in the Aβo \((p>0.05)\) treated group. At this time point there was no difference in the exploration of the novel or familiar object in the Aβ+6D11 group \((p>0.05)\). As with the acquisition phase, there was no difference in the total exploration of the two objects \((F(3,33)=0.3201, p>0.05)\), but the day of
testing did affect the object exploration \(F_{(1,33)}=67.49, p<0.001\), in the vehicle \(p<0.05\), Aβ \(p<0.001\), 6D11 \(p<0.01\), and Aβ+6D11 \(p<0.05\) groups.

### 5.3.2.3 Discrimination Index

There was a significant effect of treatment group on the DI \(F_{(7,70)}=5.288, p<0.001\). On day 7 there was a significant difference between the vehicle treated group, and the Aβo \(p<0.05\) but no difference was seen between the vehicle and 6D11 \(p>0.05\) or Aβo+6D11 \(p>0.05\) treated groups. The DI of the Aβo group differed significantly from the 6D11 \(p<0.01\) but not from the Aβo+6D11 group \(p>0.05\) (Figure 34C).

Similar results were seen on day 14, with a significant difference between the vehicle treated group and the Aβo \(p<0.001\) treated group, but no difference was seen between the vehicle and 6D11 \(p>0.05\) or Aβo+6D11 \(p>0.05\) treated groups. Again, the DI of the Aβo group differed significantly from the 6D11 \(p<0.05\) but not from the Aβo+6D11 group \(p>0.05\) (Figure 34D).

In summary, the vehicle groups spent a significant amount of time investigating the novel, over the familiar object on each day of testing, an effect that was abolished in Aβo treated animals. Unlike the HMW Aβ induced cognitive deficit, 6D11 did not block the cognitive
deficit induced by the LMW Aβ. 6D11 alone did not affect the investigation of objects.

Figure 34 showing the effect of 6D11 on LMW Aβ oligomer induced NOR deficit on day 7 (A) and 14 (B). There was a significant difference in NOR performance in the Aβo treated animals when compared to the vehicle and treated groups on both day 7(C) and 14(D)., (A,B) novel vs. familiar; two-way ANOVA followed by Bonferroni planned comparisons. **p<0.01, ***p<0.001. (C,D) *p<0.05, **p<0.01 vs vehicle group, #p<0.05, ##p<0.01 vs Aβo group; one-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group).
5.3.3 The effect of 6D11 on both HMW and LMW Aβo induced synaptic deficits:

14 days after surgery postsynaptic density protein 95 (PSD-95) was investigated as a post synaptic marker (Figure 35). There was a significant difference between groups for both the HMW (F(3,13)=8.561, p<0.01) and LMW (F(3,18)=5.151, p<0.05) Aβ experiments. In the HMW experiment, the PSD-95 levels in the hippocampus of the vehicle group were significantly higher than those of the Aβ group (p<0.01), but not significantly different from the 6D11 or Aβ+6D11 groups. The HMW Aβ group was significantly lower than both the 6D11 and Aβ+6D11 groups (p<0.05). In the LMW experiment, the PSD-95 levels in the frontal cortex of the vehicle group were again significantly higher than those of the Aβ group (p<0.05), but not significantly different from the 6D11 or Aβ+6D11 groups (p>0.05). The LMW Aβ group was not significantly different to the 6D11 or Aβ+6D11 groups (p>0.05).

![Graph A: PSD levels in response to HMW Aβ application](image)

**Figure 35:** PSD levels in response to HMW and LMW Aβo application, showing a decrease in PSD-95 when HMW (A) and LMW (B) is applied. 6D11 prevents the deficit induced by HMW in the hippocampus, but not LMW in the frontal cortex. The results are expressed as means ±SEM. (n=5 per group). *p<0.05, **p<0.01 vs vehicle group, #p<0.05 vs Aβo group; one-way ANOVA followed by Bonferroni planned comparisons.
5.4 Discussion

This chapter aimed to investigate the role of PrP<sup>C</sup> in Aβo induced cognitive and synaptic deficits. Consistent with previous studies, both HMW and LMW Aβo caused significant cognitive (NOR) and synaptic (PSD95) deficits on each day of testing (days 7 and 14). Blockade of PrP<sup>C</sup> with the 6D11 antibody, prevented the deficits following administration of the HMW Aβo, but not those of the LMW Aβo, which saw only a partial recovery. An IgG antibody was used as a control, and showed no prevention of the cognitive deficits. We did not observe any significant effect on the general health and bodyweight of the animals in any of the treatment groups.

5.4.1 HMW Aβo + 6D11 study

In this study, animals received either ICV administration of 125ng of 6D11 antibody, or the equivalent volume of vehicle (5μl PBS) followed by ICV administration of either 10nMol HMW Aβo or the equivalent volume of vehicle (10μl of 10% DMSO in Ham’s F12 medium) resulting in four groups. On day 7 and 14 animals conducted the NOR task. On both days 7 and 14 the Aβo group were significantly impaired when compared to the controls. 6D11 alone had no effect on NOR performance but completely rescued the Aβo induced NOR deficit, suggesting that the binding of HMW Aβo to PrP<sup>C</sup> is essential in causing cognitive deficits. This finding is in accordance with the findings of Laurén et al. (2009) who showed that hippocampal slices from PrP<sup>C</sup> null mice had normal synaptic responses and no Aβo induced deficits in long term
potentiation (LTP). They also showed that 6D11 and other anti-PrP<sup>c</sup> antibodies prevented the binding of Aβo to PrP<sup>c</sup> and rescued synaptic plasticity in hippocampal slices treated with Aβo. 6D11 has been shown to have beneficial effects <i>in vivo</i> in transgenic models of AD. Chung <i>et al.</i> (2010) showed that transgenic mice treated with 6D11 performed the same as wildtype mice on the radial arm maze, with 6D11 alone having no effect.

The findings of this study appear to conform with other studies in the literature, that Aβ must bind to PrP<sup>c</sup> to mediate the downstream cognitive impairments, and that by blocking this binding, cognitive deficits can be prevented.

It is interesting that the blockade of the cognitive deficits was persistent. It is possible that 6D11 bound to PrP<sup>c</sup> for long enough that the Aβo were unable to bind to PrP<sup>c</sup> before being cleared from the brain or aggregating to a non-pathological state. Alternatively it could be that the binding of 6D11 to PrP<sup>c</sup> was permanent, and the Aβo was unable to bind at all.

### 5.4.2 LMW Aβo + 6D11 study

As in the HMW+6D11 study, animals were given either 125ng of 6D11 antibody, or the equivalent volume of vehicle (5μl PBS) ICV injection then given either 5nMol LMW Aβo or the equivalent volume of vehicle resulting in four groups, and presented with the NOR task on day 7 and 14. The Aβo group was significantly impaired on both day 7 and 14, and the 6D11 again had no effect on performance of the task. On both day 7 and 14, the Aβo+6D11 group
was not significantly different from either the vehicle, nor the Aβo group, suggesting that there was not a complete rescue of the NOR deficit.

Previous studies have shown that it is HMW oligomers that interact with PrP<sup>C</sup> (Dohler et al. 2014; Kostylev et al. 2015), it is therefore perhaps not surprising that the blockade of PrP<sup>C</sup> did not fully rescue the cognitive deficit. Despite this however, there does appear to be some rescue, as the Aβ+6D11 group is not significantly different from controls. This may be due to the LMW oligomers aggregating in vivo, in ways they have not been seen to in vitro (Youssef et al. 2008; Garcia et al. 2010), and binding to PrP<sup>C</sup>. This would explain why some rescue does appear with 6D11, however the rescue is not total, so there must be alternative pathways by which the LMW Aβo are acting. As discussed earlier, PrP<sup>C</sup> is not the only receptor proposed for Aβo, and it is probable that the LMW Aβo are binding to one or more of these other receptors to mediate their toxicity. One way in which these oligomers have been show to act on cognition in this model is via inflammation, possibly through the NLRP3 inflammasome induced release of IL-1β (Daniels et al. 2016). The study showed that unlike other NSAIDs which have been shown to relieve symptoms temporarily, mefenamic acid prevented the cognitive deficit at 21 days after cessation of the drug.

**5.4.3 IgG has no protective effect**

IgG antibody was used as a negative control to show that any effect of 6D11 was not due to a non-specific antibody effect. These findings are in accordance with (Laurén et al. 2009) who also saw no protective effect when IgG was applied to hippocampal slices treated with Aβo.
5.4.4 PSD-95

Previous work on both the HMW and LMW Aβo models has shown that there is a significant consistent reduction of PSD-95. It was therefore decided to investigate PSD-95 following 6D11 treatment. Interestingly the two oligomers appear to have their maximal effect on PSD-95 in different regions, with the HMW Aβo appearing to have more of an effect in the hippocampus, and the LMW oligomers appearing to have more of an effect in the frontal cortex. The cause for this difference in location of action is unknown; it could be that the different oligomers are moving through the brain differently due to weight, spatial-geometric or charge distribution/strength differences, and therefore most concentrated at different locations, or it may be that the receptors for the different Aβo are expressed differently in the different regions, so the Aβo have more of an effect in one when compared to the other. It is interesting that despite the different regions affected, both models have similar NOR deficits, suggesting that it could be disruption of a circuit of regions, rather than a region specific effect.

In the HMW Aβo study, there was a significant decrease in hippocampal PSD-95 in the Aβo animals 14 days after surgery. This effect was completely prevented by 6D11 application, suggesting that the blockade of Aβo binding to PrP<sup>C</sup> prevents HMW Aβo induced cognitive and synaptic deficits. This prevention of PSD-95 reduction is in support of Laurén et al. (2009), who found that blocking the binding of Aβo to PrP<sup>C</sup> prevented synaptic deficits. It has been shown that the binding of Aβo to PrP<sup>C</sup> activates fyn (Larson et al. 2012; Um et al. 2012), and fyn is suggested to phosphorylate PSD-95 (Du et al. 2009).
In accordance with the behavioural work, the rescue of LMW Aβo induced PSD-95 by 6D11 is not complete. As discussed in a previous section, the reason for a partial rescue is not clear, but it may be that the LMW Aβo bind to PrP^C with less affinity than the HMW Aβo and act on other receptors, or it could be that some of the LMW Aβo oligomerise in vivo and bind to PrP^C, but not all of them. Alternatively, it may be that 6D11 acts preferentially in the hippocampus and not the frontal cortex. As the synaptic deficit following the administration of LMW Aβo oligomers is seen primarily in the frontal cortex, 6D11 may not be able to have the full effect seen with the HMW Aβo at the concentrations used.

In summary, this chapter has investigated the role of PrP^C in Aβo induced cognitive and synaptic impairments by blocking the binding of Aβo to PrP^C in vivo. It was found that in previously characterised models of HMW Aβo and LWM Aβo that blocking the binding of Aβo to PrP^C had differential effects, suggesting they act differently. Both HMW and LMW Aβo induced cognitive and synaptic deficits, however only the HMW Aβo induced deficits were completely prevented via pre-treatment with 6D11.
Chapter 6: The Effects of the Rho-kinase Inhibitor, Fasudil, on Aβo Induced Deficits
6.1 Introduction

One of the key points of the amyloid cascade hypothesis is the ability of amyloid-β (Aβ) to cause degenerative downstream changes in tau (Seward et al. 2013; Bloom 2014), however the pathway that links the two proteins in Alzheimer’s disease (AD) is yet to be fully elucidated. One mechanism which has shown some potential is Wnt signalling, of which there are three major pathways. Wnts play a crucial role in cell adhesion and cell determination in vertebrate and invertebrate development (Wodarz & Nusse 1998). They are regulated by a variety of secreted molecules, including inhibition by Dickkopf-1 (Dkk-1) via a mechanism upstream of frizzled (Krupnik et al. 1999). Dkk-1 has been found to antagonise Wnt signalling along the canonical pathway (Kawano & Kypta 2003). In the canonical pathway, Wnt ligands bind to both Frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), forming the Fz-LRP5/6 complex via Dishevelled (Dvl), promoting LRP6 phosphorylation via Glycogen synthase kinase 3 (GSK3) and CK1 (MacDonald et al. 2007) The activation of the canonical pathway leads to the inhibition of GSK3β (Alvarez et al. 2004), a protein which has been implicated in formation of neurofibrillary tangles (NFTs) in AD (Hooper et al. 2008; Kremer et al. 2011; Hernandez et al. 2012). The canonical pathway inhibitor Dkk-1 has been shown be increased in AD brains (Guo et al. 2016) and it has been shown to regulate Aβ induced pathology in rodent and cell models of AD (Purro et al. 2012; Killick et al. 2014). Therefore it is plausible that Aβ induced elevated Dkk-1 levels in AD could be inhibiting the canonical Wnt signalling pathway and causing an increase of GSK3β and abnormal tau phosphorylation (Figure 36, top pathway).
In addition to the decrease of canonical signalling, it is important to consider the increased non-canonical signalling pathways. The wnt-planar cell polarity pathway (PCP) has been shown to be induced via an Aβ-clusterin-Dkk-1 mechanism, which caused changes in a number of genes, which when silenced prevented Aβ toxicity and tau phosphorylation (Killick et al. 2014), suggesting that it is not purely the inactivation of the canonical wnt pathway, but also the activation of the PCP pathway which causes AD pathology (Figure 36, bottom pathway).

![Figure 36 Potential pathway of Aβ induced altered Wnt signalling](image)

*Figure 36 Potential pathway of Aβ induced altered Wnt signalling. The canonical pathway is inhibited leading to the increase in GSK3β and resulting tau phosphorylation. The PCP pathway is activated, leading to abnormal phosphorylation of tau and altered APP processing.*

Drosophila studies have shown an involvement of Rho family proteins in PCP signalling (Schlessinger et al. 2009), and other studies have shown its direct involvement in the activation of c-Jun N-terminal kinase (JNK) and c-jun - two downstream proteins in the PCP
pathway - , leading to changes in synaptic stability and microtubule reorganisation (Rosso et al. 2005; Gordon & Nusse 2006) and tau phosphorylation (Yoshida et al. 2004; Tatebayashi et al. 2006; Hoffmann et al. 2008).

Rho kinase (ROCK) has been identified as a target of Rho (Matsui et al. 1996) and ROCK2, the isoform most found in the brain (Nakagawa et al. 1996), has been shown to influence the production of Aβ via alteration of APP processing (Herskowitz et al. 2011). This could provide a positive feedback mechanism, whereby an increase of Aβ leads to a Wnt/PCP dependant increase of ROCK2, which then alters APP processing and increases Aβ production (Figure 36). It is conceivable then that inhibition of the ROCK could be beneficial in AD.

Fasudil is a ROCK inhibitor approved in Japan and China, commonly used to treat cerebral vasospasm. It is hypothesised that inhibiting ROCK in our rodent model will alleviate the Aβ induced cognitive and synaptic deficits seen. Due to its approval for human use, repurposing fasudil for AD could be a suitable way to treat AD, should it have the hypothesised effects.

This study will investigate the effects of the ROCK inhibitor, fasudil, in the HMW Aβ rat model previously discussed in earlier chapters.
6.2 Methods

6.2.1 Animals

All animals were female lister-hooded rats weighing approximately 200 ±20g at the start of experimentation. See section 2.2 for housing conditions. Animals had free access to food (Special Diet Services, UK) and water throughout experimentation and all studies were compliant with the Animal Scientific Procedures act (1986) and University of Manchester ethical guidelines.

6.2.2 Reagents

Aβ1-42 was prepared as described in section 2.4.2 and 10nmol was administered. The control solution was an equal volume of the vehicle (10% DMSO in Ham’s F12 medium).

Fasudil (Selleck) was diluted in saline to 10mg/mL (roughly 0.20-0.25mL/rat/injection), aliquoted for each day of injection and stored at -20°C.

6.2.3 Experimental design

40 animals were administered vehicle (saline, n=20) or fasudil (10mg/kg, n=20) twice daily IP for 7 days. One day after the first injection animals underwent surgery to receive a 10μL ICV injection of either vehicle or 10nMol HMW Aβ oligomers (n=10 per group) into the left lateral ventricle resulting in four groups. NOR was then performed on day 7. On day 7 after the NOR
procedure, animals were sacrificed and their brains snap frozen in isopentane, and stored at -80°C.

6.2.4 Behavioural testing

6.2.4.1 NOR

The apparatus was as described in section 2.5.1. The rats were habituated prior to NOR testing (see section 2.5.2.1) and tested 7 days after surgery. For details on the testing conditions, see section 2.5.2. Behavioural assessment details can be found in section 2.5.2.5.

6.2.5 Post-mortem analysis

Animals were sacrificed after the last NOR test, and the brains were removed and homogenised as described in section 2.6. PSD-95 was then investigated with a Meso Scale assay as described in section 2.6.7.
Dkk-1 levels were determined using a DuoSet ELISA Kit (R&D Systems, DY1765). ELISA was performed as described in section 2.6.6.
6.3 Results

6.3.1 Treatment with Rho-kinase inhibitor, fasudil, prevents Aβo induced cognitive deficits

6.3.1.1 Acquisition phase

During the acquisition phase (Figure 37) there was no difference in the exploration of the left/right objects ($F_{(7,79)}=0.9446, p>0.05$). There was no difference in the total exploration of the objects between the groups ($F_{(3,39)}=2.440, p>0.05$).

6.3.1.2 Retention phase

In the retention phase there was a significant difference in the exploration of novel and familiar objects ($F_{(7,79)}=15.24, p<0.001$). The vehicle treated group spent significantly more time investigating the novel object ($p<0.001$) as did the vehicle+fasudil treated ($p<0.01$) and the Aβo+fasudil ($p<0.001$) groups. There was no significant difference in the object exploration in the Aβo ($p>0.05$) treated group. Unlike during the acquisition phase, there was a significant difference in the total exploration times ($F_{(3,39)}=8.004, p<0.001$). There was no difference between the vehicle, Aβo and Aβo+fasudil groups ($p>0.05$) but the vehicle+fasudil group differed significantly from the vehicle ($p<0.01$), Aβo ($p<0.05$) and Aβo+fasudil ($p<0.001$) groups.
6.3.1.3 Discrimination Index

There was a significant effect of treatment group on the DI ($F_{(3,39)} = 4.208, p<0.05$). The vehicle group differed significantly from the Aβo group ($p<0.05$) but not from the vehicle+fasudil or Aβo+fasudil groups ($p>0.05$). There was a significant difference between the Aβo group and the vehicle+fasudil and Aβo+fasudil groups ($p<0.05$).

In summary, the vehicle treated groups spent significantly more time investigating the novel object when compared to the familiar. An effect abolished in the Aβo treated group. Animals treated with fasudil before Aβo injection showed no NOR deficit. Fasudil alone did not affect the preference for the novel object; however total exploration times were reduced.

Figure 37 showing the effect of fasudil on Aβo induced cognitive deficits (A) novel vs. familiar; two-way ANOVA followed by Bonferroni planned comparisons. ***p<0.001. There was a significant difference in NOR performance in the Aβo treated animals when compared to the vehicle and treated groups after 7 days (B) *p<0.05, vs vehicle group, #p<0.05, vs Aβo group; one-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group).
6.3.2 Post-mortem results

7 days after surgery PSD-95 was investigated as a post synaptic marker (Figure 38). There was no significant effect on PSD-95 with any treatment in the hippocampus ($F_{(3,19)}=0.6843$, $p>0.05$), but there was in the frontal cortex ($F_{(3,18)}=4.582$, $p<0.05$). The vehicle group differed from the vehicle+fasudil group ($p<0.05$) but not from the Aβo or Aβo+fasudil groups ($p>0.05$).

![Figure 38 Aβo and fasudil effects on PSD-95. (A) showing no significant difference with any treatment in the hippocampus. (B) showing a significant decrease in the frontal cortex in the vehicle+fasudil group. The results are expressed as means ±SEM. (n=5 per group). *p<0.05, vs vehicle group, one-way ANOVA followed by Bonferroni planned comparisons.]

7 days after surgery Dkk-1 levels were investigated in the hippocampus (Figure 39). There was no significant effect on Dkk-1 with any treatment ($F_{(3,19)}=0.5628$, $p>0.05$). There was a small, non-significant increase in the levels of Dkk-1 in the Aβo treated group compared with the control group ($p=0.062$).
6.3.3 The effect of fasudil on animal health

There was a significant interaction between treatment and change in animal weight ($F_{(3,36)}=8.12, p<0.001$), with both the vehicle and $\text{A}\beta\text{o}$ group gaining weight after surgery, and the vehicle+fasudil and $\text{A}\beta\text{o}$+fasudil groups losing weight after surgery. Planned comparisons revealed no differences between groups (Figure 40).

A count of line crossings in the NOR test was used to measure locomotor activity. During the acquisition phase, there was no difference between the groups in the number of line crossings ($F_{(3,39)}=2.459, p>0.05$). There was however a significant difference seen in the
retention phase ($F_{(3,39)}=3.027, p<0.05$) with the vehicle+fasudil group being significantly less active than the vehicle group ($p<0.05$).

![Figure 40](image)

**Figure 40 Changes to markers of animal health in response to Aβo and fasudil.** (A) Showing changes in animal weight in response to treatment; two-way repeated measures ANOVA followed by Bonferroni planned comparisons. (B) Showing a decrease in total exploration time of objects in the vehicle+fasudil group during the retention phase of the NOR test; one-way ANOVA followed by Bonferroni planned comparisons. (C) Showing a decrease in line crossings in the vehicle+fasudil group during the retention phase of the NOR test; one-way ANOVA followed by Bonferroni planned comparisons. The results are expressed as means ±SEM. (n=10 per group). *p<0.05 vs vehicle group, #p<0.01, ##p<0.05, ###p<0.001 vs vehicle+fasudil group.
6.4 Discussion

This chapter aimed to investigate the effects of the ROCK inhibitor fasudil on the HMW Aβo induced cognitive and synaptic deficits seen in our rat model. Fasudil prevented the NOR deficit after 7 days, however there was no significant change seen in levels of PSD95 in the hippocampus; there was however a significant decrease in PSD-95 in the frontal cortex of the fasudil only treated animals. There was also a change in weight associated with fasudil, as well as a decrease in total object investigation times and line crossings.

6.4.1 The effect of fasudil on Aβo induced NOR deficits

In this study, animals were given an Intraperitoneal (IP) injection of fasudil (10mg/kg) or vehicle (saline) twice daily for 7 days, starting one day before surgery where they were then given either 10nMol HMW Aβo or the equivalent volume of vehicle (10μl of 10% DMSO in Ham’s F12 medium) resulting in four groups. The drug dose and administration route was chosen for several reasons. ICV injections are by necessity low volume, so the concentration of fasudil would have had to be high, however, previous data from collaborators has shown fasudil to be neurotoxic in high concentrations. For this reason IP was chosen as the route of administration, and previous studies have shown positive effects at 10mg/kg on synapse numbers in the rat (Hou et al. 2012). On day 7 animals conducted the NOR task. The Aβo group was significantly impaired when compared to the controls. Fasudil alone had no effect on NOR performance, but completely prevented the deficit in the Aβo+fasudil group,
suggesting that the inhibition of ROCK is beneficial to Aβo induced deficits. Previously, fasudil has been shown to be beneficial to cognition in aged rats (Huentelman et al. 2009) and Aβ induced hippocampal neurodegeneration, suppressing inflammatory responses (Song et al. 2013). These findings support previous studies showing the protective effects of ROCK inhibition in AD like pathology (Leuchtenberger et al. 2006; Herskowitz et al. 2013).

The findings of this study appear to agree with previous studies, that ROCK plays an important role in AD-like pathology, and that the inhibition of ROCK could be a potential therapeutic strategy. It is important, however, to acknowledge that fasudil was administered before Aβo, and so the results presented are of its protective effect. Further investigation would be needed to evaluate its potential as a reversal drug in preclinical models for patients already exhibiting AD symptoms or Aβ build up.

6.4.2 The effect of fasudil on Aβo induced synaptic deficit

Unlike previous work using this model, there was no significant decrease in PSD-95 after 7 days in the hippocampus in Aβo treated group vs vehicle group. Previously this deficit has been small but significant, increasing in later time points. There was, however, a significant decrease in the frontal cortex in the vehicle+fasudil group vs vehicle group. This was surprising as there was no change in cognition in these animals, and because fasudil has previously been shown to rescue synaptic loss, rather than cause it (Hou et al. 2012). A ROCK inhibitor has also been shown to reverse Dkk-1 induced synaptic loss in vivo (Marzo et al.
and so Dkk-1 was investigated. We saw no significant change in Dkk-1 levels, however there was a non-significant increase in the Dkk-1 levels of the Aβo treated animals vs vehicle animals ($p=0.062$). Looking at previous work in this model, and the way PSD-95 deficits appear more strongly in later time points, it is plausible that if the synaptic loss is due to elevated Dkk-1 levels and that this might be seen 14+days after surgery.

6.4.3 Animal health

To measure the effects of fasudil on general health of the animals, they were weighed one week before, at the initiation, and one week after fasudil treatment. The movement and total investigation during the NOR test was also recorded. Following fasudil treatment, there was a significant change associated with treatment, with both the vehicle+fasudil and Aβo+fasudil groups losing weight, and both the vehicle and HMW Aβo groups gaining weight. This could be due to a number of reasons, perhaps the fasudil reducing appetite due to sickness or decreasing water retention. There was however no apparent symptoms or evidence of food refusal. It is unlikely to be caused by muscle atrophy, as fasudil has previously been shown to prevent this (Bowerman et al. 2012). Further work such as monitoring of food intake would be required to determine the cause. The vehicle+fasudil animals also moved less and investigated the objects less compared to all other groups in the NOR task. This could also be caused by adverse reaction to fasudil.
To summarise, the behaviour results seen in this study appear to support existing literature. The post mortem results however are not in full support. Fasudil administration was discontinued one day before NOR testing to prevent a direct effect being seen, such as a short term cognitive boost or decrease caused by the injection. It could be that this period was not long enough, and that could be the cause of the disparity between cognitive and post-mortem results. The post-mortem data do trend towards a suggestion that Aβ is causing a decrease in PSD95 and increase in DKK-1 in the hippocampus, which could be investigated further in a more comprehensive study with multiple time points. A dose response study would also be beneficial to investigate the body weight changes in the rats in response to fasudil.

The cognitive improvement seen in this experiment is robust. Fasudil is well tolerated in humans (Jiang et al. 2015; Vicari et al. 2005) and therefore despite the reduction in body weight and locomotion seen in the rats, further investigation into its potential for AD therapy is warranted.
Chapter 7: General Conclusions
7.1 Main Conclusions

The overall aim of this project was the establishment of two preclinical models of Aβ-induced cognitive damage, with relevance to Alzheimer’s disease (AD). Both models were produced in rats by injection of β-amyloid oligomers (Aβo) into the left ventricle of the brain. One model used low molecular weight (LMW) oligomers and the other used (HMW) oligomers. In both models, recognition memory was shown to be impaired early (days after surgery: LMW 4; HMW 7) and both repeatedly and persistently until the last day of testing (up to day: LMW 70; HMW 50). Whilst deficits developed after 5nMol doses in both models, the speed of onset of the deficits was related to molecular weight. The HMW Aβo required a dose of 10nMol to manifest before 35 days, whereas the LMW Aβo injected rats presented a deficit at 4 days with only 5nMol. HMW Aβo injected animals induced an NOR deficit in both male and female animals, in line with previous work on the LMW Aβo injected animals. Working memory was not seen to be impaired when tested by the 16-Holeboard maze task in the LMW Aβo model, nor Y-maze in the HMW model. There were no negative general health effects of Aβo injection when looking at animal weight or activity levels.

Synaptic deficits were seen in both models, however in different areas. The LWM Aβo injection caused a deficit in PSD-95 in the frontal cortex in line with previous studies with this model, whereas the HMW Aβo injection caused a deficit in the hippocampus but not the frontal cortex.
6D11, an antibody to PrP\textsuperscript{c}, was administered ICV to both models prior to A\textbeta o injection. In the HMW A\textbeta o injected animals 6D11 treatment completely prevented both the A\textbeta o induced NOR and PSD-95 deficits. In the LMW A\textbeta o injected animals, 6D11 treatment had only a partial rescuing effect, suggesting that the different aggregations of A\textbeta have differing effects.

Finally, the Rho-kinase 2 (ROCK2) inhibitor Fasudil was administered to the HMW A\textbeta o injected animals. This caused a prevention of the NOR deficit, however there was only a trend towards A\textbeta o induced synaptic deficits and fasudil rescue in the hippocampus. There was also a trend towards an A\textbeta o associated increase in Dkk-1 and which was reduced by fasudil.

The lack of correlation between cognitive deficits, and the brain regions implicated in the literature is puzzling. The 1 minute ITI in the NOR is thought to involved the perirhinal cortex and not the hippocampus (Antunes & Biala 2012), conversely the HMW model found an NOR deficit associated with a hippocampal synaptic deficit, and the LMW model found the NOR deficit associated with the frontal cortex. Additionally, the Y-maze task, used to investigate working memory, which is thought to involve the hippocampus (Yoon et al. 2008), showed no deficit in the HMW model, despite the synaptic deficit seen in the hippocampus in this model. It is unclear why the results of this study show no hippocampal related deficits similar to those summarised in the introduction. It could be that the particular strain of rat used in this model is less susceptible to working memory deficits, and can use compensatory methods, or the tests were not sensitive enough to pick them up. It should be noted that most studies investigating the brain regions involved with such tasks are lesion studies,
whereby the brain region of interest is completely disrupted. This relies upon the assumption that the areas of a damaged brain function identically to an undamaged brain, \textit{i.e.} with no compensatory or projection mechanisms. It is possible that with a complete lesion, the brain will find a compensatory mechanism to achieve similar results to an undamaged brain, whereas a more subtle deficit, such as the synaptic deficit seen in this study, the brain continues to act with no compensation mechanisms, but hindered by the deficit. One explanation for this could be that the damaged brain causes a disruption, either by disinhibition or over excitation its projections sites. Disinhibition of the hippocampus has been shown to cause cognitive impairments usually associated with projection sites, such as prefrontal cortex (Heath et al. 2015). As the hippocampus is known to be both directly linked with the perirhinal cortex (Liu & Bilkey 1996) and interact with the prefrontal cortex (Preston & Eichenbaum 2013), a similar explanation, of projection disruption, could be the cause of the cognitive deficits seen in the model in this study.

7.2 Limitations

With these models, along with the other A\textbeta{0} injection models discussed in the introduction, it is important to consider the physiological relevance. In AD A\textbeta{} is secreted over many years and gradually builds up, rather than in the acute way used in these models. In this regard the transgenic models of AD are more physiologically relevant to the disease. What the A\textbeta{} injection models provide is a platform with which to study the effects of A\textbeta{} and its associated pathology. They can be used to investigate the validity of the Amyloid Cascade
hypothesis, and as a model with which to test downstream targets of Aβ with a view for AD therapy.

7.3 Further work

Only recognition memory was shown to be inhibited by the ICV Aβo injections with no deficit seen with the Y-maze or 16-holeboard maze – thought to be tests of working memory. In both cases it could be that the task is not sensitive enough to pick up any deficit, and so further, more intricate tests of working memory and executive function could be used. Such tests include the radial-8 arm maze, similar to the Y-maze but with 8 baited arms, rather than 3 unbaited arms, or set shifting where the animal must adapt to changing rules in order to retrieve the reward. This would allow us to determine whether working memory is indeed unaffected, or if the tasks used were just not appropriate.

The post-mortem results seen in chapter 6 are worthy of further investigation. Although no significance level was reached there is a trend to suggest that the levels of Dkk-1 may increase with Aβo administration. The samples used for testing were taken 7 days after Aβo injection. Previously we have seen a PSD-95 deficit in the hippocampus at 14 days post injection, so it would be worth investigating whether a stronger deficit would also show a protective result following fasudil administration. It could be that the levels of Dkk-1 would continue to rise beyond day 7, so a time-course study could be conducted to investigate this. Additionally a dose response study would be useful to investigate whether the levels of
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Elevation of Dkk-1 are dose dependent. It could also be interesting to apply Dkk-1 rather than Aβo to see whether similar effects are observed. If Dkk-1 is elevated as a response to Aβo and a downstream target of the Amyloid cascade, it could show results mimicking that of Aβo administration. This has been explored with a transgenic mouse with inducible Dkk-1 overexpression, which showed that when Dkk-1 is expressed in the hippocampus, cognitive deficits and synaptic impairments are seen. These effects are completely reversed when Dkk-1 is then switched off (Marzo et al. 2016).

Further markers should also be investigated. In previous chapters it has been considered that Aβo injection may cause an alteration to APP processing, and so measuring the fragments of cleaved APP, APPα and APPβ would be beneficial to elucidating whether there is an effect of Aβo on APP processing in this model. The role of inflammation has also not been explored. As mentioned in the introduction, there is evidence that inflammation plays a role in AD, although whether this is a cause of symptoms or a response to pathology is unclear. Nevertheless, inflammatory markers could provide insight into the benefit of therapeutic strategies investigated with these models, and thus the effects of Aβo on inflammatory markers should be characterised. In addition to using immunoblotting or assays such as ELISA or Meso Scale, immunohistochemistry (IHC) should be used to investigate cell morphology such as microglia and astroglia, as well as allowing for the examination of protein localisation, such as tau mislocalisation or Aβ aggregation.

Finally, the purpose of the development and characterisation of these models is ultimately to pursue novel therapeutic strategies. In a similar way to 6D11 in Chapter 5, and fasudil in
Chapter 6, further work will involve using this model to validate the potential of therapeutic targets or drug candidates.

In summary, this project has succeeded in the validation of two similar models of Aβo toxicity with relevance to AD. With the different Aβo aggregations used, these models can be used to investigate the different effects caused, as well as their response to potential therapies.
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Investigating therapeutic strategies in a preclinical model for Alzheimer’s Disease

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Appendix

I. Conferences Attended

- Alzheimer Research UK conference – Manchester (UK) – March 2016
- Alzheimer’s Drug Discovery Foundation International Conference – New Jersey (USA) October 2015
- Dementia Research Day / Manchester Institute for Collaborative Research on Ageing (MICRA) – Manchester (UK) – June 2015
- Alzheimer Research UK conference – London (UK) – March 2015
- Immune influences on brain & behaviour – Brighton (UK), June 2014

II. Publications and Awards

- Alzheimer’s Drug Discovery Foundation Young investigator of the year award (2015)
- Successful Grindley grant application to fund travel to a conference.
- Contribution towards a successful President’s Doctoral Scholar Award for a new PhD student.

III. Disseminations and teaching

- Invited speaker at Swansea University – 2016
- Speaker at Alzheimer Research UK conference PhD day – Manchester (UK) – March 2016
- Poster presentation at Alzheimer Research UK– Manchester (UK) – March 2016
- Poster presentation at Alzheimer’s Drug Discovery Foundation International Conference – New Jersey (USA) October 2015
- Speaker and poster presentations at internal conferences 2013-2016
- Teaching assistant – Manchester Pharmacy School, The University of Manchester – 2013-17
IV. Posters

Copies of posters created during this theses are presented.

- As first author


- As co-author

William Watremez, V Fasolino, M Daniels, C Lawrence, J Jackson, G Galea, J C Neill, T Pillot, D Brough, MK Harte. **Establishment of a rat model of relevance to sporadic Alzheimer’s Disease research: birth and development of a collaborative project.** *University of Manchester Postgraduate Research Summer Showcase. Manchester (UK) 2015*

Investigating therapeutic strategies in a preclinical model for Alzheimer’s Disease
ROCK inhibitor Fasudil prevents β-amyloid-induced cognitive deficits in vivo

The University of Manchester

Investigating therapeutic strategies in a preclinical model for Alzheimer’s Disease

Background

Alzheimer’s disease (AD) is driven by β-amyloid (Aβ), which initiates a cascade of events resulting in abnormal phosphorylation of tau and cognitive impairment. Soluble Aβ oligomers (Aβo) are held to be the toxic Aβ species.

Performance in the Novel Object Recognition (NOR) task is as effective as the Morris Water Maze for evaluating cognitive performance in rodents, with the advantages of causing little stress and requiring no water.

Fasudil is the only ROCK inhibitor approved for clinical use, currently prescribed for cerebral vasospasm and post-ischaemia recovery, and has been shown to protect against age-dependent cognitive decline in wild type rats. Our main cell line based data indicate ROCK is involved in Aβo driven synapse loss. As such, we tested Fasudil in a rat/NOR model of Aβo induced cognitive deficit.

Methods

Seventy-five male (randomly bred) C57Bl/6J mice (Clea Japan) were housed in individually ventilated cages, fed standard mouse food and water ad libitum. Mice were randomly assigned to the experimental conditions, and were acclimated to the housing environment for one week prior to being tested. The animals received all procedures in accordance with the local Animal Ethics Committee guidelines and the current guidelines from the National Institute of Health.

RESULTS

1. Fasudil treatment significantly improved the performance of mice in the NOR task, as measured by the percentage of novel object exploration time compared to the control group. The results were statistically significant (p < 0.05).

2. The ROCK inhibitor Fasudil prevented the Aβo-induced cognitive deficits in the NOR task, as evidenced by increased novel object exploration time.

Cognitive deficit is blocked by Fasudil

Conclusions

1. Aβo oligomers cause cognitive deficit in the NOR test in rats. Previous work has shown that such deficits are sustained indefinitely. This acute model is then useful for investigating the early mechanisms of Alzheimer’s disease, and provides a platform for testing novel therapeutics.

2. Here we show that Fasudil prevents acute Aβo-induced cognitive deficits in this model, and that it also lowers soluble Aβo levels in frontal cortex of the 5x Tg mouse model of AD.

3. These findings add to our data from studies in primary neurons where we demonstrate that Aβo-driven synaptic loss is dependent upon a DMPK-Mt-PCP-KvA/ROCK pathway that can be blocked by Fasudil and other ROCK inhibitors, and that the synaptic-protective effects of ROCK inhibition extend in vivo to protect cognition, and also impact upon Aβo production in brain.

References


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