

**Investigating cognition and  
neuropathological markers  
following administration of  
amyloid- $\beta$  oligomers:  
  
Developing a rat model for  
Alzheimer's disease research**

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# ABSTRACT

The University of Manchester, Manchester Pharmacy School

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*Investigating cognition and neuropathological markers following administration of amyloid- $\beta$  oligomers: Developing a rat model for Alzheimer's disease research.*

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With current treatments for Alzheimer's disease (AD) only providing temporary symptomatic benefits and an ageing population, disease modifying drugs are urgently required. This approach relies on improved understanding of the early pathophysiology of AD. Ongoing research suggests that soluble amyloid- $\beta$  oligomers (A $\beta$ <sub>o</sub>) and inflammatory processes are among the early factors that contribute to the pathogenesis and progression of AD, by initially triggering synaptic deficits and subsequent cognitive impairment. The aim was to investigate cognitive and synaptic function, as well as neuroinflammatory markers, following an acute intracerebroventricular (ICV) injection of stabilised low-n A $\beta$ <sub>o</sub> in the rat.

This thesis first investigates the different existing rat models of A $\beta$ -peptide administration to better understand the mechanisms involved, neuropathological changes observed and cognitive deficits assessed. From this review, I determined the cognitive and neuropathological markers that would be investigated in the current model.

After presenting all the methods used, the following results chapter studied the reproducibility and duration of the cognitive deficits, the effect of different doses of oligomers and sex differences. An early (4 days after surgery) and lasting (up to 70 days after surgery) memory deficit in the novel object recognition (NOR) task has been found. This deficit was triggered by doses of acutely administered A $\beta$ <sub>o</sub> ranging from 0.5 to 5nmol, and equally affected female and male rats. Investigated neuropathological markers showed a synaptic deficit, raised neuroinflammatory cytokines levels and decreased density of parvalbumin-positive cells in the frontal cortex, but not the hippocampus. The NOR deficit could be rescued by treatment with the phosphodiesterase-4 inhibitor rolipram, an effect that stopped after cessation of the treatment. Treatment with the non-steroidal anti-inflammatory drug mefenamic acid prevented the NOR deficit and had an added protective effect up to 21 days after cessation of the treatment.

Taken together, these results suggest that acute ICV administration of A $\beta$ <sub>o</sub> may be a useful model to study the early mechanisms involved in AD and may provide researchers with a platform for testing novel therapeutic approaches targeting the mechanisms that contribute to AD, and particularly the early neuroinflammatory phenomenon.

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*I wish to dedicate my thesis to Patrick Carroll-Fogg who has been a real support,  
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## LIST OF ABBREVIATIONS

- 8-ARM: 8-Arm Radial Maze
- AChE: AcetylCholineEsterase
- AChEIs: AChE Inhibitors
- AD: Alzheimer's Disease
- AICD: APP IntraCellular Domain
- apoE: apolipoprotein E
- APP: Amyloid Precursor Protein
- APP-CTF: APP-CarboxyTerminal Fragment
- APPs: soluble APP
- A $\beta$ : Amyloid- $\beta$
- A $\beta$ <sub>o</sub>: A $\beta$  oligomers
- BACE:  $\beta$ -APP Cleaving Enzyme
- BBB: Blood-Brain Barrier
- BDNF: Brain-Derived Neurotrophic Factor
- BSA: Bovine Serum Albumin
- cAB: capture AntiBody
- ChAT: Choline AcetylTransferase
- COX: CycloOXygenase
- cPLA<sub>2</sub>: PhosphoLipase A<sub>2</sub>
- CSF: CerebroSpinal Fluid
- dAB: detection AntiBody
- DAMPs: Damage-Associated Molecular Patterns
- DI: Discrimination Index
- ELISA: Enzyme-Linked ImmunoSorbent Assay
- GABA:  $\gamma$ -AminoButyric Acid
- GFAP: Glial Fibrillary Acidic Protein
- H: Horizontal coordinates (on stereotaxic frame)
- HPLC: High-Performance Liquid Chromatography
- ICV: IntraCerebroVentricular
- IL: InterLeukin
- iNOS: induced Nitric Oxide Synthase
- IP: Intra-Peritoneal
- ITI: Inter-Trial Interval
- LTD: Long-Term Depression
- LTP: Long-Term Potentiation
- MCI: Mild Cognitive Impairment

- mGluR: metabotropic GLUtamate Receptor
- MWM: Morris Water Maze
- NAA: N-Acetyl-L-Aspartic acid
- nAChR: nicotinic AcetylCholine Receptor
- NF: Nuclear Factor
- NFTs: NeuroFibrillary Tangles
- NLRP-3: Nucleotide-binding oligomerisation domain-Like Receptors family, Pyrin domain containing 3
- NMDA: N-Methyl-D-Aspartate
- NMDAR: NMDA Receptor
- NOR: Novel Object Recognition
- NSAID: Non-Steroidal Anti-Inflammatory Drug
- pCREB: phospho-cAMP Response Element-Binding
- PDEs: PhosphoDiEsterases
- PKC: Protein Kinase C
- PO: Per Os
- PrP: PRion Protein
- PSD-95: Post-Synaptic Density 95
- PV: ParValbumin
- RD: Reagent Diluent
- ROS: Reactive Oxygen Species
- RT: Room Temperature
- SNAP-25: SyNaptosomal Associated Protein 25kDa
- SPs: Senile Plaques
- TNF: Tumour Necrosis Factor
- Tr: Transverse coordinates (on stereotaxic frame)
- V: Vertical coordinates (on stereotaxic frame)
- vs.: versus
- WWWhich: What-Where-Which

## Chapter 1 GENERAL INTRODUCTION

## 1.1 EPIDEMIOLOGY OF ALZHEIMER'S DISEASE: A WORLDWIDE AND INCURABLE FORM OF DEMENTIA

In their recent report on dementia, the World Health Organization and the Alzheimer's Disease International Group described dementia as: "a syndrome, usually of a chronic or progressive nature, caused by a variety of brain illnesses that affect memory, thinking, behaviour and ability to perform everyday activities" (World Health Organization, 2012). Among those brain illnesses, Alzheimer's disease (AD) has been estimated as the most common in patients over 65-year-old (Mayeux and Stern, 2012). It is nowadays a worldwide health concern, with an estimated 24.3 million cases in 2005 (Ferri et al., 2005), and it remains an incurable disease leading the patient to a state of depersonalisation and complete dependence on caregivers.

AD is a progressive pathology which will evolve from asymptomatic preclinical stages to serious cognitive and behavioural impairment (Figure 1). The first stages of the disease feature underlying biological changes with no overt clinical symptoms, rendering diagnosis nearly impossible in the current state of knowledge. The following mild-cognitive impairment (MCI) stage involves subtle cognitive changes while daily living activities are usually preserved (Petersen, 2003). Progression of the pathology will then be accompanied by cognitive and behavioural decline. The patient will progressively lose the ability to get involved in social activities, suffering from forgetfulness, learning impairment, speaking and understanding difficulties (Jalbert et al., 2008, World Health Organization, 2006, World Health Organization, 2012).

Anxiety, aggressiveness, depression and apathy are usually associated with the cognitive symptoms (Gillette-Guyonnet et al., 2011). Alongside mental decline, there is an associated decline in motor-function (incontinence, difficulties to move), resulting in the patient becoming more and more dependent on the carers to perform everyday tasks (National Institute for Health

and Clinical Excellence, 2006). Finally, as AD mainly affects people over 65-year-old, several age-related comorbidities can be associated with the patient's condition.

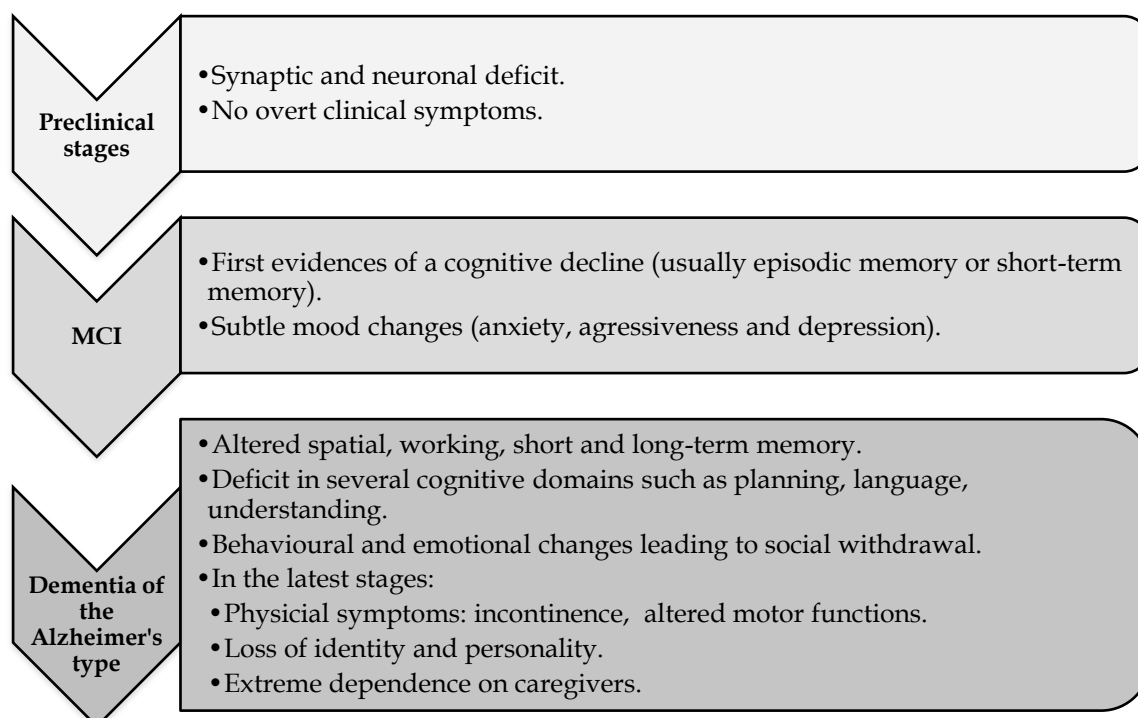


Figure 1: Continuum of Alzheimer's disease: From asymptomatic preclinical stages to progressive mental and physical decline.

Although a number of mechanisms have yet to be discovered, findings from neurochemistry analyses, genetics, behaviour studies and imaging have helped in understanding the biological progression of AD, and lead to the development of the first generation of treatments. From brain structure changes and cholinergic neuronal loss to earlier prodromal mechanisms, current knowledge enables researchers to go back up the neuropathological pathways.

## 1.2 MECHANISMS: FROM AMYLOID PRECURSOR PROTEIN TO COGNITIVE IMPAIRMENT

### 1.2.1 *Cholinergic hypothesis and first generation of treatments*

Since the first documented cases of AD, characteristic brain abnormalities have been identified during post mortem analyses: senile plaques (SPs) and neurofibrillary tangles (NFTs) (Graeber and Mehraein, 1999). SPs are formed by aggregates of amyloid- $\beta$  ( $A\beta$ ) peptides. These peptides, naturally found in the human brain, are produced by the proteolysis of the amyloid precursor protein (APP) into different fractions. The cleavage of APP can follow two main pathways: (1) cleavage by an  $\alpha$ -secretase and a  $\gamma$ -secretase which will lead to a non-amyloid protein, or (2) cleavage by a  $\beta$ -secretase (referred as  $\beta$ -APP cleaving enzyme: BACE) and a  $\gamma$ -secretase which will produce  $A\beta$  peptides (Figure 2). The multiplicity of cleavage sites, and mutations on both the APP cleavage sites and secretases, explain the multiplicity of  $A\beta$  peptides lengths that can be produced, such as the main two toxic species  $A\beta_{1-40}$  and  $A\beta_{1-42}$  which are later reviewed in this Chapter 2. Little data is available concerning the role of soluble APP (APPs), APP intracellular domain (AICD) and p3 fragments and their functions remain controversial although increasing the generation of p3 fragments, as a non-amyloidogenic pathway, is being studied (Yamada and Nabeshima, 2000, De Strooper, 2010, Benilova et al., 2012, Crouch et al., 2008). Mutations of BACE and  $\gamma$ -secretase can result in different forms of  $A\beta$  peptide being produced (Yamada and Nabeshima, 2000, Crouch et al., 2008). The involvement of the APP gene has been extensively studied and reviewed by (Walsh and Selkoe, 2007) who highlighted that: (1) the APP gene is situated on chromosome 21 and its overexpression in Down's syndrome is linked to AD-like symptoms; (2) mutations in the APP gene can cause early-onset of AD; (3) aggressive forms of AD are observed in patients with mutations on the presenilin

1 and 2 genes, which both play a role on the ratio of different forms of A $\beta$  peptides; (4) transgenic mice expressing human APP suffer from age-related AD-like symptoms.

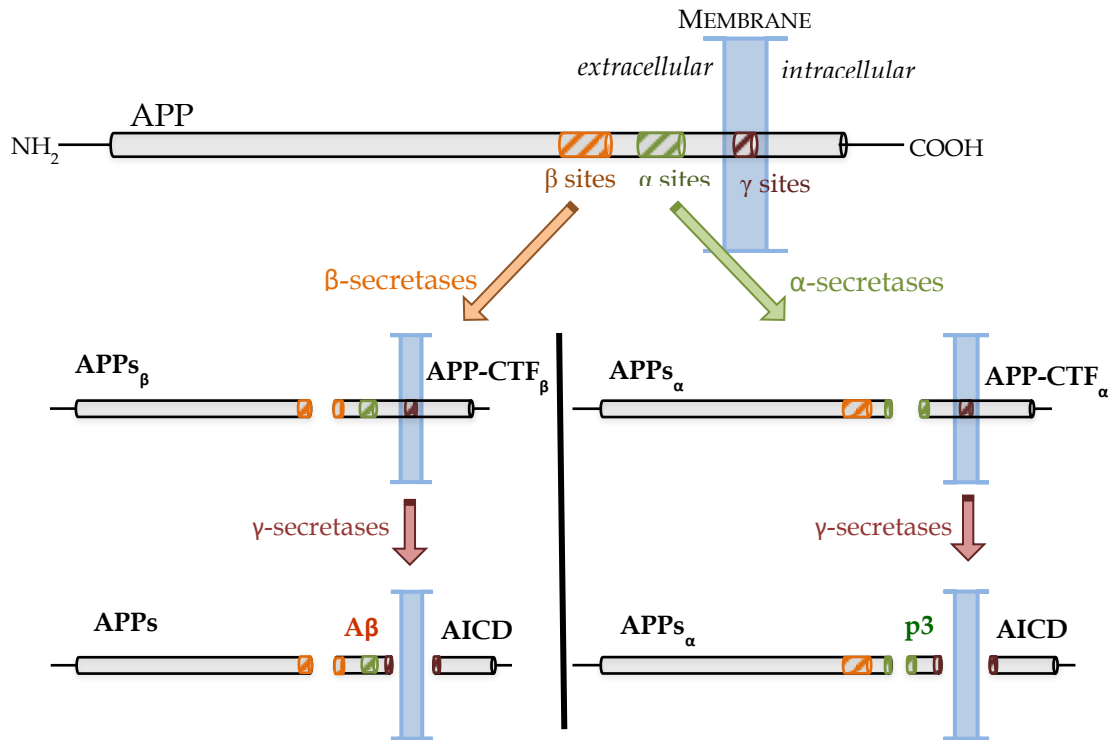


Figure 2: APP proteolysis. The cleavage by  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretases will lead to different types of peptides; several amino-acid have been identified as cleavage sites for each secretase resulting in a multitude of potential fragments. Cleavages by  $\alpha$ - and  $\beta$ -secretases will produce the soluble extracellular APPs fragments and carboxyterminal fragments (APP-CTF). Further cleavage by  $\gamma$ -secretase will lead to the production of either p3 peptide (right panel, non-amyloidogenic pathway) or amyloid- $\beta$  (A $\beta$ ) (left panel, amyloidogenic pathway), and APP intracellular domain (AICD).

SPs are found in most cases of AD and contribute to the definitive diagnosis (McKhann et al., 1984). However, SPs are not pathognomonic of AD and no correlation has been found between the density of SPs in the brain and the extent of cognitive deficit (Braak and Braak, 1991). A recent review suggests that SPs are not a direct cause of neuronal loss but will generate an aggregate stress which triggers NFTs formation (Karran et al., 2011). NFTs are pathogenic fibrils which result from the binding of hyperphosphorylated tau which, in physiological condition, are associated to microtubules and contribute to maintaining the cell cytoskeleton (Alberts et al., 2002). When hyperphosphorylated, tau can no longer promote the assembly of microtubules;

this leads to a degeneration of the cell cytoskeleton and to neuronal loss. Hyperphosphorylated tau will instead assemble into NFTs whose toxicity has directly been linked to neuronal loss (Hutton et al., 1998, Iqbal and Grundke-Iqbal, 2008). More recent findings have suggested a role of tau in neuronal migration *in vitro* (Sapir et al., 2012) but more importantly showed a direct involvement in normal synaptic functions. Tau and phosphorylated tau have been showed to be localised at both pre- (Harris et al., 2012) and post-synaptic (Tai et al., 2012) sites. Although the mechanisms still remain unclear, oligomeric tau have been found to be involved in synaptic toxicity, with a role in synaptic excitability, alteration of PSD-95 levels and impairment of mitochondrial functions (see (Pooler et al., 2014) for review).

Findings from biochemical analysis first showed that neuronal loss mainly affected cholinergic neurons – acetylcholine being the associated neurotransmitter – leading to the establishment of the cholinergic hypothesis of AD (Coyle et al., 1983, Muir, 1997, Francis et al., 1999). Hippocampal acetylcholine has indeed been shown to play a role in memory consolidation (Stanley et al., 2012, Power et al., 2003). Following this hypothesis, drug development focused on restoring the cholinergic loss by inhibiting the enzyme responsible for the degradation of acetylcholine. In the early 1990s, the acetylcholinesterase inhibitors (AChEIs) donepezil, galantamine, and rivastigmine have been developed as the main pharmacological treatment (Lecanu and Papadopoulos, 2013). Despite clinical trials suggesting a positive effect of AChEIs on cognition, a recent review questioned the methodological validity of those trials (Kaduszkiewicz et al., 2005). These results are supported by previous findings (Loveman et al., 2006) who showed mixed results on reviewing the clinical effectiveness of AChEIs from several selected studies. It has been as well suggested that AChEIs had no impact on mortality, with their effect being only symptomatic rather than curative (Bond et al., 2012).



As there is evidence for the role of calcium and the glutamatergic system in neuronal loss (Cacabelos et al., 1999), the non-competitive N-methyl-D-aspartate (NMDA) antagonist memantine has been used for the treatment of AD. It is thought that it can stop excitotoxic neuronal cell death by preventing NMDAR hyperactivity (Hempel et al., 2011). Although proof of its efficacy remains low, its potential synergy with AChEIs makes it the best current option for the treatment of AD (Loveman et al., 2006, Areosa et al., 2005).

Current treatments, associated with non-pharmacological (e.g. cognitive, physical, artistic) therapies, are thus helpful to delay the cognitive and functional decline. However, they provide only symptomatic effects and do not act on the underlying causes of AD. Understanding the early mechanisms of the disease to develop disease modifying drugs, led researchers to further investigate the role of A $\beta$  peptides, before the onset of SPs formation.

### ***1.2.2 Amyloid- $\beta$ oligomer synaptotoxicity***

Alongside its involvement in SPs formation, a toxic role of A $\beta$  peptides has been postulated since their first observations in cerebrospinal fluid (CSF) and post mortem tissues from AD patients; and accumulating evidence of a correlation between soluble A $\beta$  peptides levels and AD has been found in human studies (McDonald et al., 2010). Soluble oligomeric A $\beta_{1-40}$  and A $\beta_{1-42}$  and their relative ratio are the two main suggested forms of neurotoxic A $\beta$  peptides (Karran et al., 2011, Findeis, 2007); 1-40 and 1-42 referring to the length of the A $\beta$  peptides, while "oligomeric" means low-n non aggregated peptides (usually monomers to tetramers). Over the past 15 years, accumulative evidence of a causative role of soluble A $\beta$  oligomers (A $\beta$ o) in the early onset of AD has emerged, as they are thought to trigger a synaptic deficit – see (Selkoe, 2002) for review.

Synaptic structural modifications have been observed by electron microscopy in human case studies as early as the 1960s (Gonatas et al., 1967) and have

been confirmed by a loss of synapses, greater than neuronal loss, in the frontal and temporal lobe of AD patients (Davies et al., 1987). Following these results, autopsy studies of the left neocortex (namely midfrontal, rostral superior temporal and inferior parietal areas) of 15 patients with AD versus 9 matched controls directly correlated synaptic loss to impaired cognition (Terry et al., 1991). Significant decreases of the synaptic density (from 38 to 43%) was reported in the three regions studied. Moreover, the synaptic deficit in the midfrontal cortex was shown to significantly correlate with the patient's poor performances in the psychometric tests used. There were no correlations in relation to SPs density. Synaptic proteins have thus been suggested as a better marker of cognitive decline in AD, particularly in the hippocampus and frontal cortex where significant deficits in the immunoreactivity of the presynaptic vesicle protein synaptophysin has been shown to correlate with the progression of the disease (Sze et al., 1997, Dickson et al., 1995, Masliah et al., 2001).

In preclinical studies, natural heterogeneous human A $\beta$  species secreted by living cells have been proven to impair rat hippocampal long-term potentiation (LTP), a phenomenon linked to synaptic plasticity and memory (Townsend et al., 2006, Walsh et al., 2002). The decrease in LTP can be explained by a shifting of the balance of NMDAR-dependent signalling cascades toward long-term depression (LTD) (Shankar et al., 2007, Li et al., 2009, Shankar et al., 2008). Moreover neurons overexpressing APP have shown decreased excitatory transmission following the same pathway (Kamenetz et al., 2003). While LTP is linked to spine growth and stabilisation, LTD can be a sign of decreased dendritic density and synaptic loss in hippocampal pyramidal neurons. Induction of LTP or LTD results from the influx of post-synaptic calcium, following NMDAR activation. It has been shown that A $\beta$ o can reduce the calcium influx by approximately 27% (Shankar et al., 2007). In mice, intracerebroventricular (ICV) administration of human A $\beta$ o has been shown to inhibit the induction of LTP in an age-dependent

way (Townsend et al., 2006). Human extracts of A $\beta$  led to enhanced LTD and impaired LTP in mouse hippocampal slices associated to reduced cognitive function in rats. These effects were specifically linked to soluble A $\beta$  peptides but not to insoluble A $\beta$  peptides (Shankar et al., 2008). These results suggest that A $\beta$  affect the LTP/LTD balance rather than directly impairing LTP induction, following loss of dendritic spines and synapses, as confirmed in neurons overexpressing human APP (Hsieh et al., 2006). It has been shown that human-derived A $\beta$  specifically bind to post-synaptic sites and were co-localised with the post-synaptic density 95 (PSD-95) marker (Lacor et al., 2004). The ability of A $\beta$  to directly target post-synaptic neuron terminals (Jarosz-Griffiths et al., 2016) and disrupt synaptic functions is supported by its capacity to reduce synaptic cell adhesion molecule functions, damaging synaptic stability and structure (Leshchyn'ska et al., 2015).

These findings have orientated drug discovery strategies on inhibiting the formation of A $\beta$  to prevent LTP/LTD imbalance (Walsh et al., 2005). However, the aggregation state of A $\beta$  is of particular importance. Therapeutic molecules must degrade oligomers to less toxic monomers but not degrade fibrils to harmful low-n oligomers (Crouch et al., 2008), low-n A $\beta$  being 10 times more toxic than fibrils and 40 times more toxic than non-aggregated A $\beta$  species *in vitro* (Dahlgren et al., 2002). An alternative strategy could hence be to promote A $\beta$  aggregation, i.e. from oligomers to larger less-toxic aggregates. Comparison of different mouse strains that overexpress A $\beta$ , with their non-transgenic littermates, found better performance in cognitive tests in mice expressing a factor promoting the assembly of A $\beta$  to protofibrils and plaques (Cheng et al., 2007). *In vivo* studies showed that accelerating A $\beta$  aggregation decreases the time-frame whereby soluble and toxic A $\beta$  could damage brain structures (Bieschke et al., 2012). These results suggest that drug discovery strategies should focus on soluble A $\beta$  and not on SPs, as aggregation of A $\beta$  into SPs may

be a detoxification pathway. Taken together these studies open new perspectives to AD treatment but also highlight the risk of A $\beta$  fibril degradation strategies, which could lead to more toxic oligomers.

### ***1.2.3 Oxidative stress and neuroinflammation***

The hypothesis that oxidative stress is one of major features of AD has been suggested since the 1980s (Martins et al., 1986). Excess of reactive oxygen species (ROS) leads to a cascade of harmful events including lipid peroxidation, protein oxidation, nucleic acid damage, and alteration of the cytoskeleton; resulting eventually in cell death (Gotz et al., 1994). Analysis of post mortem brains of AD patients have highlighted brain damages resulting from oxidative stress when compared to age-matched control (Mecocci et al., 1994, Smith et al., 1991, Smith et al., 1996) suggesting that the pathological mechanisms of AD could involve an increased production of ROS (Nunomura et al., 2001) or a weakening of the defence systems – especially in elderly patients (Sparkman and Johnson, 2008).

Oxidative stress in A $\beta$  pathology has been shown to play a role in mitochondrial malfunction (Gotz et al., 1994, Gutteridge, 1994), including increased mitochondria degradation in hippocampal pyramidal cells (Hirai et al., 2001). It has been particularly stressed that mitochondria are altered in AD brains as a consequence of A $\beta$  toxicity (Iijima-Ando et al., 2009, Butterfield et al., 2013) and later emphasised by the tau-pathology (Quintanilla et al., 2012). Mutations of mitochondrial DNA have also been reported to be a risk factor for AD, meaning that mitochondrial malfunction potentially has additional causative effects in AD – see (Yan et al., 2012) for review. These data are further supported by the capacity of A $\beta$  to increase the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), an effect that could be prevented by antioxidants (Behl et al., 1994, Butterfield et al., 1994, Schubert et al., 1995, Mattson and Goodman,

1995). Oxidative stress has been shown to play a role as well in the alteration of the permeability of the blood-brain barrier (BBB), the modulation of  $\beta$ - and  $\gamma$ -secretases and the release of pro-inflammatory cytokines, which then further contribute to A $\beta$  synthesis and deposition – see (Cai et al., 2011) for review.

Oxidative stress and neuroinflammation are closely linked to neuronal pathology. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is an early pro-inflammatory cytokine produced after brain injury or infection (Dinarello, 1988), which has been found to be increased in the temporal lobe (Griffin et al., 1989), frontal cortex, hippocampus (Cacabelos et al., 1994), and CSF (Cacabelos et al., 1991) in patients with AD. *In vitro* IL-1 $\beta$  promotes APP gene expression (Goldgaber et al., 1989, Donnelly et al., 1990). IL-1 $\beta$  is thought to play a role in neurotoxicity and A $\beta$  generation properties – see (Shaftel et al., 2008, Candore et al., 2010) for review – and to impair hippocampal dependent memory, especially LTP – see (Rachal Pugh et al., 2001) for review. In a series of studies, Pugh et al. demonstrated that *in vivo*, direct administration of IL-1 $\beta$  into the ventricles or its stimulation by different infectious factors (Lipopolysaccharide, human immunodeficiency virus—1 glycoprotein gp120) impairs elements of hippocampal-dependent memory in fear conditioning tests but not hippocampal-independent aspects (Pugh et al., 1998, Pugh et al., 1999, Pugh et al., 2000). Regarding AD, it is thought that misfolded proteins such as A $\beta$  stimulate the secretion of IL-1 $\beta$  by microglia (Masters and O'Neill, 2011). It would thus appear that A $\beta$  cause inflammation in the brain and trigger the production IL-1 $\beta$  which in turn feeds back by increasing the production of APP and subsequently A $\beta$ . Sterile neuroinflammation – i.e. inflammation independent from external infection – thus appears to play an important role in AD as both cause and consequence of A $\beta$  production and contributing to synaptic and neuronal damages – see (Heppner et al., 2015) for review.

## 1.3 CURRENT CHALLENGES AND AIMS OF THE PROJECT

### 1.3.1 Development and limitations of AD models

Despite the current knowledge on AD and the underlying mechanisms, available treatments only provide symptomatic relief and do not tackle the causative mechanisms of the disease (O'Brien et al., 2011). As such there is a strong unmet need for new treatments that could target the underlying mechanisms and prevent or treat AD at much early stages (Selkoe, 2012). Added to the unmet need of disease modifying treatment, a second challenge is the development of biomarkers for early diagnosis. Currently, the diagnosis of AD mainly relies on cognitive impairment such as the mini-mental state examination (Folstein et al., 1975) and, where possible, by additional biomarkers (Dubois et al., 2007). However, as discussed above, cognitive symptoms are a late feature of AD that can appear years after the underlying pathological and synaptic deficits occur (Figure 3). As such the development of reliable and early biomarkers is thus another unmet need that the research field faces.

The drug development pipeline is hence now focusing on targeting earlier stages of the disease. However the past decades have seen many clinical failures (Mullane and Williams, 2013, Rosenblum, 2014). Indeed, despite many animal models existing and helping in the understanding of the pathological mechanisms, none has yet been able to select and develop an effective disease modifying treatment which could succeed to prove its efficacy and lack of serious side effects in clinical trials (Huang and Mucke, 2012, Braidy et al., 2012, Van Dam and De Deyn, 2011). There is hence an urgent need to rethink which animal models to use and how to design preclinical research (Golde et al., 2011). Since the early 1980s when the lack of reliable *in vivo* AD models was first highlighted (Vogel, 1980), AD animal models have evolved alongside the understanding of the pathology (Figure 4).

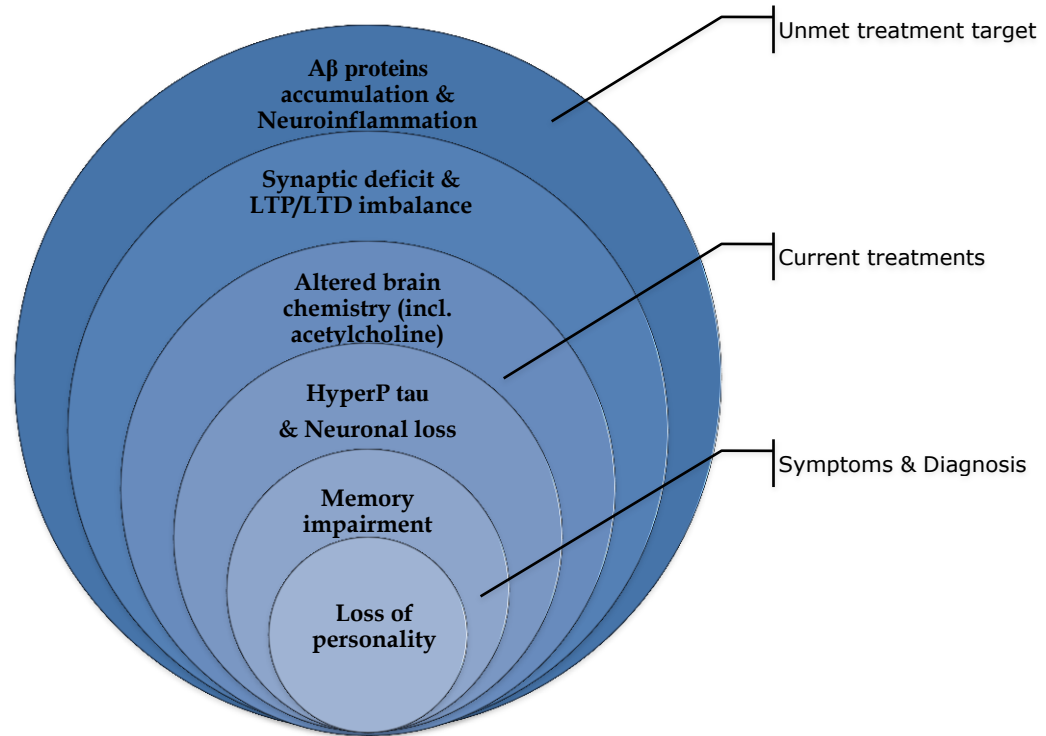


Figure 3: Current treatments are given too late in the disease progression. There is an unmet need for the development of new drugs which could tackle the early stages of the disease. HyperP: Hyperphosphorylated.

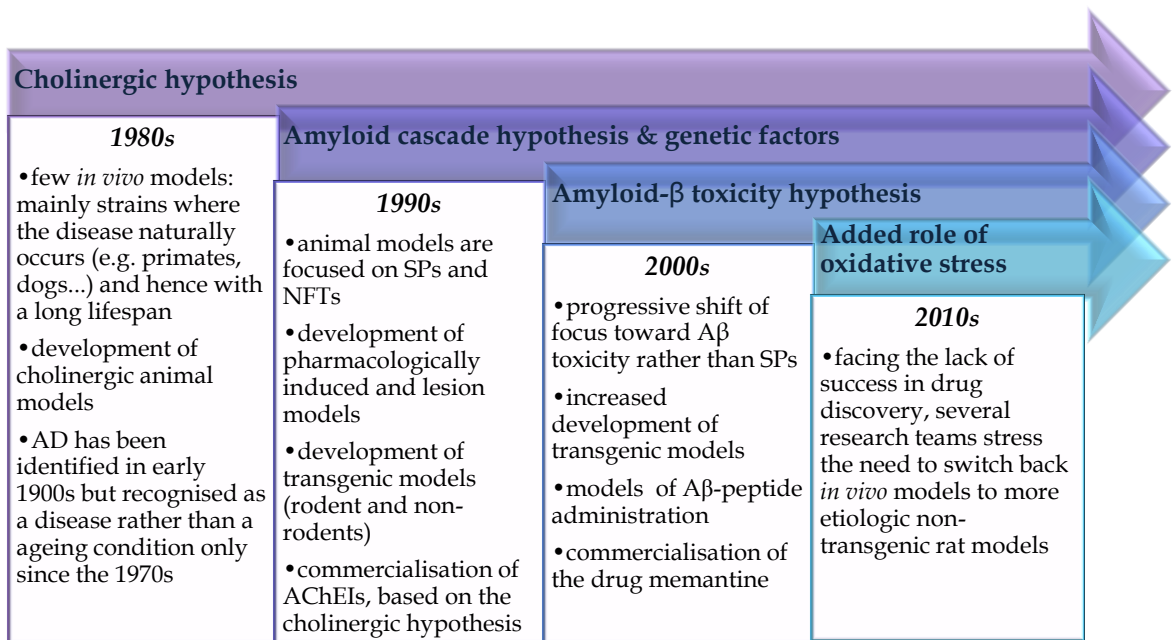


Figure 4: Timeline of *in vivo* AD models, from naturally occurring models toward the different hypotheses on pathological mechanisms.

In the 1980s, the first rodent models were based on the cholinergic hypothesis (Nakahara et al., 1988) and led to the development of AChEIs. The 1990s saw the emergence of transgenic models – mainly mouse models with mutations on the APP, PSEN1 and/or tau genes – which are still used (Braidy et al., 2012). These models have proved useful in understanding some of the molecular mechanisms, but genetic AD counts for less than 5% of the cases and to date these models have not yielded any successful treatments for the clinic (Lecanu and Papadopoulos, 2013, Braidy et al., 2012). Meanwhile, development of rat models has shifted from the cholinergic hypothesis to the amyloid cascade hypothesis, which focuses on the burden of SPs (Karran et al., 2011) and then – in the 2000s – to the toxicity of low-n soluble A $\beta$ . These pharmacological based models have been shown to induce a memory deficit or a learning impairment following administration of A $\beta$  peptides. These models showed conclusive results both in behavioural studies and histopathologic analyses, but no complete model of AD has been found and it is likely that advances will come from considering and testing both transgenic and non-transgenic models (Delacourte and Buee, 2005). Taking into consideration the known mechanisms of AD and current drug discovery challenge, I aimed to establish and validate a rat model of relevance for AD research based on the following criteria:

- Validating a non-transgenic and more ethologic rat model (Lecanu and Papadopoulos, 2013).
- Using soluble low-n A $\beta$  as a trigger of synaptic deficit and neuroinflammation rather than plaque burden (Selkoe, 2002, Walsh and Selkoe, 2007).
- Targeting the early stages to improve the efficacy of treatments and have a better impact on morbidity and mortality (Prince et al., 2011, Selkoe, 2012).
- Assess the behavioural and neurobiological impact of A $\beta$  to link cognitive decline to potential biomarkers that can be translated to the clinic.



### 1.3.2 Rationale of the model

The main purpose of the model is to investigate the cognitive alterations and associated neurobiological changes caused by A $\beta$  pathology. **Thus the model cannot be qualified as a model of AD but more specifically as a model of cognitive and neuropathological changes induced by specific A $\beta$ <sub>1-42</sub>.** The aims of the model are: (1) to better understand the neuropathological pathways of A $\beta$  toxicity; (2) to investigate and link cognitive decline to brain biomarkers; (3) to provide a platform for drug screening of relevance for A $\beta$ -related pathologies and AD.

It has been decided to build the model in rats as a relevant species for preclinical cognitive tests. Moreover, the team I am working with has a strong expertise in investigating rat models of brain pathologies (Neill et al., 2010, McLean et al., 2010, McLean et al., 2012, Barnes et al., 2012) as well biochemical analyses (McKibben et al., 2010, Harte et al., 2005). This pharmacological model will be induced by an acute ICV injection of soluble low-n A $\beta$ <sub>1-42</sub> synthesized by the company SynAging (<http://synaging.com/>). This company already has characterised the effects of these A $\beta$  *in vitro* and *in vivo* in a mouse model (Youssef et al., 2008, Desbene et al., 2012, Pillot et al., 1999a, Garcia et al., 2010, Drouet et al., 2001).

### 1.3.3 Outline of the project

It has thus been highlighted that there is an urgent need to develop disease modifying medicines. Over the past decade, the role of A $\beta$  induced toxicity and neuroinflammation have been demonstrated as early and important features of the disease (Youssef et al., 2008, Cai et al., 2011, Butterfield et al., 2013, Quintanilla et al., 2012, Shankar et al., 2007). Succeeding in understanding, blocking and reversing A $\beta$  toxicity could lead to the development of preventive and curative treatments. The rationale that led to establishing a pharmacological

rat model is presented in Figure 5. It is important to stress that the model presented in this thesis aims to reproduce A $\beta$  toxicity and not the entire spectrum of AD mechanisms and symptoms.

In order to decide on the most relevant way of establishing and testing the model, a systematic review of current non-transgenic pharmacological rat models of A $\beta$ -peptide/oligomer administration will be presented (Chapter 2).

In Chapter 3, the methods selected to establish the model and assess cognitive functions and biomarkers will be outlined. Chapter 4 investigates the consequence of A $\beta$  administration on memory function; time-course, dose-response and influence of gender have also been examined. Chapter 5 will present different biomarkers and additional cognitive tests investigated. Chapter 6 investigates potential reversal of the cognitive deficits and neuropathology by the anti-inflammatory drug mefenamic acid.

This model aims to increase the understanding of the early stages of AD and of any other A $\beta$  toxicity related pathology. A valid model would be of use in preclinical research for screening and testing preventive or curative drugs.

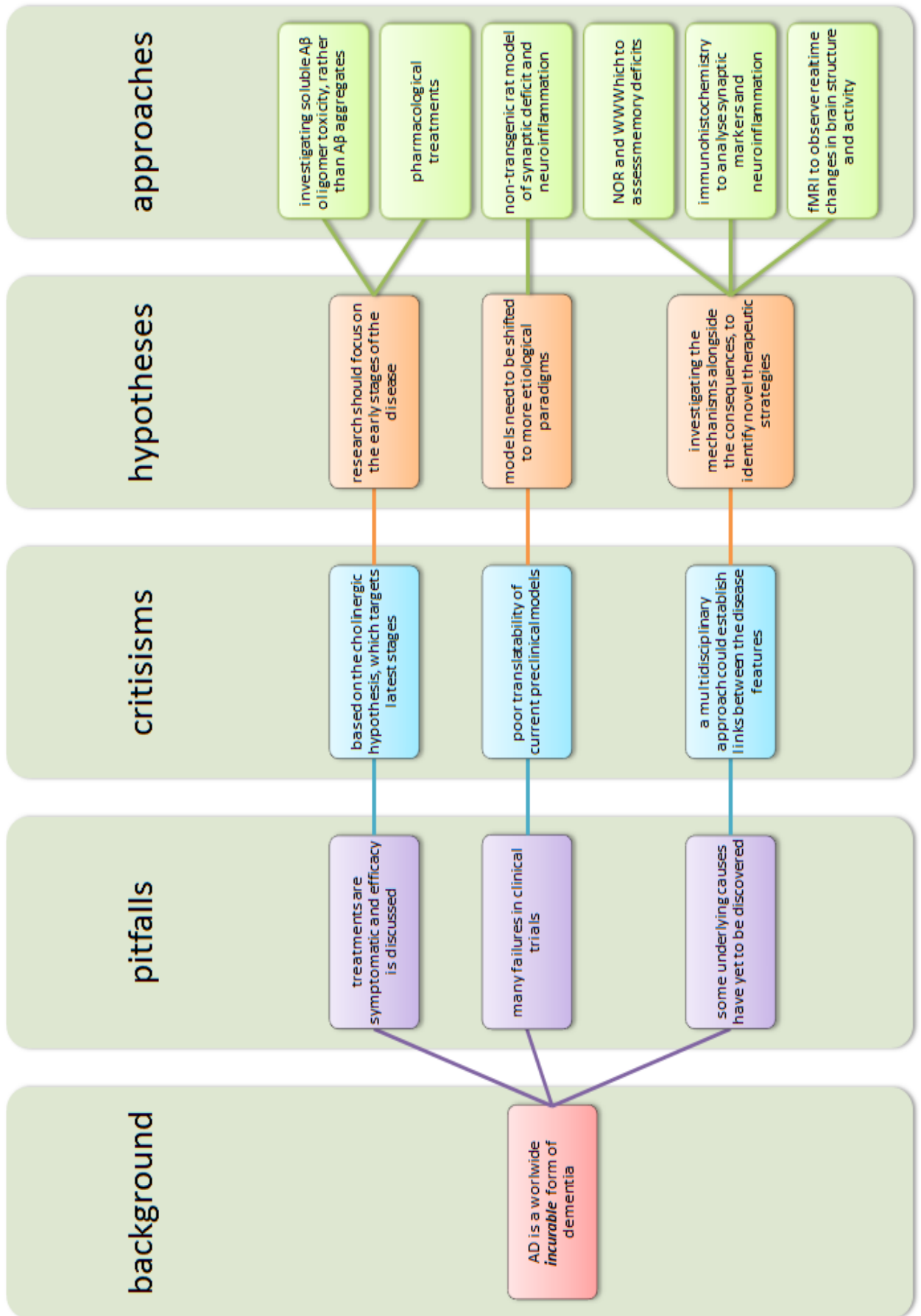


Figure 5: Project proceeding

## Chapter 2 SYSTEMATIC REVIEW OF RAT MODELS BASED ON THE ADMINISTRATION OF AMYLOID-B PEPTIDES

## 2.1 INTRODUCTION

AD is the main cause of dementia, with increasing incidence rates above the age 65, and remains nowadays an incurable worldwide disease (Mayeux and Stern, 2012). In the clinic, it is characterised by a progressive loss of memory and personality, as a result of neuronal dysfunction and neuronal loss (Braak and Braak, 1991, Jack et al., 2010, Jack et al., 2013). Although plaques of aggregated A $\beta$  proteins are recognised as a hallmark of AD, no correlation could be established between plaque burden and the severity of neuronal loss and cognitive symptoms (Gomez-Isla et al., 1997, Price et al., 2009, DaRocha-Souto et al., 2011). However non-aggregated forms of A $\beta$ , and particularly low number soluble A $\beta$  (monomers to tetramers, Figure 6), have shown increased toxicity compared to plaques (McLean et al., 1999, Kreplak and Aebi, 2006) and better correlation with the severity of AD (Mc Donald et al., 2010). Soluble A $\beta$  oligomers are now believed to play a central and early – if not causative – role in AD (Hardy and Selkoe, 2002). These oligomers have shown toxic effects in particular on synaptic plasticity, dendritic spine density and LTP (Walsh et al., 2002, Townsend et al., 2006, Shankar et al., 2008, Selkoe, 2008), see (Walsh and Selkoe, 2007, Karran et al., 2011, Benilova et al., 2012) for review.

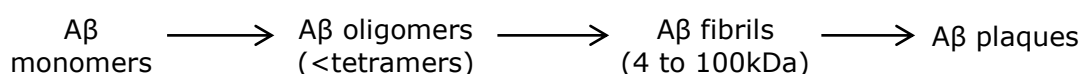


Figure 6: Aggregation of A $\beta$  forms from monomers to plaques

This review aims to centralise work on rat models that investigated the toxicity of A $\beta$ -peptide/oligomer administration. There are a number of similar models in that field with subtle changes in the protocol (e.g. species of oligomers used, dose administered, markers investigated) and this work gives an overview of the behavioural tasks tested, markers measured and their main outcomes. Pharmacological models based on A $\beta$ -peptide administration are

numerous and, although their results are usually converging, none of them can present a comprehensive set of tests. Any research team should adapt their model to their resources, planned outcomes and objectives. Pharmacological models based on A $\beta$ -peptide administration have been proposed as a relevant and complementary alternative to transgenic rodent models. Firstly this approach allows the researcher to administrate a known quantity of a clearly identified species of A $\beta$  peptides. Secondly, administrating A $\beta$  peptides to healthy animals isolates the effects due to the A $\beta$  peptides from other comorbidities that can be found in patients. Although these two points are an obvious limit to the construct validity of a model of AD, they present a clear advantage when it comes to studying the mechanisms and effects of the early A $\beta$  driven pathology, helping us better understand its implication and potential drug targets. Altogether, transgenic models have helped researchers make significant discoveries regarding the disease mechanisms – see (Benedikz et al., 2009) – but disease modifying treatments that have demonstrated clinical benefit in patients are still to be approved. Despite this significant improvement made in understanding AD pathology, it has been suggested that a complimentary approach to include pharmacological models based on A $\beta$ -peptide administration could help drug discovery by complementing discoveries from genetic models and providing insights into early A $\beta$  driven pathology (Lecanu and Papadopoulos, 2013).

The current review focuses on pharmacological models based on A $\beta$ -peptide administration in rats, which can be traced to the early 1990s. Other animal strains, genetic models, lesion models and pharmacological models where A $\beta$  peptides are not administrated into the brain of the rats, have been excluded. Preliminary *in vitro* data have been included where they were clearly supported by *in vivo* data in the same model. Models were classified following the type of A $\beta$  peptide used: A $\beta$ <sub>1-40</sub> peptides, A $\beta$ <sub>1-42</sub> peptides, A $\beta$ <sub>35-45</sub> peptides

and A $\beta$  peptides extracted from human brains. Each section is further subdivided by the type of administration: acute or chronic. In Appendix V (p188), articles are referenced by strain of rats used, an important but often neglected factor. Although a third classification by site of administration seemed relevant, the multiplicity of sites made it too complex to be presented here. While most sites of administration are ICV and intra-hippocampal, some other sites include: the septum, nucleus basalis and entorhinal cortex. Within these sub-types of administration sites, different locations are used and usually reported in relation to Bregma coordinates.

This review looks at the different pharmacological models based on A $\beta$ -peptide administration in rats and their resulting behavioural and neurochemical investigations (Figure 7). These models have proved of relevance for AD research, both from a drug discovery perspective and to better understand the underlying mechanisms of this A $\beta$  driven pathology. For other relevant rodent models, refer to the reviews from (Yamada and Nabeshima, 2000, Stephan and Phillips, 2005, Van Dam and De Deyn, 2011, Braidy et al., 2012, Lecanu and Papadopoulos, 2013) and Part IV (Chapter 12 to 24) in the book "Animal models of dementia (2011), De Deyn, P. P. & Van Dam, D. (editors), Neuromethods Vol. 48, Humana Press" .

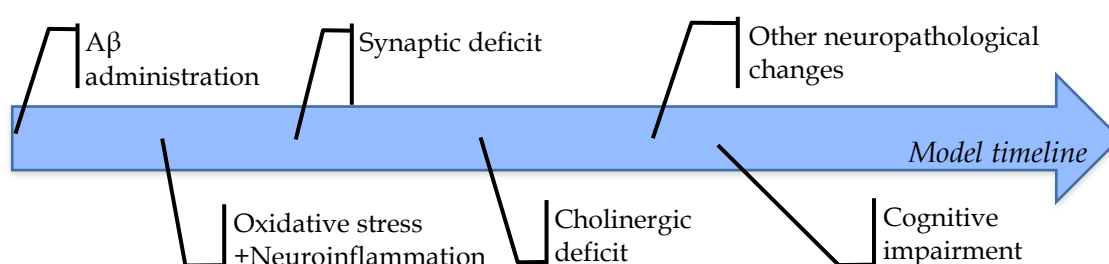


Figure 7: Proposed timeline of the deficits observed in A $\beta$ -peptide administration models. This figure represents a simplified view of the appearance of the neuropathological features and it is important to note that the different alterations can be concomitant and interlinked.

Most models described in this review used scrambled or a reverse sequence of the A $\beta$  peptides as a control group; in order to obtain damages due to the structure and properties of the peptide itself rather than simply due to its primary structure (sequence of peptides). The use of reversed or scrambled sequence as a control peptide is now widely accepted. Early *in vitro* and *in vivo* studies showed that only A $\beta_{1-40}$  peptides could aggregate in fibrils comparable to the one found in AD while A $\beta_{40-1}$  peptides aggregated into an amorphous and less toxic structure (Giordano et al., 1994). Reverse or scrambled sequence of A $\beta$  peptides have since sometimes be used as a non-toxic control instead of vehicle in some experiments (Nitta et al., 1994, Giovannelli et al., 1995, Yamada et al., 1998, Yamada et al., 1999a, Yamada et al., 1999b, Tang et al., 2000, Nakamura et al., 2001, Rosales-Corral et al., 2004, Ryu et al., 2004, Zand et al., 2005, McLarnon et al., 2006, Ryu and McLarnon, 2006, Perez et al., 2010, Carrero et al., 2012, Zussy et al., 2013, Ryu et al., 2015, Nell et al., 2015).

## 2.2 PHARMACOLOGICAL MODELS BASED ON A $\beta_{1-40}$ ADMINISTRATION

### 2.2.1 Neuroinflammation and oxidative stress

Neuroinflammation is a feature present in most current A $\beta_{1-40}$  peptides administration models. Increased glial fibrillary acidic protein (GFAP) immunoreactivity – a marker of astroglia – in the CA1 hippocampal area, has been found following 14-day ICV administration of A $\beta_{1-40}$  peptides at 3, 30 or 300 pmol/day (Nitta et al., 1997). Kinetic studies of neuroinflammatory markers following acute intra-hippocampal administration of fibrillar A $\beta_{1-40}$  peptides at 1 mM (Rosales-Corral et al., 2004) found a rapid increase in the levels of the pro-inflammatory cytokine IL-1 $\beta$  in the first 12 hours, followed by transient increase in IL-6 and TNF- $\alpha$  levels, a finding that was confirmed by (Xuan et al., 2012). Thus it appears that there is an early inflammatory phenomenon in response to



the administration of  $A\beta_{1-40}$  peptides. Involvement of IL-1 $\beta$  has been further supported by (Schmid et al., 2009) who demonstrated that treatment with IL-1 $\beta$  antagonist could partially reverse the impairment of LTP as assessed by excitatory postsynaptic potential *in vivo* in the hippocampus (Schmid et al., 2009). Neuroinflammation in the hippocampus has also been reported by (Malin et al., 2001) who showed gliosis, following 14-day intra-hippocampal administration of  $A\beta_{1-40}$  peptides (3 nmol in each of 7 sites) (Malin et al., 2001). Studies investigating mitochondrial activity in relation to markers of oxidative stress following 14 days of ICV administration of  $A\beta_{1-40}$  peptides found increased hydrogen peroxide  $H_2O_2$  generation and decreased levels of enzymes responsible for its degradation (Kaminsky and Kosenko, 2008).

### 2.2.2 Synaptic deficit

(Ahmed et al., 2010) reported a decrease in the synaptic markers, PSD-95 and synaptophysin following acute intra-hippocampal administration of  $A\beta_{1-40}$  peptides. Although in this study,  $A\beta_{1-40}$  peptides were co-administrated with ibotenic acid, a chemical known to have neurotoxic effects (discussed in more detail in Section 2.5.2).

### 2.2.3 Cholinergic markers

Acetylcholine is the active neurotransmitter of cholinergic neurons. It is synthesised by choline acetyltransferase ChAT and metabolised to its inactive form choline by AChE. In light of the approval of AChE inhibitors for the treatment for AD a number of the earlier studies focused on measuring markers of the cholinergic transmitter system following  $A\beta$  administration. Following a 14-day ICV administration of  $A\beta_{1-40}$  peptides at 0.003, 0.03 or 0.3 nmol/day, decreased ChAT activity was found in the frontal cortex, highlighting a deficit in cholinergic neurotransmission and cholinergic fibres loss (Nabeshima and Nitta,

1994, Nitta et al., 1994, Nitta et al., 1997). This deficit was also found in the hippocampus for the highest dose 0.3 nmol/day (Nabeshima and Nitta, 1994, Nitta et al., 1994, Nitta et al., 1997, Yamada et al., 1998, Nag et al., 1999a, Nag et al., 1999b). However, modifications of ChAT activity have not always been reproducible (Tanaka et al., 1998). This could be due to the age of the rats which has been reported to play a role in the sensitivity of cholinergic neurons following A $\beta$ -peptide administration (Gonzalo-Ruiz et al., 2005, Gonzalo-Ruiz et al., 2006) although contradictory findings exist (Nag and Tang, 2001). A decrease in acetylcholine release in the hippocampus has been confirmed by microdialysis following acute administration of A $\beta_{1-40}$  peptides, 21 days after administration of 3 nmol in the septum (Abe et al., 1994) and up to 30 days after administration of 10  $\mu$ g in the nucleus basalis (Giovannelli et al., 1995). Involvement of the cholinergic system is further supported by the co-administration of A $\beta_{1-40}$  fibrils with AChE which accelerated the aggregation and depositions of A $\beta_{1-40}$  fibrils, increased astroglial activation (and hence neuroinflammation) and induced local neuronal loss (Reyes et al., 2004).

Modulation of acetylcholine levels will in turn affect activity of nAChR, which has been link to modulation of LTP formation and amplitude in the hippocampal CA1 region. However, depending on the subtype of nAChR studied, this modulation can result in potentiation or depression of LTP (Itoh et al., 1996, Nabeshima and Itoh, 1998, Itoh et al., 1999, Chen et al., 2006, Wu et al., 2008). Immunohistochemistry studies found that nAChR-containing cell number was reduced in the CA1 and dentate gyrus of the hippocampus following acute administration of 2  $\mu$ g of A $\beta_{1-40}$  fibrils in the retrosplenial cortex (Arevalo-Serrano et al., 2008). Direct action of acetylcholine on nAChR activity is further supported by *in vivo* and *in vitro* studies on nicotinic modulated release of the neurotransmitters aspartate, glutamate and  $\gamma$ -aminobutyric acid (GABA). It has been found that various concentrations of A $\beta_{1-40}$  peptides would result on

stimulation of different subtypes of nAChR and could promote or inhibit the release of the 3 neurotransmitters (Mura et al., 2012, Salamone et al., 2014). The decrease in acetylcholine release in both the frontal cortex and hippocampus has been found as well to be concomitant with a decrease in dopamine levels in the striatum (Itoh et al., 1996). Although clear mechanisms are not yet fully understood, and the link between acetylcholine and dopamine could not be elucidated, it appears that the acetylcholine deficit will have an effect on LTP and neurotransmitter release, particularly in the hippocampus and the frontal cortex, two areas heavily involved in memory.

### 2.2.4 Others neuropathological markers

*In vivo* the amplitude and duration of glutamatergic NMDAR-mediated LTP was reported to be reduced following ICV administration of 0.4 and 3.4 nmol of  $A\beta_{1-40}$  peptides (Cullen et al., 1997), an effect that was prevented by treatment with an NMDAR antagonist (Cullen et al., 1996). Further investigation of LTP showed that administration of  $A\beta_{1-40}$  peptides (200 nmol/L) on hippocampal slides modified NMDAR-mediated field potentials but was not directly linked to reduced LTP induction, implying the involvement of other second messenger systems (Raymond et al., 2003). Among them, protein kinase C (PKC) has been suggested because of its role in the induction of LTP and its down-regulation following 14-day ICV administration with  $A\beta_{1-40}$  (Olariu et al., 2002). Involvement of the glutamatergic system is further supported by evidence that co-administration of  $A\beta_{1-40}$  with NMDAR agonist ibotenic acid (Morimoto et al., 1998, Li et al., 2004, Nakamura et al., 2006, Ogino et al., 2014) or kainate receptor agonists (Morimoto and Oda, 2003) increases neuronal loss in the hippocampus and potentiate learning deficit (MWM and Y-maze). These effects could be prevented by treatment with corresponding antagonists. Neuronal loss has been reported in these early rat models, especially at the site of

administration. A reduction in cresyl-violet cell number was found in cortical sections 7 days following an acute administration of 3 nmol of  $A\beta_{1-40}$  peptides (Kowall et al., 1991) and 14 days following intra-hippocampal administration (21 nmol) (Malin et al., 2001). Moreover, 14-day ICV administration of  $A\beta_{1-40}$  peptides (0.3 nmol/day) has been reported to increase ciliary neurotrophic factor levels in the frontal cortex, hippocampus and cerebellum, suggestive of a potential defence mechanism. However levels were reduced in the brain stem (Yamada et al., 1995).

Another sign of hippocampal damage was shown following 14-day ICV administration of  $A\beta_{1-40}$  peptides (0.3 nmol/day) by decreased activity of glucagon-like peptide , which is linked to eating behaviour and hippocampal activity; although there is not yet a direct link established with cognition (Oka et al., 1999). Following a 14-day ICV administration of  $A\beta_{1-40}$  peptides (300pmol/day), biochemical analyses demonstrated a decrease in somatostatin levels, an hormone inhibiting the growth-hormone release (Nag and Tang, 2001).

### 2.2.5 Cognitive tasks

Hippocampal dysfunction has been found to be consistently associated with deficits in the MWM (measure of learning and spatial memory). Dose dependent deficits in the MWM were reported following 14-day ICV administration of  $A\beta_{1-40}$  peptides at 0.003, 0.03 or 0.3 nmol/day (Nabeshima and Nitta, 1994, Nitta et al., 1994, Nitta et al., 1997). However, further investigation of hippocampal function using the Y-maze task showed inconsistent deficits. The deficit in the MWM was shown to be reproducible at 0.3 nmol/day, and independent of the age of the animals tested (Nag and Tang, 2001).

In a series of experiments, spatial memory consolidation was assessed following  $A\beta_{1-40}$  peptide administration. Using the Y-maze, rats were trained to either explore a baited arm or avoid an aversive arm (electric shock). Following training rats then received an intra-hippocampal administration of  $A\beta_{1-40}$  peptides and were tested a week later in the same learning task. Despite a similar performance in the training trials pre-surgery, rats receiving intra-hippocampal administration of  $A\beta_{1-40}$  peptides showed poorer performances than controls in the task (McDonald et al., 1994). Testing in the standard 8-arm radial maze (8-ARM) maze revealed no deficit following acute or chronic  $A\beta_{1-40}$  peptides administration over 26 days (McDonald et al., 1994). However, performance of the  $A\beta_{1-40}$  peptides group were significantly lower than control if a distraction phenomenon was added (rat is taken out of the maze for 30s between the 4<sup>th</sup> and 5<sup>th</sup> arm entry), highlighting a potential role of attention/distraction (Sweeney et al., 1997). These tests showed an impairment of memory consolidation but not a full impairment of spatial memory following administration of  $A\beta_{1-40}$  peptides, which is in agreement with the inconsistent deficit found in the Y-maze. This implies a partial deficit in spatial cognition which has been showed to rely on hippocampal function (Hartley et al., 2014) and partially on prefrontal function (Lalonde, 2002).

Similarly, the role of attention, in relation to distraction and decreased performance following  $A\beta_{1-40}$  peptide administration, could be a consequence of a deficit in cholinergic prefrontal function (Dere et al., 2007). Attention is also known to play a role in visual recognition memory impairment in AD patients (Alescio-Lautier et al., 2007). Attention and visual recognition can be assessed in rodents with the novel object recognition (NOR) task which has been found to be impaired following a 14-day ICV administration of  $A\beta_{1-40}$  peptides at 0.3 nmol/day (Nag et al., 2001), and 2 months after an acute administration of  $A\beta_{1-40}$  peptides (10  $\mu$ g) in the nucleus basalis (Giovannelli et al., 1995). This task is

sensitive to deficits of attention but can as well highlight damages in the perirhinal cortex (Barker et al., 2007).

Transient deficits in the passive avoidance test have also been reported, following a 14-day ICV administration of A $\beta$  peptides at 0.3 nmol/day (Nabeshima and Nitta, 1994, Nitta et al., 1994, Nitta et al., 1997, Oka et al., 1999).

### ***2.2.6 Pharmacological treatment***

Data indicating a role of neuroinflammation and oxidative stress in rat models orientated drug screening toward antioxidant and anti-inflammatory drugs. The antioxidant drugs melatonin, vitamin C, and vitamin E lowered levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Rosales-Corral et al., 2003). Similarly, cognitive deficits in several A $\beta$ <sub>1-40</sub> peptides administrated models could be partially prevented by treatment with the flavone luteolin (Yu et al., 2015), the cyclooxygenase (COX)-2 inhibitor harpagoside (Li et al., 2015), and the anti-oxidant salidroside (Zhang et al., 2013a)

Huperzine A, an AChE inhibitor, has been used to treat the cholinergic deficit. Following ICV administration of A $\beta$ <sub>1-40</sub> peptides (2.4 nmol over 11 days), it has been found that huperzine A (0.2 mg/kg, intra-peritoneal (IP), daily for 12 days) could prevent cognitive deficits in the MWM and the reduction in ChAT activity (Wang et al., 2001). In another model where 10  $\mu$ g of A $\beta$ <sub>1-40</sub> peptides were administrated into the nucleus basalis, huperzine A (0.18 mg/kg, per os (PO), daily for 21 days) prevented the decrease in levels of acetylcholine in the medial prefrontal cortex (Liang et al., 2008). In the later study, the authors also highlighted a potential link with the noradrenergic and dopaminergic systems. Reduced levels of monoamines (which could be a direct consequence of the A $\beta$ <sub>1-40</sub> peptides or a consequence of the cholinergic deficit) were also rescued

following treatment with huperzine A. Huperzine A has went on to be assessed in clinical trials and demonstrated some improvements in cognition with few adverse effects, although the methodology used in the trials have led to warnings about the potential misinterpretation of its beneficial effects (Li et al., 2008, Wang et al., 2009, Yang et al., 2013). However it is still used in a number of rat models, as a positive control (Zhang et al., 2013a, Liang et al., 2015).

Drug studies found that the deficit in the MWM could be prevented by chronic treatment with NC-1900 (SC 1 ng/kg), an analogue of arginine-vasopressin that acts to increase ChAT activity (Tanaka et al., 1998). In the same model, chronic oral dosing with propentofylline (10 and 25 mg/kg), which is thought to stimulate nerve growth factor synthesis, reversed the Y-maze impairment (Yamada et al., 1998). Both studies were conducted following 14-day ICV administration of A $\beta$  peptides at 0.3 nmol/day. A number of studies have demonstrated a reversal of A $\beta_{1-40}$  peptides induced cognitive deficits following treatment with different compounds. These include the novel compound stemazole (Han et al., 2011), the neuropeptide galanin (Li et al., 2013), the oestrogen receptor agonist genistein (Bagheri et al., 2011) and the traditional Chinese medicine extract Tong Luo Jiu Nao (Shi et al., 2015).

## 2.3 PHARMACOLOGICAL MODELS BASED ON A $\beta_{1-42}$ ADMINISTRATION

### 2.3.1 *Neuroinflammation and oxidative stress*

In line with findings following A $\beta_{1-40}$  peptides administration, neuroinflammation and oxidative stress are two features commonly reported in A $\beta_{1-42}$  peptides administration models. Increased production of the pro-inflammatory cytokine IL-1 $\beta$  has been shown to appear as early as 24 hours after acute administration of 5  $\mu$ g of A $\beta_{1-42}$  peptides in the nucleus basalis. This inflammatory phenomenon was associated with increased COX and iNOS immunoreactivity, as well as

increased microglia and astroglia activation 7 days after administration (Giovannini et al., 2002). In a similar model (4  $\mu\text{g}$  of  $\text{A}\beta_{1-42}$  peptides administrated in the nucleus basalis) protein oxidation levels were found to be increased at the site of administration and spread across the cortex and the hippocampus (Boyd-Kimball et al., 2005). Increased COX immunoreactivity, microglial and astroglia activation were again confirmed in a series of experiments following acute administration of 1 nmol of  $\text{A}\beta_{1-42}$  peptides in the hippocampus (Ryu et al., 2004, McLarnon et al., 2006, Ryu et al., 2015). These experiments also revealed an increased permeability of the BBB (Ryu and McLarnon, 2006) and angiogenesis which is sometimes found in chronic inflammatory diseases (Zand et al., 2005). Glial activation and BBB leakage could be reduced by treatment with the antibiotic and anti-inflammatory drug minocycline and an iNOS inhibitor (Ryu et al., 2004, Ryu and McLarnon, 2006). The results above have been reproduced by other research teams where glial activation (Perez et al., 2010, Li et al., 2010a), increased COX immunoreactivity and increased levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Li et al., 2010a, Carrero et al., 2012, Zhang et al., 2013b) have all been reported. Administration of  $\text{A}\beta_{1-42}$  peptides in these models were varied: 2  $\mu\text{g}$  in the retrosplinal cortex (Perez et al., 2010, Carrero et al., 2012), 10  $\mu\text{g}$  ICV, (Zhang et al., 2013b) and 2.2 nmol ICV (Li et al., 2010a).

Changes in markers of oxidative stress have been reported following acute ICV administration of  $\text{A}\beta_{1-42}$  peptides (10  $\mu\text{g}$ ), with elevated levels of glutathione, lipid peroxidation, caspase-3 (involved in apoptosis) in aged rats (Cetin and Dincer, 2007, Cetin et al., 2013). Intra-hippocampal administration of 5  $\mu\text{g}$  of  $\text{A}\beta_{1-42}$  peptides increased lipid peroxidation and reactive oxygen species production with reduced antioxidant enzyme activity (Turunc Bayrakdar et al., 2014). However chronic ICV administration of  $\text{A}\beta_{1-42}$  peptides 0.3



nmol/day over 14 days did not modify lipid peroxide levels in the hippocampus, assessed 19 days after A $\beta$ <sub>1-42</sub> peptides administration (Yamada et al., 1999a).

Increasing evidence highlights the involvement of the nuclear factor (NF)- $\kappa$ B, a protein complex involved in inflammation and cell apoptosis. Activation of the NF- $\kappa$ B pathway in A $\beta$ <sub>1-42</sub> peptide rat models has also been supported by several articles (Carrero et al., 2012, Ashabi et al., 2013, Turunc Bayrakdar et al., 2014).

### 2.3.2 *Synaptic deficit*

Synaptic activity has been shown to be impaired with reduced levels of synaptophysin 15 days following ICV (2 nmol of A $\beta$ <sub>1-42</sub> peptides (Frezza et al., 2013)) or intra-hippocampal administration (Frautschy et al., 2001). In the latter case, levels of PSD-95 were also decreased. ICV administration (10  $\mu$ g (Zhang et al., 2013b), 20  $\mu$ g (Tong et al., 2015), 2.2 nmol (Li et al., 2010a), 5 nmol (Wang et al., 2015)) was also found to inhibit LTP. In the CA1 - CA3 hippocampal regions, it has been found that the inhibition of LTP and potentiation of LTD involved several glutamatergic pathways including a role for ionotropic NMDA receptors (Hu et al., 2014, Klyubin et al., 2014b, Xi et al., 2015) and metabotropic glutamate receptor 5 (mGluR5) (Hu et al., 2014, Klyubin et al., 2014b).

### 2.3.3 *Cholinergic markers*

The cholinergic system appears to be affected in A $\beta$ <sub>1-42</sub> peptide models, an effect usually observed a couple of weeks following administration of the peptides. Acute administration (0.6 nmol in the nucleus basalis) caused a deficit in ChAT and a small but significant deficit in AChE activity in the frontal cortex, 14 days after surgery. This deficit was associated with the degeneration of cholinergic neurons projecting to the frontal cortex (Harkany et al., 1995b, Harkany et al.,

1995a), and reproduced in a similar model (Giovannini et al., 2002). These deficits could be partially prevented by treatment with a tetrapeptide, thought to be an antagonist of  $A\beta_{1-42}$  peptides (Harkany et al., 1999a), and the NMDAR antagonist MK-801 (2.5 mg/kg) (Harkany et al., 1999b), suggesting a role of the glutamatergic system (Harkany et al., 2000). ICV administration of  $A\beta_{1-42}$  peptides (20  $\mu$ g over 3 days) was found to cause a time-dependant decrease in ChAT activity with no difference from control 25 days after surgery but a significant decrease in the hippocampus and the striatum after 84 days (Nakamura et al., 2001).

### 2.3.4 Other neuropathological markers

Chronic ICV administration of  $A\beta_{1-42}$  peptides (0.3 nmol/day over 14 days) caused an increase in brain-derived neurotrophic factor (BDNF) expression in the hippocampus on days 3 and 7 after the start of  $A\beta_{1-42}$  administration (Tang et al., 2000).

Neuronal damages, as assessed by cresyl-violet staining, was found in the CA1 area of the hippocampus, 85 days after ICV administration of  $A\beta_{1-42}$  peptides (20  $\mu$ g over 3 days) (Nakamura et al., 2001). Damages in the CA1 were further supported by potentiated LTD in this region following acute ICV administration of  $A\beta_{1-42}$  (0.5 nmol) (Kim et al., 2001).

### 2.3.5 Cognitive tasks

Similar to  $A\beta_{1-40}$  peptides, ICV administration of  $A\beta_{1-42}$  peptides (0.3 nmol/day over 14 days (Yamada et al., 1999a), 10, 20  $\mu$ g over 3 days (Nakamura et al., 2001) or 20  $\mu$ g acute (Tong et al., 2015)) caused memory impairment in both the both MWM and Y-maze. Impairment in the MWM was confirmed following ICV administration (10  $\mu$ g (Zhang et al., 2013b) 2.2 nmol (Li et al., 2010a)). In female Wistar rats, the memory impairment appears to be potentiated by

following deprivation of oestrogens, although the role of oestrogen in women suffering from AD remains unclear (Yamada et al., 1999c).

Fibrillar forms of  $A\beta_{1-42}$  peptides have been shown to have an effect on cognition with impaired performances in the NOR task (Sipos et al., 2007, Kreutz et al., 2013). However, the Y-maze was not impaired and the MWM was only partially impaired (Kreutz et al., 2013), suggesting a higher toxicity of soluble forms.

Interestingly, it has been shown that acute administration of 0.6 nmol of  $A\beta_{1-42}$  peptides in the nucleus basalis caused anxiety in the elevated plus-maze, linked to an altered serotonergic system (Harkany et al., 1999b, Harkany et al., 2001). Increased anxiety has been later supported by tests in the open field and burrowing behaviour tasks (Salgado-Puga et al., 2015). Although not widely studied in AD, involvement of the serotonergic system is further supported by chronic treatment with Neu-P11, a melatonin and serotonin agonist, which prevented cognitive deficits in the NOR task and prevented neuronal loss in the CA1 region; following an acute administration of 6  $\mu\text{g}$   $A\beta_{1-42}$  peptides into the hippocampus (He et al., 2013).

### **2.3.6 Pharmacological treatment**

Levels of inflammatory markers (IL-1 $\beta$ , IL-6, TNF- $\alpha$  and glial activation) and MWM impairment have been partially rescued following daily treatment with hydrogen-rich saline, a free-radical scavenger (Li et al., 2010a, Wang et al., 2011) or a 3-week oral administration with atorvastatin (Zhang et al., 2013b). Atorvastatin is a statin used in humans for the treatment of hyperlipidaemia, which can have anti-inflammatory properties at high doses. It has also been shown to reduce microglial activation in another rat model (Clarke et al., 2007). Further tests on potential positive effects of anti-inflammatory drugs have found

that IL-1 $\beta$  production, COX and iNOS expression could be prevented by administration of the COX inhibitor rofecoxib (Giovannini et al., 2002). Ibuprofen has also been shown to transiently reverse the cognitive deficits and prevent astroglia activation (Richardson et al., 2002). When assessing cognition,  $\alpha$ -tocopherol (150 mg/kg) and idebenone (20 mg/kg, PO, over 23 days) reversed the deficit in the MWM and the Y-maze (idebenone only), supporting again a role for neuroinflammation (Yamada et al., 1999a).

Nefiracetam (3 mg/kg, PO from day 7 to day 20) has been shown to reverse the deficit in the MWM induced by chronic ICV administration of A $\beta$ <sub>1-42</sub> peptides (0.3 nmol/day over 14 days), and increased ChAT activity in the hippocampus (Yamada et al., 1999b). Activation of the cannabinoid receptor CB1 has been found to rescue the cognitive deficit in the passive avoidance test and to restore LTP function in the CA1 hippocampal region (Haghani et al., 2012), supporting a protective role of CB1 in AD rat models (Altobelli et al., 2015).

Following acute ICV administration of A $\beta$ <sub>1-42</sub> (0.4 nmol), (Cioanca et al., 2014) showed that inhalation of volatile coriander oil (60 min/day for 21 days) reduced anxiety in the elevated-plus maze and depressive behaviour in the forced swimming test when compared to non-treated rats. This treatment also resulted in an improvement in cognitive function in the Y-maze (Cioanca et al., 2013), along with a reduction in markers of oxidative stress (assessed by superoxide dismutase, lactate dehydrogenase and glutathione peroxidase levels). Similar encouraging results on cognition and markers of oxidative stress were found following treatment with both Juniper oil (Cioanca et al., 2015) and extracts of Piper nigrum fruit (Hritcu et al., 2014, Hritcu et al., 2015). Similar to A $\beta$ <sub>1-40</sub>-peptide models, treatment with plant extracts have shown promising preliminary data on rescuing the cognitive deficits and alterations in markers of oxidative stress: tetrandrine (He et al., 2011), naoerkang (Li et al., 2011),

Bushenyisui (Cui et al., 2012), Cistanche tubulosa (Wu et al., 2014), the flavones baicalin (Ding et al., 2015), pratenseine (Liang et al., 2015, Wei et al., 2015), soybean (Xi et al., 2015) and berberine (also a potential cholinesterase inhibitor) (Haghani et al., 2015). Chronic but not acute treatment with curcumin showed a dose-dependent rescue of  $A\beta_{1-42}$  peptide-induced memory deficits in the MWM and Y-maze. Chronic treatment with curcumin was also found to increase BDNF levels (Zhang et al., 2015c, Zhang et al., 2015b), and rescue the deficits in both synaptophysin and PSD-95 levels (Frautschy et al., 2001).

## 2.4 PHARMACOLOGICAL MODELS BASED ON $A\beta_{25-35}$ ADMINISTRATION

### 2.4.1 *Neuroinflammation and oxidative stress*

Similar to results with  $A\beta_{1-40}$  peptides, increased mitochondrial generation of  $H_2O_2$  and decreased metabolising enzyme activity was found following chronic administration of  $A\beta_{25-35}$  peptides (ICV, 0.3 nmol/day for 14 days) (Kaminsky and Kosenko, 2008). Disruption of mitochondrial metabolism prior to administration  $A\beta_{25-35}$  peptides increased their toxicity (as assessed by measuring lesions volume by cresyl-violet staining) on hippocampal neurons (Arias et al., 2002).

Administration of  $A\beta_{25-35}$  peptides also caused an increase in astroglia activation, an effect that could be prevented by treatment with an NMDAR antagonist, further highlighting the involvement of the glutamatergic system (Harkany et al., 2000, Morimoto et al., 1998). Increased astroglia activation in the hippocampus, associated with neuronal shrinkage and loss, were also found following administration of  $A\beta_{25-35}$  peptides (5 nmol) into the amygdala (Sigurdsson et al., 1996, Sigurdsson et al., 1997a, Sigurdsson et al., 1997b, Sigurdsson et al., 2000). Microglia activation in the forebrain was found 21 days following ICV administration of  $A\beta_{25-35}$  peptides (21.2  $\mu\text{g}/\mu\text{L}$ ) in 6 and 9 but not

3-month old rats (Nell et al., 2015). Increased astroglia (as measured by GFAP reactivity) in the CA1 area of the hippocampus, increased levels of IL-1 $\beta$ , and activation of caspase-3 have also been reported 7 days after ICV injection of A $\beta$ <sub>25-35</sub> peptides (Jin et al., 2005, Xu et al., 2014). The increase in astroglial activation and levels of IL-1 $\beta$  were potentiated by ICV co-administration of TNF- $\alpha$  (Stepanichev et al., 2003). In the same model, the inflammatory phenomenon was associated with lipid peroxidation, increased NO production and cell loss in CA1 (Stepanichev et al., 2004, Stepanichev et al., 2006, Stepanichev et al., 2008). (Zussy et al., 2013) also reported increased caspase-3 activation and astroglia activation in the frontal cortex.

#### 2.4.2 Synaptic deficit

*In vivo* studies demonstrated an impairment of hippocampal LTP, 5 min and 1 hour following ICV administration of 10 or 100 nmol of A $\beta$ <sub>25-35</sub> peptides (Freir et al., 2001) and 2 weeks following bilateral intra-hippocampal administration of 5 nmol of A $\beta$ <sub>25-35</sub> peptides (Cai et al., 2014).

#### 2.4.3 Cholinergic markers

Microdialysis study revealed a decrease in acetylcholine release in the hippocampus one week after acute administration of A $\beta$ <sub>25-35</sub> peptides (5 nmol) in the septum (Abe et al., 1994). Levels of nicotine-induced acetylcholine release were also lowered following chronic administration of A $\beta$ <sub>25-35</sub> peptides (ICV, 0.3 nmol/day for 14 days) (Olariu et al., 2001). Cholinergic neuron loss was dependent on the age of the rats, with higher loss reported in 6 and 9-month old rats when compared to 3-month old rats (Nell et al., 2015). ChAT activity (measured by radioactivity) was reduced in the hippocampus, and ChAT immunoreactivity was reduced in the medial septum 8 days after ICV administration of A $\beta$ <sub>25-35</sub> peptides (15 nmol) (Yamaguchi and Kawashima, 2001).

Reduction of ChAT-positive cells was also found 3 weeks after ICV administration of A $\beta$ <sub>25-35</sub> peptides (10  $\mu$ g) (Zussy et al., 2013).

Interestingly a specific isoform of AChE (G1) was found to be increased in both the cortex and the CSF, while total AChE activity was unchanged, following a 7-day ICV administration of A $\beta$ <sub>25-35</sub> peptides (20  $\mu$ g per day) (Saez-Valero et al., 2002). These findings suggest that different isoforms of AChE could be involved in AD and that the physiological levels of AChE sometimes reported in rat models could mask altered modifications of specific isoforms.

#### **2.4.4 Other neuropathological markers**

Similar to the study by (Nag and Tang, 2001) following chronic A $\beta$ <sub>1-40</sub> peptide administration, decreased levels of the hormone somatostatin were also found following chronic administration of A $\beta$ <sub>25-35</sub> peptides (ICV, 0.3 nmol/day for 14 days) (Aguado-Llera et al., 2004). This study was performed in ovariectomised female rats but was also later confirmed in male rats, following both acute and chronic administration (Hervas-Aguilar et al., 2005), resembling the deficits reported in human AD brains (Krantic et al., 1992). These changes in somatostatin could be prevented by the administration of minocycline (Burgos-Ramos et al., 2008, Burgos-Ramos et al., 2009).

Interestingly, 6 weeks after ICV administration of A $\beta$ <sub>25-35</sub> peptides (10 $\mu$ g), levels of APP (measured by Western Blot) were markedly increased in the frontal cortex, amygdala and hypothalamus but decreased in the hippocampus. Levels of C99 (a marker of the amyloidogenic pathways) were increased in the frontal cortex, amygdala and hippocampus, suggesting a modification of APP processing toward pathological A $\beta$  (Zussy et al., 2013). Acute bilateral administration of A $\beta$ <sub>25-35</sub> peptides (5 nmol) into the amygdala was shown to increase tau levels (Sigurdsson et al., 1997a, Sigurdsson et al., 1997b) but not tau-mRNA levels

(Chambers et al., 2000). Changes in tau-phosphorylation varied depending on the area of the brain and tau-epitope investigated (Zussy et al., 2013).

Disruption in the permeability of the BBB was investigated following the administration of  $A\beta_{25-35}$  peptides into the carotid artery. Immunohistochemistry analysis revealed elevated amounts of T-lymphocyte cells in the central nervous system, suggesting a higher permeability of the BBB (Farkas et al., 2003).

Cresyl-violet staining showed a decrease in cell density in the CA1, CA2 and CA3 (but not DG) areas of the hippocampus, 3 weeks after acute ICV administration of  $A\beta_{25-35}$  peptides (10  $\mu$ g) (Zussy et al., 2013).

#### 2.4.5 Cognitive tasks

As with the previous models described, the two main cognitive tasks investigated and found to be impaired are the MWM and the Y-maze, following administration of  $A\beta_{25-35}$  peptides: ICV, 0.3 nmol/day for 14 days (Tang et al., 2000, Olariu et al., 2001), ICV 10  $\mu$ g (Zussy et al., 2013), both dorsal hippocampi (Chen et al., 1996). However, the deficit in the MWM has sometimes been shown to be age dependent, appearing in 6 but not 3-month old rats tested at the same time-point following surgery (Nell et al., 2015). Deficits in the NOR task was observed following 7 and up to 60 days following acute administration of  $A\beta_{25-35}$  peptides (10  $\mu$ g) in the nucleus basalis (Giovannelli et al., 1995).

Increased anxiety, assessed using the elevated-plus maze, was also found following chronic administration of  $A\beta_{25-35}$  peptides (ICV, 0.3 nmol/day for 14 days) (Olariu et al., 2001).



### ***2.4.6 Pharmacological treatment***

Drug screening on anti-oxidant molecules found that S-allylcysteine reduced formation of reactive oxygen species and lipid peroxidation, and restored performances in the 8-ARM (Perez-Severiano et al., 2004). Z-ligustilide has shown an anti-inflammatory effect (reduced TNF- $\alpha$  levels) and restored cognition in the MWM (Kuang et al., 2009). The impairment in LTP was improved following treatment with arginine vasopressin (Jing et al., 2009); it has been shown that arginine metabolism was altered following ICV administration of aggregated A $\beta_{25-35}$  peptides in the prefrontal cortex and hippocampus (Liu et al., 2011, Bergin et al., 2015). Deficits in LTP were also reversed following treatment with erythropoietine (Tazangi et al., 2015), lixisenatide (Cai et al., 2014), humanin (Guo et al., 2010) and its derivatives colivelin (Wu et al., 2015) and rattin (Wang et al., 2014). Deficits in the MWM were reversed by the essential oil, *Zataria multiflora* Boiss (Majlessi et al., 2012), and the plant extracts scutellarin (Guo et al., 2013), bajijiasu (Chen et al., 2014a), and Ropren<sup>®</sup> (polyprenols extracted from conifer) (Fedotova et al., 2012).

## **2.5 OTHER AB-PEPTIDE ADMINISTRATION MODELS**

### ***2.5.1 Extracted and secreted natural forms of A $\beta$ peptides***

In the early models, extracts from amyloid plaques from patients were directly administrated into the rat brain. Although these models present a better translatability to the human pathology, they lack reproducibility. The variability in the qualitative and quantitative composition of the extracts is an obstacle to the use of these models for drug discovery purposes and to understanding the mechanisms attributable to each form of A $\beta$  peptide. They offer however a better insight to the downstream pathways.

Administration in the cerebral cortex and hippocampus caused neuronal loss after 1 month, along with hyperphosphorylated tau, close to the sites of injection (Frautschy et al., 1991) and A $\beta$  deposition in brain vessels (Frautschy et al., 1992). *In vitro*, extracted oligomers impaired LTP and enhanced LTD, which resulted *in vivo* in deficit in cognition (passive avoidance test) in rats (Shankar et al., 2008). Inhibition of LTP by A $\beta$  peptides from AD brain extracts appear to be linked to the prion protein (PrP) receptor and can be prevented by administration of PrP antagonists (Klyubin et al., 2014a, Hu et al., 2014).

Another way to investigate the effect of human A $\beta$  peptides was to use Chinese ovary cells expressing human APP to secrete A $\beta_{1-40}$  and A $\beta_{1-42}$  oligomeric forms. These oligomers present the advantage of being highly potent at lower concentrations than synthetic A $\beta$  peptides (Walsh et al., 2002). Blockade of hippocampal LTP *in vivo* was reported following ICV administration of 1.5  $\mu$ L of cell media containing the secreted A $\beta$  oligomers. This effect was dependent on the presence of oligomers and was not seen following administration of monomers or fibrils (Walsh et al., 2002). Impairment of LTP by cell-secreted A $\beta$  oligomers was confirmed by (O'Hare et al., 2010, O'Hare et al., 2014), and was found to be associated with synaptic loss (Shankar et al., 2007). When assessing cognition, ICV administration of these oligomers induced a rapid but transient deficit in the learned alternating lever cyclic ratio task (Cleary et al., 2005, Poling et al., 2008).

### 2.5.2 Comorbidities models

(Frautschy et al., 2001) studied an intra-hippocampal co-administration model of both A $\beta_{1-40}$  peptides and A $\beta_{1-42}$  peptides (ratio 20  $\mu$ g / 5  $\mu$ g in a group and 20  $\mu$ g / 30  $\mu$ g in a different group). This model showed increased lipid peroxidation, activated microglia, decreased synaptic markers levels (synaptophysin and PSD-95) and impaired performances in the MWM. Unfortunately, this model has not

been compared to administration of any of the A $\beta$  peptides species alone. In this model, treatment with curcumin rescued both synaptophysin and PSD-95 levels and reduced A $\beta$  deposition. Treatment with the non-steroidal anti-inflammatory drug (NSAID) ibuprofen reduced microglial activation but had no effect on deficit in synaptophysin levels (Frautschy et al., 2001).

(Shin et al., 1997) found that A $\beta_{1-40}$  peptides but not A $\beta_{1-42}$  peptides were forming fibrils after administration in the hippocampus or cortex of the rat. In this study however, A $\beta_{1-42}$  peptides could form fibrils *in vitro* suggesting a different processing of both proteins in the rat brain. It can then be hypothesised that A $\beta_{1-42}$  toxicity could be linked to a longer soluble state when compared to A $\beta_{1-40}$  peptides.

As seen previously, A $\beta_{1-40}$  peptides effects can be potentiated by co-administration with the glutamate analogue ibotenic acid. In this model, curcuminoids have shown a dose-dependent beneficial effect on cognitive (MWM) and neuropathological (neuronal and synaptic markers) deficits (Ahmed et al., 2010). When co-administrated with A $\beta_{1-42}$  peptides, ibotenic acid was found to impair LTP in the CA1 region and performance in the MWM. Both deficits could be reversed by the NSAID aspirin and partially by the NSAID sodium salicylate (Mohammadpour et al., 2015). Co-administration of ibotenic acid with A $\beta_{25-35}$  peptides was also shown to potentiate neuronal loss in the hippocampus (Morimoto et al., 1998).

Interestingly, the role of diet has been investigated following findings implicating the involvement of cholesterol in AD (Sponne et al., 2004, Florent et al., 2006). Acute frontal and cingulate cortex administration of fibrillar A $\beta_{1-40}$  peptides (2  $\mu$ g) associated with high-cholesterol or high-fat diet caused an age-dependant loss of cholinergic neurons, particularly marked in the high-cholesterol (but not high-fat) group (Gonzalo-Ruiz et al., 2005, Gonzalo-Ruiz et

al., 2006). Administration of A $\beta$ <sub>1-42</sub> peptides to animals with a diet rich in cholesterol showed a potentiation of the deficit in the MWM, along with increased levels of astroglia and decreased nicotinic receptors when compared to A $\beta$ <sub>1-42</sub> peptides alone. High-cholesterol diet alone increased the levels of astroglia but did not impair cognition (as assessed using the MWM) (Liu et al., 2008). In link with the previous cholesterol model, (Lecanu et al., 2010) showed that specific forms of cholesterol have modulating, and sometimes protective, effect on the MWM deficit in their model.

Another interesting model where A $\beta$ <sub>1-42</sub> peptide was administered with Fe<sup>2+</sup> buthionine-sulfoximine ("FAB" model, with potentiated oxidative properties), reported impaired performance in the MWM. Tau hyperphosphorylation, neuronal loss in the hippocampus, amyloid deposits and an increase in inflammatory markers was also found. However, in this model, A $\beta$ <sub>1-42</sub> peptide administration (0.015 nmol/ $\mu$ L) alone did not trigger AD-like features.

Chronic ICV administration over 14 days of A $\beta$ <sub>1-42</sub> peptide coupled with a chronic psychosocial stress – induced by swapping rat littermates – showed impaired spatial memory, potentiated LTD, decreased LTP, and increased levels of APP when compared to A $\beta$ <sub>1-42</sub> peptides or stress alone (Srivareerat et al., 2009, Tran et al., 2011). It is interesting to remark however that stress alone has been showed to potentiate the expression of APP in the brain (Rosa et al., 2005, Sayer et al., 2008).

## 2.6 DISCUSSION

This article intended to give a global, yet comprehensive, overview of rat A $\beta$ -peptide administration models. Research teams seem to agree on utilising three main species of peptides: A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-42</sub> and A $\beta$ <sub>1-35</sub> peptides. A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> peptides are the most commonly produced species in AD (Haass et al., 1992, Seubert et al., 1993, Selkoe, 1998). Although it is unclear if the A $\beta$ <sub>25-35</sub>-peptide form is naturally found in human, this sequence has shown its damaging effects *in vitro* and *in vivo* as shown in this review, and is believed to be the toxic domain of A $\beta$  peptides (Yankner et al., 1990). This choice of A $\beta$ -peptide species tested raises the first difficulty regarding inter-lab variability of A $\beta$ -peptide models' results. Repeatability and comparability of results is not always achievable due to three main differences: (1) A $\beta$  peptides (type of amyloid used, preparation and characterisation, aggregation state), (2) protocol (site of injection, concentration and volume injected), (3) outcome measured (behavioural tasks, biomarkers tested and time points). While a variability of protocols allow scientists to test a wider range of possibilities and enhance scientific knowledge, the lack of a consensus model and level of details presented in research papers make comparisons and discussions across labs hardly achievable, as discussed by (Benilova et al., 2012).

It is however possible to draw some common outcomes.

Firstly, whatever the type of A $\beta$  peptide used, rats show signs of neuroinflammation: increased levels of IL-1 $\beta$  after a few hours and increased levels of IL-6 and TNF- $\alpha$  within the first few days following A $\beta$ -peptide administration. However, the increase in the levels of these 3 markers seems to be transient. Biochemical analysis shows signs of microglia and astroglia activation. This inflammation may be associated with oxidative stress: lipid peroxidation, protein oxidation, a decrease in mitochondrial activity and H<sub>2</sub>O<sub>2</sub>

metabolism. Increased caspase 3 activity and activation of the NF- $\kappa$ B pathway have also been shown and could be linked to cell apoptosis and neuronal death.

Alongside the direct effects on cell viability, synaptic activity is also affected in the early stages, with decreased synaptic markers such as synaptophysin and PSD-95. Decreased ChAT activity is a common feature and is associated with changes in the glutamatergic system, particularly ionotropic NMDAR and metabotropic mGluR5. All these events lead to an imbalance of LTP/LTD in the hippocampus and results in the appearance of memory deficits.

In the later stages, neuronal damage, especially in the hippocampus, plaque deposition and tau-hyperphosphorylation can be found, although these features are not present in every model presented.

Results from the behavioural tests show clear short-term, learning and working memory impairment. This is mainly assessed using the MWM, a task that relies on hippocampal function. The passive avoidance test and Y-maze are two commonly used tests but impairment in these tests are not always repeatable. Although widely used in other models, little is published regarding the NOR test in A $\beta$ -peptide administration models, but it seems that rats cannot perform this test when compared to controls. It is worth noting that a few teams have assessed emotional states of the animal and found increased anxiety in the elevated plus-maze, which could be associated with brain area-dependent changes in levels of serotonin and dopamine. In each case, all these outcomes appear quickly (within weeks).

Markers investigated	Hours	Days	Weeks
<b>Neuro inflammation and oxidative stress</b>	↑IL-1 $\beta$	↑IL-1 $\beta$ levels ↑IL6 levels ↑TNF $\alpha$ levels ↑ astroglia immunoreactivity ↑ caspases-3 levels	↑ protein oxidation ↑ lipid peroxidation ↑ astroglia immunoreactivity ↑ microglia immunoreactivity ↓ mitochondrial metabolism ↑ H <sub>2</sub> O <sub>2</sub> generation ↑ caspase-3 levels ↑ COX immunoreactivity ↑ iNOS immunoreactivity
	<b>Synaptic deficit and LTP/LTD</b>		↓ synaptophysin levels ↓ PSD-95 levels LTP inhibition LTD potentiation
<b>Cholinergic markers</b>		↓ ChAT levels ↓ induced acetylcholine release	↓ ChAT levels ↑ AChE levels ↓ induced acetylcholine release
<b>Other markers</b>		↑ neuronal loss	↑ neuronal loss ↑ BBB permeability
<b>Cognitive tasks</b>		NOR impairment	MWM impairment NOR impairment (± Y-maze impairment) ↑ anxiety

Table 1: Markers commonly investigated in A $\beta$ -peptide administration models

Whereas the A $\beta$ -peptide species used differ, it appears that the pathological pathways and consequences remain similar. Indeed, despite differences between A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-42</sub>, and A $\beta$ <sub>25-35</sub> peptides it appears that their toxicity and their ability to bind to receptors is more linked to their aggregation state rather than peptide length, (Jarosz-Griffiths et al., 2016). The main concern remains the concentration of A $\beta$  peptides used. Firstly, it is not yet possible to precisely quantify the concentration and rate of diffusion of the peptides in the different areas of the rat brain, making direct comparison between models almost impossible. Secondly, no clear measure of A $\beta$  peptides, and especially soluble forms, is currently achievable in AD patients. Hence no clear translatability of A $\beta$ -peptide concentrations can be achieved in rat administration models.

## 2.7 CONCLUSION

A $\beta$ -peptide administration models thus appear to provide a useful insight into AD pathogenesis. Despite their relevance for AD research and AD drug discovery, many caveats remain. When looking at the face validity of these models, it appears that they can model cognitive symptoms, signs of neuroinflammation and oxidative stress, synaptic deficits and neuronal damages. However, they do not fully mimic the symptomatology of AD and plaque formation or neurofibrillary tangles are often not present. AD is a complex multifactorial disease for which no comprehensive preclinical model has been yet developed. It appears more accurate to present A $\beta$ -peptide administration models as model of amyloid pathology, emphasizing that only this aspect of AD is investigated. The same can be applied to the construct validity. A $\beta$ -peptide administration models lack the time progression of the disease since the peptides are injected over a short period rather than slowly building up to toxic levels. Moreover, most research teams test only one species of A $\beta$  peptides which does not reflect the entire pool of different peptides found in the brain, nor their relative concentrations. It is thus important to clearly define the outcomes and objectives before choosing a model. A $\beta$ -peptide administration models remain nonetheless of relevance for AD research and I am confident that – associated with other *in vivo* and *in vitro* models – they will help discovering new drugs.



## Chapter 3 MATERIALS AND METHODS

## 3.1 INTRODUCTION

### 3.1.1 *Choice of the tests*

Data gathered in Chapter 1 (General introduction) and Chapter 2 (Systematic review of rat models based on the administration of amyloid- $\beta$  peptides) oriented the choice for behavioural and biochemical tests in order to validate and characterise the model presented in this project. Studies have been designed with the main aims of complying with animal welfare and statistical relevance, following the "Guidelines for the design and statistical analysis of experiments using laboratory animals" (Festing and Altman, 2002), and the 3R rule: Reduce, Refine and Replace. All the protocols run are detailed below.

### 3.1.2 *Power calculation and statistical analysis*

A free online power analysis software has been used to calculate the sample size required for each group of animals (Charan and Kantharia, 2013) (<http://www.biomath.info/power/index.htm>). Calculations were based on the most restrictive test, behavioural tests usually requiring a larger effect size than biochemical analyses. The effect size and standard deviation were estimated from previous studies performed by our team on different projects. Although a Type 1 error alpha of 5% ( $p=0.05$ ) is accepted for behavioural tests, power calculations were based on a Type 1 error of 1% ( $p=0.01$ ). A power of 80% was set, the direction of the effect was two-tailed and statistical analysis was based on the Student's t-test and ANOVA. It has been hence calculated that 10 animals were required per group in order to obtain relevant and significant results in the behavioural tests.

Statistical analysis tests used vary depending on the behavioural or biochemical analysis performed and will be detailed for each experiment. All

statistical tests were performed using "IBM® SPSS® Statistics" software. Graphs were made on "GraphPad Prism" (La Jolla California USA) software.

### 3.1.3 General outline of the studies

Rats receive acute ICV administration of 10  $\mu$ L of either Vehicle or A $\beta$ <sub>1-42</sub> (5 nmol), both provided by SynAging (France). The day of surgery is referred to as Day 0. Behavioural tests are performed on one or several selected time points, more than one behavioural test can be performed. Rats are culled and biochemical analysis is conducted on brains extracted on the last day of the experiment; alternatively, brains from selected rats in each group were extracted at different time points. Brains were always extracted after behavioural testing so behavioural and biochemical data could be compared. When required, a pharmacological treatment was administered, either preventively (before surgery), chronically (over time) or acutely on one or several selected time points. An overview of the design of the different studies performed is given below (Figure 8).

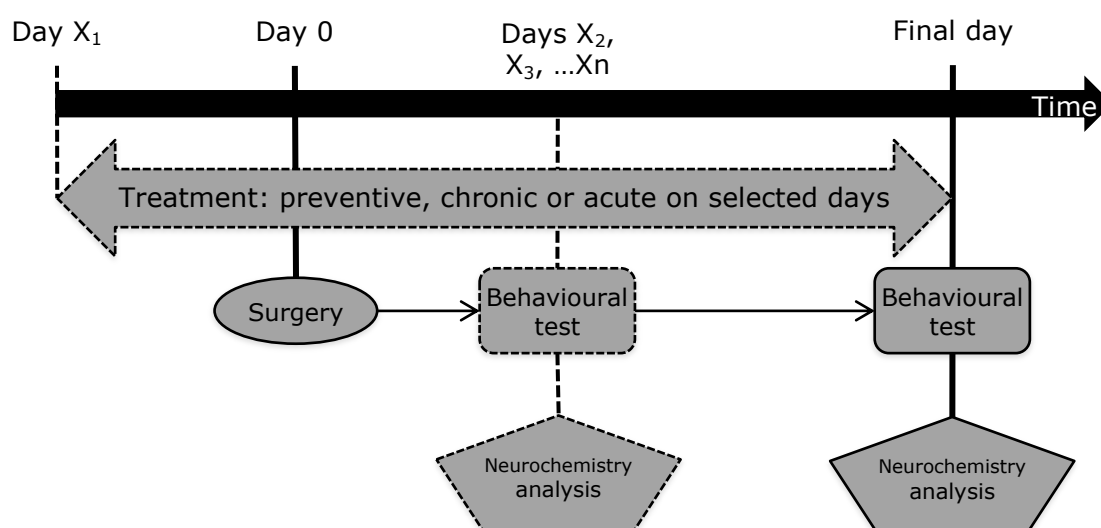


Figure 8: Outline of the studies. Surgery is performed on Day 0. Behavioural tests are performed on one or several selected time points, on Days X<sub>n</sub>. Rats are culled and neurochemistry analysis is conducted on brains extracted on the Final day. Treatment can be chronic or acute.

## 3.2 SURGERY AND EXTRACTION OF THE BRAINS

### 3.2.1 Intracerebroventricular administration of A $\beta$

#### 3.2.1.1 Materials

Name	Company	Reference #	Store at
Alcoholic iodine solution (iodine 2.5% w/v, potassium iodine 2.5% w/v)			RT
EMLA <sup>®</sup> cream: 2.5% w/w of lidocaine and 2.5% w/w of prilocaine	AstraZeneca		RT
Lacri-lube <sup>®</sup> : paraffin and wool alcohol	Allergan		RT
Vetergesic <sup>®</sup> : Buprenorphine 0.3 mg/mL	Alstoe		RT
Orbenin dry cow <sup>®</sup> : Cloxacillin 500mg	Pfizer		RT
Sterile saline solution			RT

#### 3.2.1.2 Equipment

Name	Company	Reference #
Rat stereotaxic frame		
Clippers, Scalper, Kelly forceps, cotton buds (all sterile)		
Hand drill	Silfradent	Microspeed 317in
10 $\mu$ L syringe	Hamilton	701 N
Suturing: P-3, 13 mm, 3/8 circle needle; undyed braided suture, size 5-0 absorbable Coated Vicryl <sup>®</sup> plus polyglactin 910 suture	Ethicon	VCP493H
Heating pad/Temperature controller	CMA	CMA150

### 3.2.1.3 Protocol

- Anaesthesia was induced at 4% isoflurane in O<sub>2</sub>.
- The scalp was then shaved before the start of the surgery procedure.
- The rat was placed on a stereotaxic frame, on a heating pad; and gaseous anaesthesia maintained at 2-3% isoflurane in O<sub>2</sub>.
- The head was secured with blunt ear bars previously coated with a topical local anaesthetic (EMLA<sup>®</sup> cream) (Figure 9).
- An ocular lubricant (Lacri-lube<sup>®</sup>) was applied on the eyes and the scalp was swabbed with alcoholic iodine solution.
- A sub-cutaneous injection of analgesic (Vetergesic<sup>®</sup>) was given, at 0.1 mg/kg.
- A midline sagittal incision on the skin overlying the surface of the skull was performed.
- Horizontal (H) and transverse (Tr) coordinates of Bregma were localised, and the coordinates of the left ventricle were calculated as following:  $H_v = H - 0.8\text{mm}$ ;  $Tr_v = Tr - 1.5\text{mm}$  (Paxinos and Watson, 1998).
- A hole was gently and slowly drilled at the new calculated coordinates.
- Using the implanter device from the frame, the Hamilton syringe containing 10  $\mu\text{L}$  of the drug was positioned at the surface of the skull, above the hole, giving the vertical coordinate (V).
- The depth of the ventricle was calculated as following:  $V_v = V - 4.5\text{mm}$  (Paxinos and Watson 1998).
- The syringe was lowered to the calculated coordinate and the drug was injected at a rate of 5  $\mu\text{L}$  per 2 minutes.
- The skin was then sutured back and a topical antibiotic (Orbenin dry cow<sup>®</sup>) was applied on the wound.
- 1.5 to 2.5 mL of saline solution were injected IP.
- The rat was then left to recover in a recovery chamber ( $\sim 28^\circ\text{C}$ ).

*Remark: The accuracy of the procedure has been checked by administering a dyed solution in the brain, during a non-recovery procedure. The solution has been shown to fill the ventricle (Figure 10A, B) and surround the hippocampus (Figure 10C, D).*

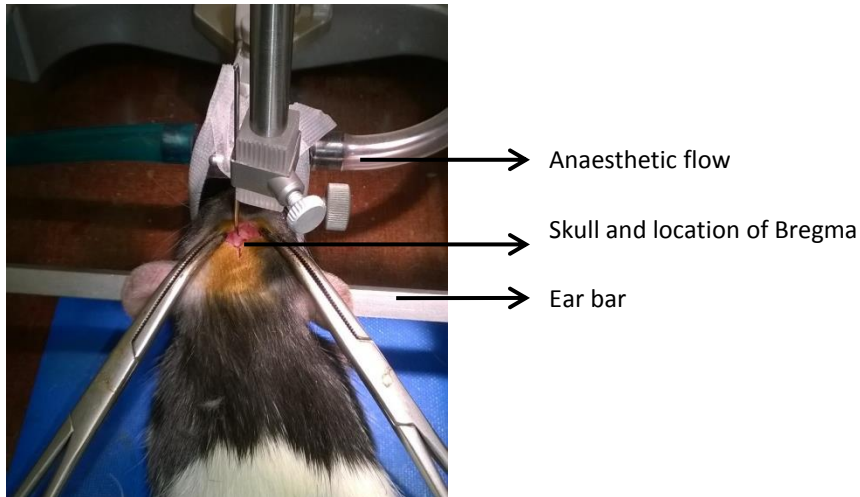


Figure 9: ICV administration surgery.

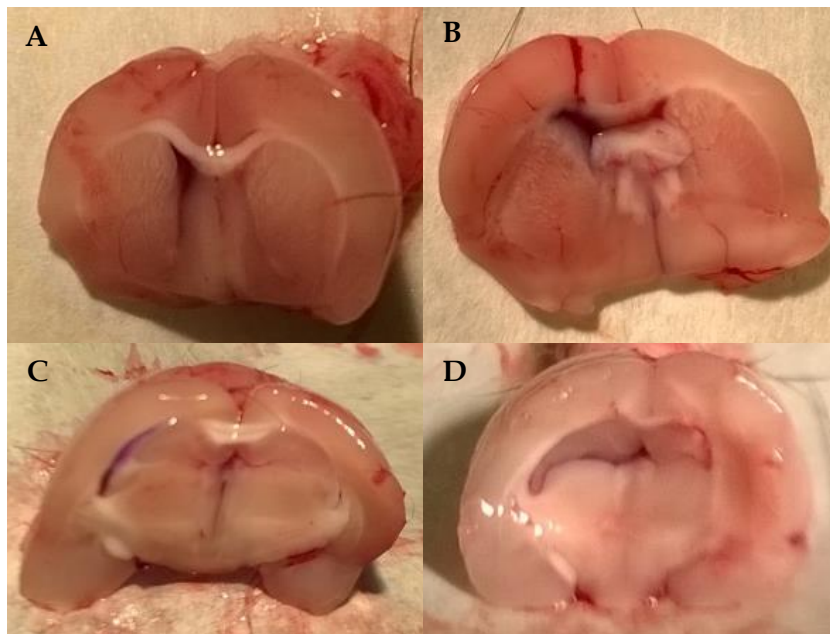


Figure 10: Rat brain slides following administration of a purple dyed solution. The solution has been administered in the ventricle (A, B) and surrounds the dorsal hippocampus (B, C). The needle track has been filled with blood and can be seen above the left ventricle (B).

### 3.2.2 Brain perfusion

#### 3.2.2.1 Materials

Name	Company	Reference #	Store at
Sucrose [CAS #57-50-1]	Sigma-Aldrich	<a href="#">S0389</a>	RT
PBS CA2+/Mg2+ free 10 X (diluted to a working concentration of 1 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT

#### 3.2.2.2 Equipment

Name	Company	Reference #
Peristaltic pump	Waston-Marlow	505S
Butterfly winged needle infusion set 21 G	Hospira	
Scissors, Kelly forceps, Forceps, Bone gouge forceps		

#### 3.2.2.3 Protocol

- The solution of PBS was kept on ice throughout the entire procedure.
- The rat was anaesthetised with an overdose of isoflurane (5% in O<sub>2</sub>, 2 L/min) and maintained at this concentration during the entire procedure.
- Blinking reflex was checked to ensure deep anaesthesia before any non-recovery step.
- An incision was made in the skin below the xiphoid cartilage.
- The skin and muscles were cut from the incision toward the left and right forepaws, taking care to avoid underlying structures.
- The diaphragm was then cut from left to right to allow the ribcage to be reflected upward, exposing the heart.
- The right auricle was cut to allow the blood to flow out.
- A butterfly needle was inserted in the left ventricle and secured with Kelly forceps (Figure 11).
- The pump was set at 20 mL/min (6 rpm) and the perfusion run for 6 min or until the flow coming out of the heart was clear.
- Once the procedure complete, both pump and anaesthetic were switched off.
- The head was then cut and the brain was extracted from the skull (Figure 12).

- The brain was dissected immediately and frozen in  $-80^{\circ}\text{C}$  isopentane (see 3.2.3 Brain dissection ).

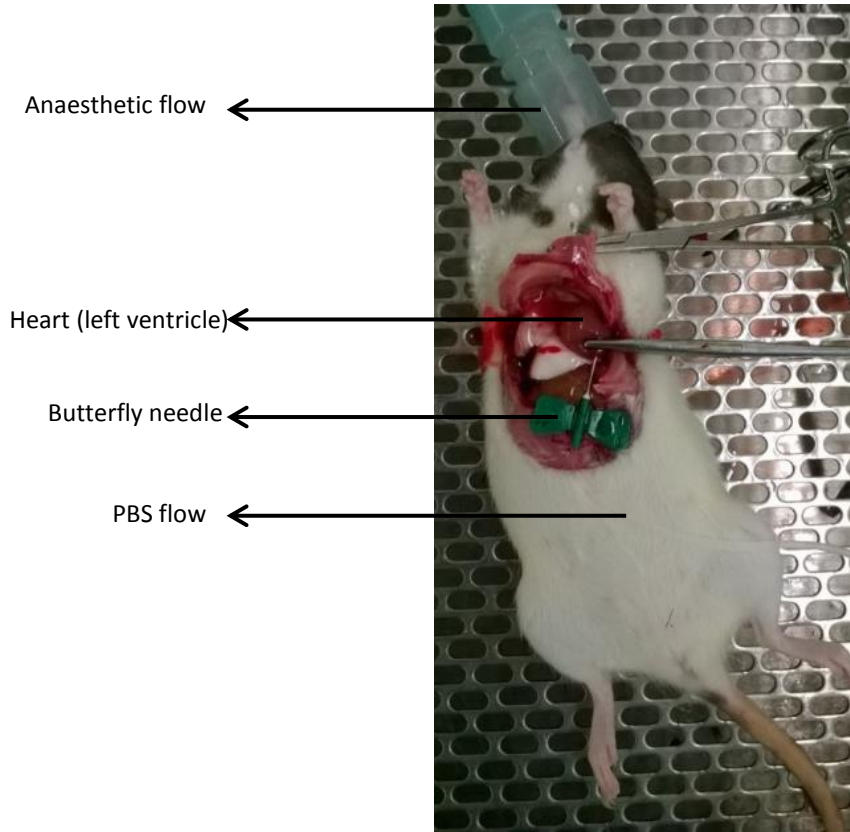


Figure 11: Brain perfusion through the heart on anaesthetised rat.

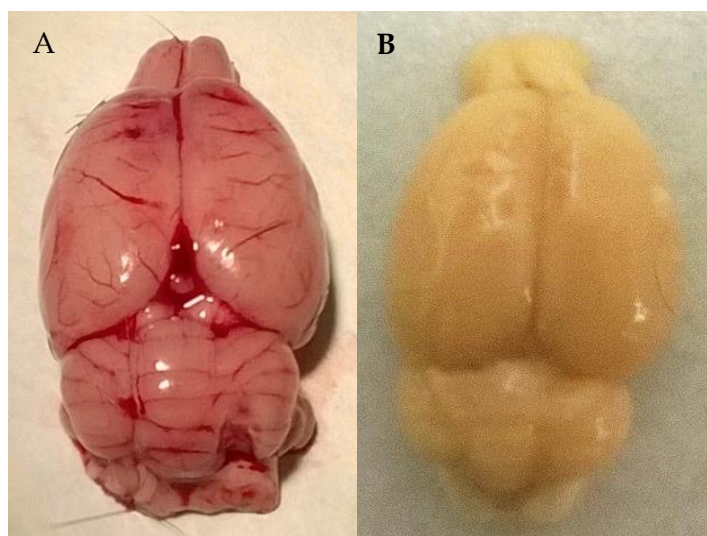


Figure 12: Extracted whole rat brains. (A) Non-perfused brain compared to (B) PBS perfused brain.



### 3.2.3 Brain dissection

#### 3.2.3.1 Materials

Name	Company	Reference #	Store at
Isopentane [CAS #78-78-4]	Fisher Scientific	<a href="#">10121820</a>	RT
PBS CA <sup>2+</sup> /Mg <sup>2+</sup> free 1 X (Reused from perfusion)			RT

#### 3.2.3.2 Equipment

Name	Company	Reference #
Petri dish and filter paper		
Dry ice		
Razor blade, scalpel, scissors, Hull forceps, curved tweezers		
1.5 mL centrifuge tubes	Eppendorf	

#### 3.2.3.3 Protocol

- Alternating the use of scissors and Hull forceps, the skull was gently removed away, making sure not to damage the brain tissue.
- The brain was extracted with tweezers and gently put onto a filter paper soaked in PBS (Figure 13A).
- The olfactory bulbs and cerebellum were removed and the brain was separated into left and right hemisphere (Figure 13B).
- On the left hemisphere, the frontal+prefrontal cortex was first extracted and put in a centrifuge tube before being frozen in  $-80^{\circ}\text{C}$  isopentane.
- On the left hemisphere, the midbrain was removed from the rest of the brain, using the blunt parts of curved tweezers.
- On the left hemisphere, the hippocampus was peeled of and gently put in a centrifuge tube before being frozen in  $-80^{\circ}\text{C}$  isopentane.
- The previous steps were then repeated on the right hemisphere.

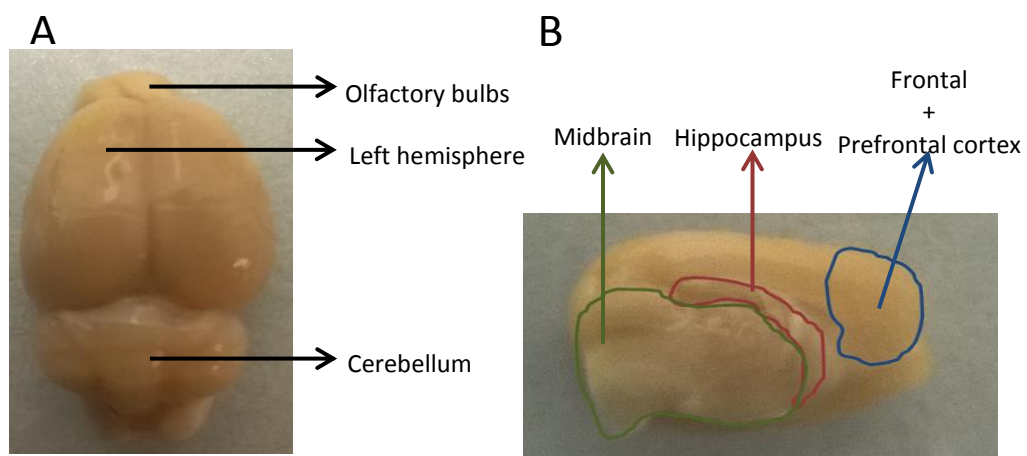


Figure 13: Perfused rat brain. (A) Whole brain, top view; (B) Left hemisphere and areas of interest.

### 3.3 COGNITIVE TASKS

#### 3.3.1 Novel object recognition task

##### 3.3.1.1 Purpose

The NOR task was used to assess short term visual memory in rats by measuring the time spent exploring different objects over two 3-min phases. This test relies on the inquisitive behaviour of rats and their preference for novelty. The rat is first presented with two identical versions of a same object which it is left free to explore for 3 min. It is expected that the rat will spend an equal amount of time exploring both identical objects. After an inter-trial interval (ITI), the rat is presented with an identical version of the previous object ("Familiar" object) and a new different object ("Novel" object), and is left free to explore them for 3min. It is expected that a control rat will spend more time exploring the novel object than the familiar one, due to its preference for novelty; while a rat with short term memory impairment is expected to spend an equal amount of time exploring both objects. This test has originally been published by (Ennaceur and Delacour, 1988).

##### 3.3.1.2 Equipment

Name	Company	Reference #
PVC test arena: <ul style="list-style-type: none"> <li>• Length: 52 cm; width: 52 cm; height: 31 cm.</li> <li>• Black PVC walls. White PVC floor with a grid pattern delimiting the area into 9 squares</li> </ul>		See Figure 14
4 objects – 3 similar versions of the same object, and 1 different object – from the list below: <ul style="list-style-type: none"> <li>• Object B: brown glass bottle filled with water – 12.5x6.5 cm</li> <li>• Object C: can of Coca Cola® – 12.5x6.5 cm</li> <li>• Object M: bottle of Frijj® filled with water – 12.5x6.5 cm</li> <li>• Object P: a terracotta pot – 12.5x5 cm</li> </ul>		See Figure 15
Cameras and recording system		
Surface disinfectant: Anistel	Tristel Solutions	



Figure 14: Example of a NOR setting with object B and C, retention session



Figure 15: From left to right: object B, object C, object P, object M

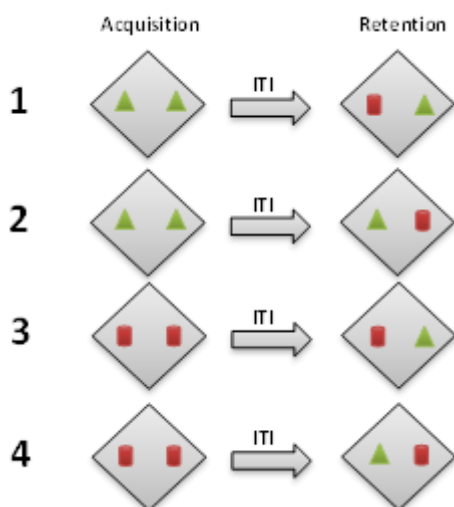


Figure 16: Possible object configurations

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### 3.3.1.3 Protocol

- **Habituation phase**
  - Two days before the task, rats were left free to explore the NOR box for 10min, with their cage mates.
  - On the day before the task, rats were left free to explore the NOR box for 10min, this time individually.
- **Acquisition phase**
  - The tested rat was placed at the bottom corner of the test arena, facing the wall.
  - It was then left free to explore two identical versions of the same object in the left and right corner of the test arena, 6 cm away from the walls, at the intersection of two lines, for 3 min.
- **ITI**
  - During the 2 min ITI, the rat was single housed, while the acquisition phase objects were swapped for the retention phase objects (Figure 14).
  - Urine and faeces were removed from the test arena, but no surface disinfectant was used between the two phases.

Remark: the objects used, their left/right position and their familiarity/novelty were balanced between animals (Figure 16).

- **Retention phase**
  - At the end of the ITI, the tested rat was placed in the same location and position as it was at the beginning of the acquisition phase, and left free to explore the arena and the two objects freely for 3 min.

---

### 3.3.1.4 Scoring

- The scoring was done from the recorded video, with the experimenter blind to the treatment and to the familiarity/novelty of the objects.
- Scoring consisted of the time spent exploring the left and right object during both the Acquisition and the Retention phases.
  - Were considered as "exploring": sniffing, licking, touching, and staring at the object from a distance of less than 2 cm.
  - Were not considered as "exploring": sitting on the object and touching the object without looking at it.

- Rats were excluded if:
  - Single object exploration was < 2s.
  - The rat escaped the test arena or made an object fall.

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### 3.3.1.5 Data analysis

- For each treatment group, the following score were compared:
  - Left vs. Right object exploration time, in seconds;
  - Familiar vs. Novel object exploration time, in seconds;
  - Total exploration time (Left + Right or Familiar + Novel)
  - Discrimination index (DI):  $DI = \frac{novel-familiar}{novel+familiar}$
- **Statistical analysis of object exploration time**
  - 1-way ANOVA on repeated measures with "object" as within-subject factor and "treatment" as in-between subject factor. 2-way ANOVA when comparing more than one variable (treatment + day).
  - In case of significance ( $p < 0.05$ ), individual paired samples Student's t-tests were run in each group, comparing the exploration time of both objects.
- **Statistical analysis of total exploration time**
  - 1-way ANOVA between groups followed by Bonferroni post-hoc. 2-way ANOVA when comparing more than one variable.
  - Independent samples Student's t-test if only two groups were tested.
- **Statistical analysis of DI**
  - Univariate Student's t-test of DI vs. 0 in each group.
  - 1-way ANOVA between groups followed by Bonferroni post-hoc. 2-way ANOVA when comparing more than one variable.
  - Independent samples Student's t-test if only two groups were tested.

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### 3.3.1.6 Validation of NOR objects

In order to verify that rats do not have a natural preference for either object or location in the test arena, each couple of objects was first validated with naïve rats, as presented in Appendix VI Validation of NOR objects (p192).

### 3.3.2 What-where-which task

#### 3.3.2.1 Purpose

The what-where-which (WWWhich) task was used to assess episodic-like memory. This task is similar to the NOR task with an added acquisition phase (Figure 19). Rats are expected to identify a novel combination of object/place/context instead of novel object only.

#### 3.3.2.2 Equipment

Name	Company	Reference #
<ul style="list-style-type: none"> <li>Context ①: similar to NOR</li> <li>Context ②: similar test arena, walls taped with texturized and coloured vinyl plaques (30.5x30.5 cm); floor covered with a vinyl plaque (45.7x45.7 cm) and clean litter. Top end of each wall is rubbed with vanilla essential oil.</li> </ul>	Custom made	See Figure 17 and Figure 18
<ul style="list-style-type: none"> <li>Objects: similar to NOR</li> </ul>		See Figure 15
Cameras, recording system		
Surface disinfectant: Anistel	Tristel Solutions	

#### 3.3.2.3 Protocol

- Similar to NOR protocol, with an extra Acquisition phase.
  - Habituation: similar to NOR habituation phase; performed in both contexts.
  - Acquisition phase 1: first context, two different objects.
  - ITI 1 (2 min).
  - Acquisition phase 2: second context, same objects as Acquisition phase 1 in reversed locations.
  - ITI 2 (2 min).
  - Retention phase: first or second context, with two identical versions of one of the objects.

#### 3.3.2.4 Scoring and data analysis

- Scoring and data analysis are similar to the NOR task.



Figure 17: Example of a WWWhich setting, Acquisition 1, with object B and C, Context ①



Figure 18: Example of a WWWhich setting, Acquisition 2, with object B and C, Context ②



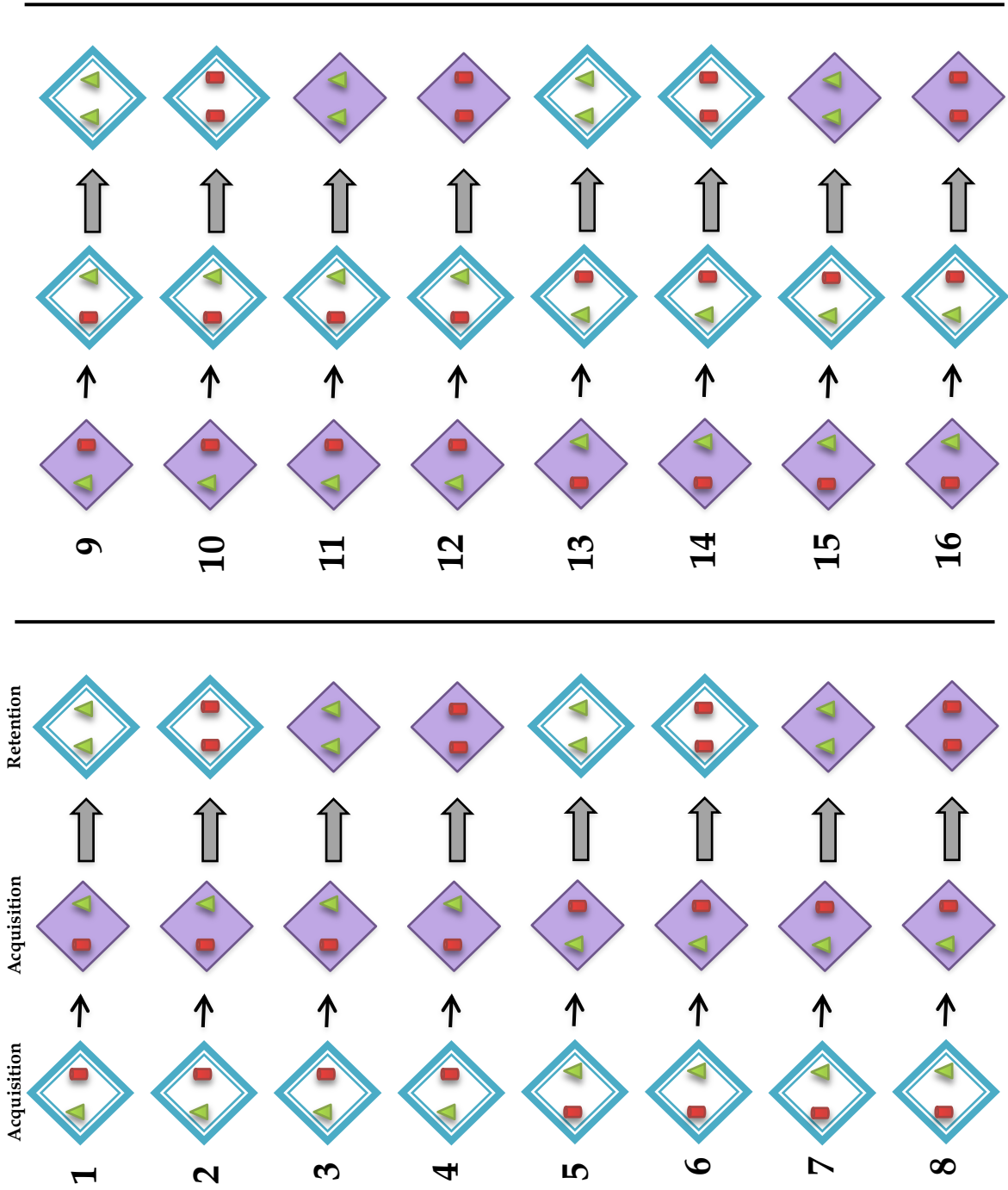


Figure 19: WWWhich task possible configurations

### 3.3.3 Y-maze task

#### 3.3.3.1 Purpose

This task was used to assess short term spatial memory. The index measured was the percentage of alternation in arm entries in a Y-maze with three equal arms referred as "A", "B" and "C".

#### 3.3.3.2 Equipment

Name	Company	Reference #
PVC test arena: radial 8-arms maze (only 3 arms open), each arm has the following dimensions: <ul style="list-style-type: none"> <li>• Length: 60 cm</li> <li>• Width: 10 cm</li> <li>• Height: 29.5 cm</li> <li>• Black PVC walls</li> <li>• White PVC floor</li> </ul>	Custom made	See Figure 20
External cues outside the maze		
Camera above the maze		
Surface disinfectant: Anistel	Tristel Solutions	

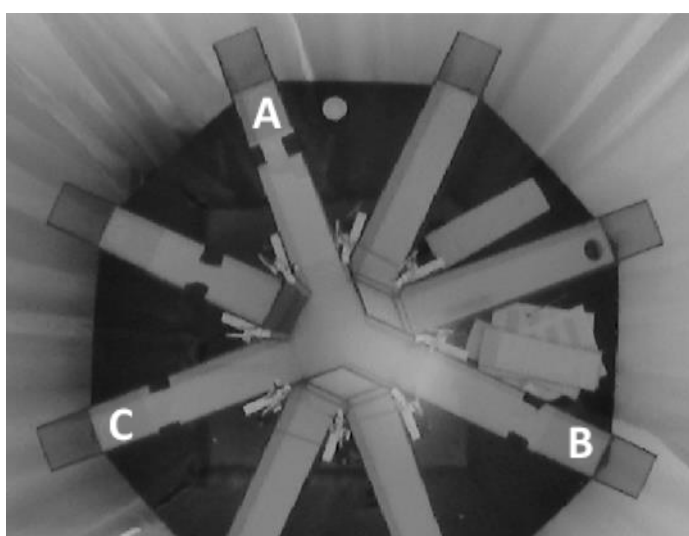


Figure 20: Screenshot of the Y-maze, made from a modified 8-arms radial maze. In the Y-maze configuration, only the 3 arms labelled "A", "B", and "C" are open and can be explored by the rat from the central platform

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### **3.3.3.3 Protocol**

- The rat was placed at the end of one arm, facing the wall. The starting arm was randomly assigned and balanced between animals.
- The rat was left free to explore the maze for 8 min.

---

### **3.3.3.4 Scoring and data analysis**

- Scoring of arm entries sequence was done live, from the computer screen.
- Was considered as an alternation: three successive arm entries in different arms; i.e. ABC or ACB or BAC or BCA or CAB or CBA.
- % alternation was calculated as following:

$$\%Alternation = \frac{\text{number of alternations}}{\text{number of arm entries} - 2} \times 100$$

- Statistical analysis of % alternation was made by 1-way ANOVA between groups followed by Bonferroni post-hoc.

## 3.4 BIOCHEMICAL ANALYSIS

### 3.4.1 Synaptosomal preparation from rat brain tissue

#### 3.4.1.1 Materials

Name	Company	Reference #	Store at
Trizma <sup>®</sup> base [CAS #77-86-1]	Sigma-Aldrich	<a href="#">T1503</a>	RT
Sucrose [CAS #57-50-1]	Sigma-Aldrich	<a href="#">S9378</a>	RT
EDTA [CAS #6381-92-6]	Sigma-Aldrich	<a href="#">ED2SS</a>	RT
Protease inhibitor cocktail tablets cOmplete <sup>®</sup>	Roche	<a href="#">11836145001</a>	+2-8°C
PBS CA2+/Mg2+ free 10 X (working concentration: 1 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT
PMSF [CAS #329-98-6]	Sigma-Aldrich	<a href="#">P7626</a>	RT
Sodium orthovanadate [CAS #13721-39-6]	Sigma-Aldrich	<a href="#">S6508</a>	RT
Ethanol absolute [CAS #64-17-5]	Fisher Chemical		RT

#### 3.4.1.2 Equipment

Name	Company	Reference #
Eppendorf <sup>®</sup> micropestles	Sigma-Aldrich	<a href="#">Z317314</a>
Centrifuge 5402 – Rotor F-45-18-11 – Radius 7.2 cm	Eppendorf	

### 3.4.1.3 Preparation of the solutions

- **PMSF 0.1M stock solution – 5 mL**
  - 87.10mg of PMSF in 5 mL of ethanol absolute.
  - Stored at +2-8°C.
- **Sodium orthovanadate 0.1 M stock solution – 5 mL**
  - 91.96mg of sodium orthovanadate in 5 mL of distilled H<sub>2</sub>O.
  - Stored at +2-8°C.
- **Homogenisation buffer – 200 mL**
  - In 200 mL distilled H<sub>2</sub>O, pH adjusted to 7.4:
    - 242.28 mg Trizma<sup>®</sup> base;
    - 21.9 mg sucrose;
    - 148.9 mg EDTA.
  - Extemporaneously, 1 tablet of Protease inhibitor cocktail tablets cComplete<sup>®</sup> was added for every 50 mL.
  - 500 $\mu$ L of PMSF stock solution and 500 $\mu$ L of sodium orthovanadate stock solution were added before use (cannot be used after 30 min).

### 3.4.1.4 Protocol

- Samples were kept at +4°C during the entire procedure.
- Each sample was homogenised in 600  $\mu$ L of homogenisation buffer, using a micropestle, for 3 min.
- Samples were centrifuged at 800G (~3200 rpm), for 15 min, at +4°C.
- The pellet (P1) was discarded and the supernatant (S1) was further spun at 12000G (~12200 rpm), for 20 min, at +4°C.
- The supernatant (S2) was discarded and the pellet (P2) was resuspended with 400  $\mu$ L of PBS 1 X.

### 3.4.2 Protein assay – modified Bradford assay

#### 3.4.2.1 Materials

Name	Company	Reference #	Store at
Bovine Serum Albumin standard BSA [CAS #9048-46-8] 2 mg/mL (2 X)	BioRad	<a href="#">500-0206</a>	+2-8°C
Protein assay dye reagent concentrate	Bio-Rad	<a href="#">500-0006</a>	+2-8°C

#### 3.4.2.2 Equipment

Name	Company	Reference #
96-well plate	Corning	
Plate reader	Bio-Tek	$\mu$ quant
Plate reader software	Bio-Tek	Gen 5

#### 3.4.2.3 Preparation of the samples and standards

- Samples were prepared accordingly to the experiment run (Western Blot, ELISA, HPLC).
- The buffer was made accordingly to the lysis buffer used for the samples. The same buffer was used to serve as a blank and take into account any absorbance variation due to the buffer itself.

Standard	Concentration BSA [mg/mL]	Volume BSA	Volume buffer [ $\mu$ L]
F	1	150 $\mu$ L of BSA	150
E	0.75	75 $\mu$ L of F	25
D	0.5	50 $\mu$ L of F	50
C	0.2	20 $\mu$ L of F	80
B	0.1	10 $\mu$ L of F	90
A	0	0	100

#### 3.4.2.4 Protocol

- Before analysis, samples were diluted 1:10.
- 10  $\mu$ L of each standard and samples were run in triplicate.
- 200  $\mu$ L of dye reagent (1:5 in ultrapure H<sub>2</sub>O) was added to each well.
- The plate was then left to incubate at RT for 5 min on a shaker.
- Absorbance was measured at 595 nm and samples concentrations were calculated from the standard curve, taking into account the 1:10 dilution.

### 3.4.3 ELISA assay for PSD-95 and SNAP-25

#### 3.4.3.1 Materials

Name	Company	Reference #	Store at
Enzyme-linked Immunosorbent Assay Kit for discs, large homolog 4 (DLG4)	Cloud-Clone Corp.	<a href="#">SEG168Ra</a>	+2-8°C -20°C
Enzyme-linked Immunosorbent Assay Kit for Synaptosomal Associated Protein 25kDa (SNAP25)	Cloud-Clone Corp.	<a href="#">SEC955Mi</a>	+2-8°C -20°C
PBS CA2+/Mg2+ free (10 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT

#### 3.4.3.2 Equipment

Name	Company	Reference #
Microplate reader	BioTek	ELx800
Microplate reader software	BioTek	Gen 5

#### 3.4.3.3 Preparation of the solutions

- **Detection reagents A and B (1 X) – 11 mL**
  - 110  $\mu$ L of Detection Reagent A in 10890  $\mu$ L of Assay Diluent A.
  - 110  $\mu$ L of Detection Reagent B in 10890  $\mu$ L of Assay Diluent B.
- **Wash solution – 300 mL**
  - 10 mL of Wash buffer 30 X in 290 mL of ultrapure H<sub>2</sub>O.
- **Standards**

Standard	Concentration [ng/mL]	Volume standard [ $\mu$ L]	Standard Diluent [ $\mu$ L]
A	20	-	1000
B	10	500 $\mu$ L of A	500
C	5	500 $\mu$ L of B	500
D	2.5	500 $\mu$ L of C	500
E	1.25	500 $\mu$ L of D	500
F	0.625	500 $\mu$ L of E	500
G	0.312	500 $\mu$ L of F	500
H	0	0	500

---

### 3.4.3.4 Protocol

- Samples were obtained after synaptosomal preparation (see 3.4.1 – Fraction P2) and diluted 1:10 in standard diluent.
- Standards or samples were added in triplicate (standards) or duplicate (samples), 100  $\mu$ L per well.
- Plates were covered and incubated at 37°C for 2 hours.
- Plates were emptied but not washed.
- Detection reagent A was added, 100  $\mu$ L per well.
- Plates were covered and incubated at 37°C for 1 hour.
- Plates were washed 3 times with the wash solution.
- Detection reagent B was added, 100  $\mu$ L per well.
- Plates were covered and incubated at 37°C for 30 min.
- Plates were washed 5 times.
- Substrate solution was added, 90  $\mu$ L per well.
- Plates were covered and incubated at 37°C for 20 min.
- Stop solution was added, 50  $\mu$ L per well.
- Absorbance was measured at 450 nm and samples concentrations were calculated from the standard curve, taking into account the 1:10 dilution.

---

### 3.4.3.5 Data analysis

- Concentrations were normalised using the protein assay (see 3.4.2).
- Values are analysed by 1-way ANOVA between groups followed by Bonferroni post-hoc. Independent samples Student's t-test when only two groups were tested.



### 3.4.4 ELISA assay for IL-6, TNF- $\alpha$ and IL-1 $\beta$

#### 3.4.4.1 Materials

Name	Company	Reference #	Store at
Rat IL-6 DuoSet ELISA (coating antibody, recombinant standard, detection antibody, streptavidin HRP)	R&D system	<a href="#">DY506</a>	+2-8°C -20°C
Rat TNF-alpha DuoSet ELISA (coating antibody, recombinant standard, detection antibody, streptavidin HRP)	R&D system	<a href="#">DY510</a>	+2-8°C -20°C
Rat IL-1 beta/IL-1F2 DuoSet ELISA (coating antibody, recombinant standard, detection antibody, streptavidin HRP)	R&D system	<a href="#">DY501</a>	+2-8°C -20°C
Bovine Serum Albumin standard BSA [CAS #9048-46-8] 2 mg/mL (2 X)	BioRad	<a href="#">500-0206</a>	+2-8°C
PBS CA2+/Mg2+ free (10 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT
Sulfuric acid H <sub>2</sub> SO <sub>4</sub> [CAS #7664-93-9]	Sigma-Aldrich	<a href="#">339741</a>	RT
Tween 20 [CAS #9005-64-5]	Sigma-Aldrich	<a href="#">P1379</a>	RT

#### 3.4.4.2 Equipment

Name	Company	Reference #
Microplate reader	BioTek	ELx800
Microplate reader software	BioTek	Gen 5

#### 3.4.4.3 Preparation of the solutions

- Wash solution: 0.05% Tween20 in PBS 1 X
- Reagent diluent: 1% BSA in PBS 1 X
- Streptavidine HRP: 1:100 in reagent diluent
- Substrate solution: 1:1 reagent A + reagent B
- Standards:
  - Standards were reconstituted in 0.5 mL of RD (final concentration: IL-6 740 ng/mL, TNF- $\alpha$  160 ng/mL, IL-1 $\beta$  290 ng/mL).
  - Standard ranges for ELISA are given on the next page.

IL-6				
Standard	Concentration [pg/mL]	Volume standard [ $\mu$ L]	Reagent Diluent [ $\mu$ L]	Final volume [ $\mu$ L]
A	8000	5.4	494.6	250
B	4000	250 $\mu$ L of A	250	250
C	2000	250 $\mu$ L of B	250	250
D	1000	250 $\mu$ L of C	250	250
E	500	250 $\mu$ L of D	250	250
F	250	250 $\mu$ L of E	250	250
G	125	250 $\mu$ L of F	250	500
H	0	0	250	250

TNF- $\alpha$				
Standard	Concentration [pg/mL]	Volume standard [ $\mu$ L]	Reagent Diluent [ $\mu$ L]	Final volume [ $\mu$ L]
A	4000	6	234	120
B	2000	120 $\mu$ L of A	120	120
C	1000	120 $\mu$ L of B	120	120
D	500	120 $\mu$ L of C	120	120
E	250	120 $\mu$ L of D	120	120
F	125	120 $\mu$ L of E	120	120
G	62.5	120 $\mu$ L of F	120	240
H	0	0	120	120

IL-1 $\beta$				
Standard	Concentration [pg/mL]	Volume standard [ $\mu$ L]	Reagent Diluent [ $\mu$ L]	Final volume [ $\mu$ L]
A	4000	4	286	170
B	2000	120 $\mu$ L of A	120	120
C	1000	120 $\mu$ L of B	120	120
D	500	120 $\mu$ L of C	120	120
E	250	120 $\mu$ L of D	120	120
F	125	120 $\mu$ L of E	120	120
G	62.5	120 $\mu$ L of F	120	240
H	0	0	120	120

---

#### **3.4.4.4 Protocol**

- Samples were obtained after synaptosomal preparation (see 3.4.1 – Fraction S2) and diluted 1:2 in RD.
- Reconstituted capture antibody was added, 50  $\mu$ L per well.
- Plates were covered and incubated at RT for 16 hours.
- Plates were washed 3 times with the wash solution.
- Reagent diluent was added, 200  $\mu$ L per well.
- Plates were covered and incubated at RT for 1 hour.
- Plates were washed 3 times.
- Standards or samples were added in triplicate (standards) or duplicate (samples), 50  $\mu$ L per well.
- Plates were covered and incubated at RT for 2 hours.
- Plates were washed 3 times.
- Detection antibody was added, 50  $\mu$ L of per well.
- Plates were covered and incubated at RT for 2 hours.
- Plates were washed 3 times.
- Streptavidin HRP was added, 50  $\mu$ L of per well.
- Plates were covered and incubated at RT for 20 min.
- Plates were washed 3 times.
- Substrate solution was added, 50  $\mu$ L of per well.
- Plates were covered and incubated at RT for 20 min.
- H<sub>2</sub>SO<sub>4</sub> solution was added, without washing step, 50  $\mu$ L of per well.
- Absorbance was measured at 450 nm and samples concentrations were calculated from the standard curve, taking into account the 1:2 dilution.

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#### **3.4.4.5 Data analysis**

- Concentrations were normalised using the protein assay (see 3.4.2).
- Values are analysed by 1-way ANOVA between groups followed by Bonferroni post-hoc. Independent samples Student's t-test when only two groups were tested.

### 3.4.5 Western blot for IL-1 $\beta$

#### 3.4.5.1 Materials

Name	Company	Reference #	Store at
Protease inhibitor cocktail	Sigma-Aldrich	<a href="#">P8340</a>	-20°C
Ladder Precision Plus Protein Standards, Dual Color	BioRad	<a href="#">161-0374</a>	-20°C
RIPA buffer (1 X)	Sigma-Aldrich	<a href="#">R0278</a>	+2-8°C
Clarity Western ECL substrate kit	BioRad	<a href="#">170-5061</a>	RT
Laemmli sample buffer (2 X)	BioRad	<a href="#">161-0737</a>	RT
PBS CA2+/Mg2+ free (10 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT
Running buffer 10X Tris/Glycine/SDS	BioRad	<a href="#">161-0772</a>	RT
Tween 20 [CAS #9005-64-5]	Sigma-Aldrich	<a href="#">P1379</a>	RT
$\beta$ -mercaptoethanol HSCH <sub>2</sub> CH <sub>2</sub> OH [CAS # 60-24-2]	Sigma-Aldrich	<a href="#">M3148</a>	RT
Powder milk			
<b>Antibodies</b>			
Rat IL-1 beta/IL-1F2 (made in goat)	R&D	<a href="#">AF-501-NA</a>	-20°C
Anti-goat HRP	Abcam	<a href="#">ab97100</a>	+2-8°C
<b>Gels</b>			
Criterion® TGX Stain-Free™ Gel, 18 wells, 30 $\mu$ l: 8-16%	BioRad	<a href="#">567-8104</a>	+2-8°C
<b>Transfer pack</b>			
Trans-Blot® Turbo™ Midi PVDF	BioRad	<a href="#">170-4157</a>	+2-8°C

#### 3.4.5.2 Equipment

Name	Company	Reference #
Centrifuge 5402 – Rotor F-45-18-11 – Radius 7.2cm	Eppendorf	
Power supply: PowerPac™ HC High-Current Power Supply	BioRad	<a href="#">164-5052</a>
Tank: Criterion™ Cell	BioRad	<a href="#">165-6001</a>
Trans-Blot® Turbo™ Transfer Starter System	BioRad	<a href="#">170-4155</a>
Gel imaging system: ChemiDoc™ MP System	BioRad	<a href="#">170-8280</a>
Eppendorf® micropestles	Sigma-Aldrich	<a href="#">Z317314</a>

### 3.4.5.3 Preparation of the solutions

- **PBST – 1 L**
  - 1 mL Tween 20 (0.1%) in 1 L PBS 1 X.
- **Sample buffer – 1 mL**
  - 85  $\mu$ L  $\beta$ -mercaptoethanol in 915  $\mu$ L Laemmli Sample Buffer 2 X.
- **ECL – 12 mL**
  - 6 mL Luminol/Enhancer solution + 6mL of Peroxide solution.
- **Lysis buffer – 1 mL** (600  $\mu$ L needed per sample)
  - 990  $\mu$ L Ripa buffer + 10  $\mu$ L protease inhibitor.
- **Blocking solution – 3% milk – 15 mL**
  - 450 mg powder milk in 15 mL PBST.
- **Reconstituted primary antibody:** 0.2  $\mu$ g/mL in blocking solution
- **Reconstituted secondary antibody:** anti-Goat 1:1000 in blocking solution

### 3.4.5.4 Protocol

#### Preparation of the samples

- Samples were kept at +4°C during the entire procedure.
- Each sample was homogenised in 600  $\mu$ L of homogenisation buffer, using a micropestle, for 3 min.
- Samples were centrifuged at 12200 rpm, for 20 min, at +4°C.
- The Western Blot was performed on the supernatant; the pellet was discarded.

#### Preparation of loading samples

- Following a protein assay (see 3.4.2), samples were diluted to a final equal concentration of 20 to 40  $\mu$ g of total protein per well (25  $\mu$ L), in a 1:2 sample buffer in distilled H<sub>2</sub>O
- Samples were denatured at 90°C for 5 min.
- Samples were briefly centrifuged and re-homogenised.

#### Electrophoresis

- The electrophoresis was run accordingly to the manufacturer's instruction, at 120 V for 40 to 60 min (1 L running buffer, loading volume: 10  $\mu$ L ladder, 25  $\mu$ L samples).

### **Gel activation and transfer onto the membrane**

- Gel activation was done using the ChemiDoc™ MP System, according to the manufacturer's instruction.
- Gel transfer onto membrane was done in the Trans-Blot® Turbo™ Transfer Starter System, according to the manufacturer's instruction.
- The membrane was then analysed in the ChemiDoc™ MP System, using the stainfree blot protocol to obtain an image of the total proteins vlot, used later for normalisation of the results.

### **Immunoblotting of the membrane**

- The membrane was incubated with the blocking solution, at RT, for 1 hour.
- The membrane was washed in PBST.
- The membrane was incubated with the primary antibody solution, at +4°C, for 16 hours.
- The membrane was washed 3 times in PBST.
- The membrane was incubated with the secondary antibody solution, at RT, for 1 hour.
- The membrane was washed 3 times in PBST.
- The membrane was incubated with the ECL solution, at RT, for 5 min.
- The membrane was then analysed in the ChemiDoc™ MP System, on the High sensitivity protocol to obtain an image of the protein of interest; then on the Colorimetric protocol to obtain an image of the ladder.

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#### **3.4.5.5 Data analysis**

- Protein concentrations were normalised in each lane to the total protein concentration, comparing the images from the "High sensitivity" protocol (protein of interest) to the "Stainfree" one (total protein). For each lane, the software calculate the ratio of protein of interest per total protein concentration and then normalise these value to an arbitrary chosen control lane, giving a semi-quantitative result.
- Values are analysed by 1-way ANOVA between groups followed by Bonferroni post-hoc. Independent samples Student's t-test if only two groups have been tested.

### 3.4.6 High-performance liquid chromatography for N-acetylaspartate

#### 3.4.6.1 Materials

Name	Company	Reference #	Store at
Sodium phosphate monobasic $\text{NaH}_2\text{PO}_4$ [CAS #10049-21-3]	Sigma-Aldrich	<a href="#">71504</a>	RT
Sodium phosphate dibasic $\text{Na}_2\text{HPO}_4$ [CAS #7558-79-4]	Sigma-Aldrich	<a href="#">71642</a>	RT
Perchloric acid (PCA) $\text{HClO}_4$ [CAS #7601-90-3]	Sigma-Aldrich	<a href="#">244252</a>	RT
Sodium carbonate $\text{CNa}_2\text{O}_3$ [CAS #497-19-8]	Sigma-Aldrich	<a href="#">S7795</a>	RT
Phosphoric acid $\text{H}_3\text{PO}_4$ [CAS #7664-38-2]	Sigma-Aldrich	<a href="#">345245</a>	RT
Methanol $\text{CH}_4\text{O}$ [CAS #67-56-1]	Fisher Scientific	<a href="#">10675112</a>	RT
N-Acetyl-L-aspartic acid (NAA) $\text{HO}_2\text{CCH}_2\text{CH}(\text{NHCOCH}_3)\text{CO}_2\text{H}$ [CAS #997-55-7]	Sigma-Aldrich	<a href="#">00920</a>	RT

#### 3.4.6.2 Equipment

Name	Company	Reference #
Centrifuge 5402 – Rotor F-45-18-11 – Radius 7.2 cm	Eppendorf	
Eppendorf® micropestles	Sigma-Aldrich	<a href="#">Z317314</a>
SAX columns		
HPLC apparatus: pump, vacuum, injector, column, detector, printer		

### 3.4.6.3 Preparation of the solutions

- **Perchloric acid 0.1 M – 500 mL**
  - 7.195 mL of perchloric acid in 500 mL distilled H<sub>2</sub>O.
- **Sodium carbonate 0.2 M – 10 mL**
  - 0.212 g of sodium carbonate in 10 mL H<sub>2</sub>O.
- **Monosodium phosphate (NaH<sub>2</sub>PO<sub>4</sub>) solutions – 500 mL**
  - 1 M: 69 g of NaH<sub>2</sub>PO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
  - 50 mM: 3.45 g of NaH<sub>2</sub>PO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
  - 2 mM: 0.138 g of NaH<sub>2</sub>PO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
  - Solutions are adjusted to pH6 with disodium phosphate solutions.
- **Disodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) solutions – 500 mL**
  - 1 M: 71 g of Na<sub>2</sub>HPO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
  - 50 mM: 3.55 g of Na<sub>2</sub>HPO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
  - 2 mM: 0.142 g of Na<sub>2</sub>HPO<sub>4</sub> in 500 mL distilled H<sub>2</sub>O.
- **Phosphoric acid solutions**
  - 0.5%: 2.94 mL of phosphoric acid in 500 mL distilled H<sub>2</sub>O.
  - 0.1%: 2.35 mL of phosphoric acid in 2 L distilled H<sub>2</sub>O.
- **Standards – NAA**
  - Stock – NAA 10 mM:
    - 17.51 mg NAA in 10 mL H<sub>2</sub>O.
  - Working solution (W) – NAA 1 mM:
    - 1:10 of the stock solution in phosphate buffer 50 mM .

### 3.4.6.4 Protocol

#### Tissue homogenisation

- Samples were kept at +4°C during the entire procedure.
- Each sample was homogenised in 10 times their weigh (10 mL/g) in homogenisation buffer (PCA 0.1M), using a micropestle, for 3 min.
- Samples were centrifuged at 12000 rpm, for 5 min, at +4°C.
- 50  $\mu$ L of supernatant was added to 30  $\mu$ L CNa<sub>2</sub>O<sub>3</sub> + 920  $\mu$ L phosphate buffer 50 mM.



## Extraction

- The following solution were run to waste in the SAX columns:
  - 1 mL methanol.
  - 2 mL PhosA 0.5%.
  - 4 mL phosphate buffer 1 M.
  - 2 mL phosphate buffer 50 mM.
  - 1 mL sample.
  - 2 mL phosphate buffer 2 mM.
- 1.5 mL PhosA 0.5% was then run and collected.

## HPLC parameters

- 120  $\mu$ L of solution per vial.
- 15 min per sample and 50  $\mu$ L injected volume.
- $\lambda=215$  nm; AUFS=0.010 ; mobile phase flow: 0.5 mL/min ; printing speed at 0.5 cm/min.

## Linear standard curve

The linear standard curve was obtained from the following NAA standards:

- Unextracted NAA standards:
  - 1500  $\mu$ L 0.5% PhosA
  - 20  $\mu$ L solution W + 1480  $\mu$ L 0.5% PhosA
  - 40  $\mu$ L solution W + 1460  $\mu$ L 0.5% PhosA
  - 80  $\mu$ L solution W + 1420  $\mu$ L 0.5% PhosA
- Extracted NAA standards:
  - 1000  $\mu$ L phosphate buffer 50 mM
  - 20  $\mu$ L solution W + 980  $\mu$ L phosphate buffer 50 mM
  - 40  $\mu$ L solution W + 960  $\mu$ L phosphate buffer 50 mM
  - 80  $\mu$ L solution W + 920  $\mu$ L phosphate buffer 50 mM

### 3.4.7 Parvalbumin immunostaining

#### 3.4.7.1 Materials

Name	Company	Reference #	Store at
Methanol CH <sub>4</sub> O [CAS #67-56-1]	Fisher Scientific	<a href="#">10675112</a>	RT
Triton x-100 [CAS #9002-91-1]	Sigma-Aldrich	<a href="#">T-8787</a>	RT
PBS CA2+/Mg2+ free (10 X)	Sigma-Aldrich	<a href="#">D1408</a>	RT
Normal horse serum	Vector Laboratories	<a href="#">S-2000</a>	+2-8°C
Mouse monoclonal anti-PV antibody	Swant	<a href="#">PV235</a>	+2-8°C
Biotinylated horse anti-mouse IgG secondary antibody, rat adsorbed	Vector Laboratories	<a href="#">BA-2001</a>	+2-8°C
ABC kit (Vectastain®)	Vector Laboratories	<a href="#">PK-6100</a>	+2-8°C
DAB 3',3'-diaminobenzadine kit	Vector Laboratories	<a href="#">SK-4100</a>	+2-8°C
Ethanol absolute [CAS #64-17-5]	Fisher Chemical		RT
Histo-Clear II	National Diagnostics	<a href="#">HS-202</a>	RT
DPX mounting medium	Fisher Scientific	<a href="#">D/5319/05</a>	RT

#### 3.4.7.2 Equipment

Name
Olympus microscope interfaced to an Image Pro 6.3 (Media cybernetics) via a JVC 3-CCD video camera

#### 3.4.7.3 Preparation of the solutions

- **H<sub>2</sub>O<sub>2</sub> solution – 250 mL:**
  - 1.5 mL H<sub>2</sub>O<sub>2</sub>.
  - 25 mL methanol.
  - 0.2 5mL Triton x-100 solution.
  - 22 mL 0.1M PBS.
  - In 201.25 mL distilled H<sub>2</sub>O.
- **Protein block solution – 120 mL:**
  - 6 mL normal horse serum.
  - 0.48 mL Triton x-100 solution.
  - 11.4 mL 0.1 M PBS.
  - In 102.12 mL distilled H<sub>2</sub>O.

- **Primary antibody – 40 mL:**
  - 8  $\mu$ L mouse monoclonal anti-PV antibody in 40 mL protein block solution.
- **Secondary antibody – 1:200 – 40 mL:**
  - 0.2 mL anti-mouse antibody in 40 mL of protein block solution.
- **ABC solution and DAB solution** were made according to the manufacturer's instructions.

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#### **3.4.7.4 Protocol**

Staining was realised on 30 $\mu$ m free floating sections from perfused and formaldehyde-fixed rat brains.

- Sections were washed in 0.01 M PBS, 5 min.
- Sections were incubated in H<sub>2</sub>O<sub>2</sub> solution, 30 min, RT.
- Sections were washed in 0.01 M PBS, 5 min.
- Sections were incubated in protein block, 60 min, RT.
- Sections were washed in 0.01 M PBS, 5 min.
- Sections were incubated in primary antibody, 16 hours, +4°C.
- Sections were washed in 0.01 M PBS, 5 min, twice.
- Sections were incubated in secondary antibody, 2 hours at RT.
- Sections were washed in 0.01 M PBS, 5 min.
- Sections were incubated in ABC solution, 2 hours, RT.
- Sections were washed in 0.01 M PBS, 5 min.
- Sections were incubated in DAB solution, 15 min, RT.
- Sections were washed distilled H<sub>2</sub>O, 5 min.
- Sections were mounted on microscope slides.
- Slides were dehydrated in 70% ethanol, 5 min, RT.
- Slides were dehydrated in 90% ethanol, 5 min, RT.
- Slides were dehydrated in 100% ethanol, 5 min, RT.
- Slides were incubated in HistoClear, 5 min, RT.
- Sections were mounted with DPX.

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#### **3.4.7.5 Data analysis**

- PV-stained cells were counted in each area of interest, blind to the treatment. and results were expressed as the mean density for 6 sections per animal (number of cells per mm<sup>2</sup>).
- Values were analysed by independent samples Student's t-test between the two groups.

## Chapter 4 INVESTIGATING THE EFFECTS OF AMYLOID-B OLIGOMERS ON COGNITION IN THE RAT

## 4.1 INTRODUCTION

AD, the most common form of dementia, is a neurodegenerative disease characterised in the clinic by a progressive loss of cognitive functions, behavioural changes and depersonalisation (Mayeux and Stern, 2012). A $\beta$ -peptide toxicity is now recognised as a central and early phenomenon of the disease (Walsh and Selkoe, 2007, Crouch et al., 2008, Benilova et al., 2012, Karran et al., 2011). Rodent models built on A $\beta$ -peptide toxicity have been showing promising results in recent years as relevant models for gaining a better understanding of disease process and provide a platform for drug screening (Chambon et al., 2011, Lecanu and Papadopoulos, 2013, McLarnon, 2014) Based on the administration of low-n soluble A $\beta$ <sub>0</sub>, the SynAging lab ([www.synaging.com](http://www.synaging.com)) has developed a mouse model of relevance to AD. After a brief presentation of their findings, I will detail results obtained in a corresponding rat model ICV administrated with the same A $\beta$ <sub>0</sub>. By proving the validity and repeatability of this model in both mice and rats, the aim is to expand the knowledge on A $\beta$ <sub>0</sub> toxicity mechanisms as well as providing a relevant and translatable model for AD drug discovery, based on the early stages of the disease.

Researchers at SynAging first investigated the capacity of A $\beta$  peptides to modify the cell membrane's properties, using synthesised natural and mutated fractions of the 29-40 and 29-42 C-terminal domains as well as the 13-28 central domain (Pillot et al., 1996). The C-terminal domains but not the central domain showed fusogenic properties, which were decreased in elongated peptides (A $\beta$ <sub>22-42</sub> and A $\beta$ <sub>12-42</sub>). The C-terminal structure has hence been suggested to play a critical role in cell toxicity by modifying membrane permeability, with a high importance of its hydrophobic properties and the angle of insertion with the membrane. Although the mechanisms are not fully

understood, A $\beta$  peptides have been shown to induce both necrosis and apoptosis of rat cortical cell cultures (Pillot et al., 1999a). In this study, different fragments of A $\beta$  peptides – particularly A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>12-42</sub>, A $\beta$ <sub>29-40</sub> and A $\beta$ <sub>29-42</sub> – confirmed the importance of the membrane-destabilising properties of the C-terminal domain in inducing cell death mechanisms. The membrane fluidity appears to be a key component of oligomeric A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>29-40</sub> peptide toxicity. Thereby, an increase or deficit in neuronal plasma membrane cholesterol levels induces a protective or weakening impact on apoptosis (Sponne et al., 2004, Florent et al., 2006), suggesting a potential role of diet (Oster and Pillot, 2010, Florent-Bechard et al., 2007, Florent-Bechard et al., 2009). However, these studies showed no effect on cholesterol levels in relation to fibrillary A $\beta$ -peptide toxicity, suggesting a different mechanism of action of low-n A $\beta$ o versus fibrillar forms. Interestingly, the fusogenic properties of soluble A $\beta$ o C-terminal fragments can be inhibited by apolipoprotein E (apoE) isoforms apoE2 and apoE3 while the apoE4 isoform has no effect (Pillot et al., 1997, Lins et al., 1999). The different forms of apoEs are a large subject of study in AD research, with the apoE4 recognised as an important risk factor for AD (Corder et al., 1993, Castellano et al., 2011). It has been demonstrated *in vitro* that apoEs can form a stable complex with A $\beta$ o via their C-terminal lipid binding region (Pillot et al., 1999b, Drouet et al., 2001). By forming a complex with A $\beta$ o, apoE can inhibit their interaction with the cell membrane and reduce the toxicity of A $\beta$ o.

Further investigation of oligomeric A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>29-40</sub> revealed that early mechanisms involved included: (1) perturbation of the plasma membrane fluidity, (2) modification of the calcium homeostasis, (3) induction of oxidative stress, (4) activation of caspases 3 and 9, (5) alteration of the neuronal cytoskeleton (Sponne et al., 2003, Fife et al., 2006, Kriem et al., 2005). These mechanisms appear to involve a phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and a calcium dependant pathway. cPLA<sub>2</sub> is known to play a role in lipid membrane integrity

and neuroinflammation, and shows an increased immunoreactivity in AD brains – see (Gentile et al., 2012) for review. The importance of cPLA<sub>2</sub> has been confirmed by inhibition (Kriem et al., 2005, Malaplate-Armand et al., 2006, Desbene et al., 2012, Youssef et al., 2008) or inactivation (Desbene et al., 2012) of the cPLA<sub>2</sub> gene (cPLA<sub>2</sub><sup>-/-</sup>) in mouse cortical neurons which prevented the apoptotic mechanisms. *In vivo*, inactivation of the cPLA<sub>2</sub> gene in mice protected them against A $\beta$ <sub>1-42</sub> induced memory deficits in both the Y-maze and MWM, and prevented the deficits in the post-synaptic marker PSD-95 (Desbene et al., 2012). Toxicity of A $\beta$ o in wild type mice has been further supported by ICV administration of either A $\beta$ <sub>1-42</sub> or N-truncated A $\beta$ <sub>3(pE)-42</sub> oligomers; which equally reduced the performances of mice in both the Y-maze and MWM when compared to saline or A $\beta$ <sub>42-1</sub> injected groups (Youssef et al., 2008). *In vitro* tests confirmed the toxic effect of those oligomers with a reduction of cell viability from 25% (after 24H of incubations) to 50% (48H incubation) at a concentration of 1 $\mu$ M of soluble oligomers.

The current study focuses on characterising cognitive deficits in a rat following ICV administration of A $\beta$ o<sub>1-42</sub>. The A $\beta$  peptides injected were provided by SynAging (France) as stable oligomeric forms (monomers to tetramers - Figure 21). Studies were conducted to investigate: time-course in relation to appearance and duration of the deficits, a dose-response study, and effect of gender. Cognition was mainly assessed by testing short term visual memory, as an early clinical symptom of AD in humans (Snowden et al., 2011, Didic et al., 2013, Didic et al., 2010, Alescio-Lautier et al., 2007). In rodents, it can be assessed with the NOR task (Ennaceur and Delacour, 1988), which is equivalent to short-term memory impairment (Bertaina-Anglade et al., 2006, Bevins and Besheer, 2006), and particularly used in AD rodent models – see (Grayson et al., 2015) for review. Moreover, the NOR is a simple ethological task based on spontaneous behaviour, limiting biases from stress and other manipulations

used in certain cognitive tasks (e.g. food restriction) and has shown similar results to the MWM in mice (Zhang et al., 2012).

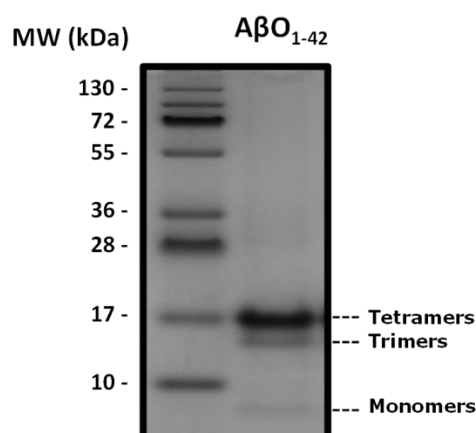


Figure 21: Preparation of  $A\beta$  provided by SynAging. The  $A\beta$  injected are exclusively  $A\beta_{1-42}$  and aggregated into oligomers, mainly tetramers as well as monomers and trimmers, after 24 hours of incubation has shown on the gel.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Animals

Unless otherwise specified, subjects were adult female Lister Hooded rats (Charles River, UK) aged 3-month old, and weighing 190-230 g at the time of surgery. Rats were housed in groups of 5, on a 12-hour light-dark cycle, with free access to food and water, in a controlled environment of temperature ( $21\pm 2^{\circ}\text{C}$ ) and humidity ( $55\pm 5\%$ ) in the Biological Services Facility at the University of Manchester. Experiments were conducted during the light cycle, in the morning. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) 1986 Act and University ethical guidelines. Surgery for administration of  $A\beta_{1-42}$  and behavioural testing (NOR and Y-maze) were performed as describe previously (Chapter 3 Materials and methods. 3.2.1, 3.3.1 and 3.3.3).



### 4.2.2 Data analysis

Results are expressed as mean  $\pm$  SEM. NOR data were analysed by 1-way ANOVA on repeated measures with object as within-subject factor and group as in-between subject factor. 2-way ANOVA was run when comparing more than one variable (group + day). In cases of significance ( $p < 0.05$ ), individual paired samples Student's t-tests were run in each group, comparing the exploration time of both objects. Total exploration time and DI were analysed by 1-way ANOVA and Bonferroni post-hoc. DI were also analysed by univariate Student's t-test versus 0. Y-maze data were analysed by 1-way ANOVA and Bonferroni post-hoc. All the statistical analyses have been calculated using IBM SPSS (version 20).

### 4.2.3 Experiments design

#### **Experiment 1: Time-course study** (Figure 22– Top)

The purpose of the first experiment was to confirm the appearance and duration of a deficit in the NOR task. NOR was assessed from 4 days up to 10 weeks following administration of A $\beta_{0-42}$ . Animals received 10  $\mu$ L of either vehicle ( $n=10$ ) or A $\beta_{0-42}$  5 nmol ( $n=10$ ). The NOR was then performed on days 4, 14, 35 and 70.

#### **Experiment 2: Dose-response study** (Figure 22 – Centre)

The second experiment looked at the effect of different doses of A $\beta_{0-42}$ . Rats received 10  $\mu$ L of either vehicle ( $n=20$ ) or A $\beta_{0-42}$  at three different doses ( $n=10$  per dose): 5 nmol, 1 nmol and 0.5 nmol. The NOR was then performed on day 35.

#### **Experiment 3: Sex-difference study** (Figure 22 – Bottom)

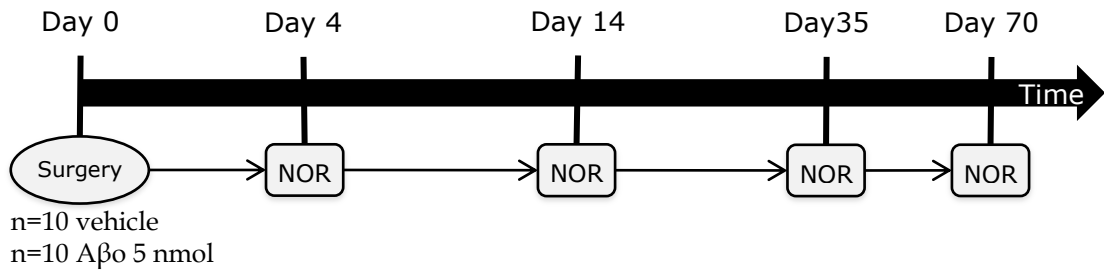
The third experiment assessed potential sex-related differences in the NOR impairment, by comparing female and male Lister Hooded rats. Animals received

10  $\mu$ L of either vehicle (n=10 females, n=10 males) or A $\beta$ <sub>1-42</sub> 5 nmol (n=10 females, n=10 males). The NOR was then performed on day 4.

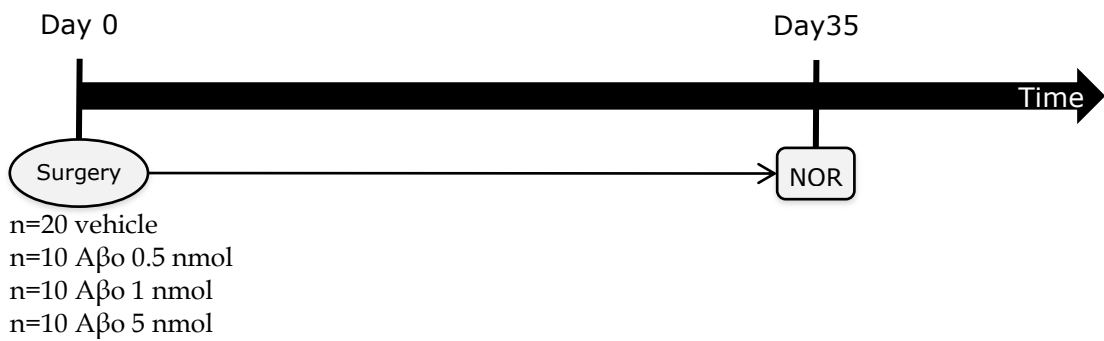
**Experiment 4: Complementary behavioural data** (Figure 22 – Bottom)

The fourth experiment investigated performances in the Y-maze (working memory), NOR with no ITI as well as bodyweight as a general health indicator. These experiments were carried on rats from Experiment 3. Bodyweight of female rats was recorded on the day of surgery and on days 4, 14 and 30; NOR with no ITI was performed on males on day 14; the Y-maze was performed in females on day 35. In this test, an extra age-matched 10 female rats, receiving an IP dose of scopolamine (0.7 mg/kg, 30 min pre-treatment) was included. Scopolamine is known to decrease acetylcholine transmission and is widely used as a control in spontaneous alternation tests (Lalonde, 2002, Klinkenberg and Blokland, 2010).

**Experiment 1**



**Experiment 2**



**Experiments 3 and 4**

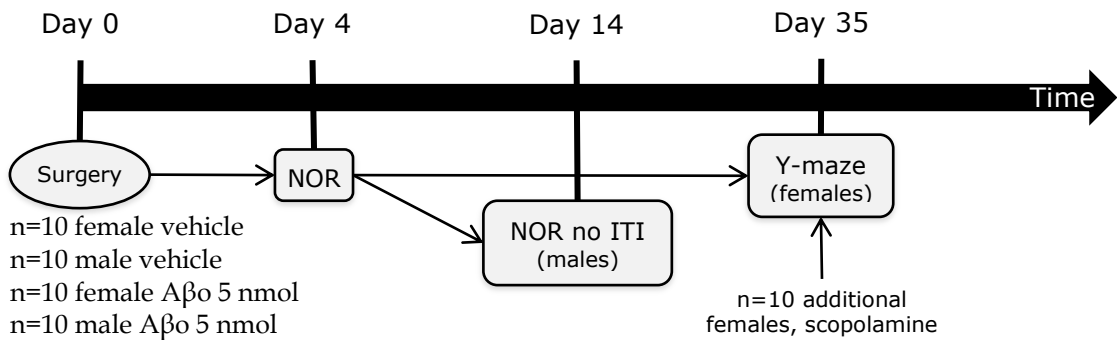


Figure 22: Outline of the behavioural experiments. Top: Time-course study. Centre: Dose-response study. Bottom: Sex-difference study, Y-maze study and NOR no ITI study.

## 4.3 RESULTS

### 4.3.1 Experiment 1: Time-course study

#### **Acquisition Phase:**

During the acquisition phase, both Vehicle and A $\beta$ o groups explored both objects equally, on each day of test. (Figure 23A-D). There was no difference in left/right object exploration [ $F_{(1,18)}=2.326$   $p>0.05$ ] regardless of group [ $F_{(1,18)}=0.806$   $p>0.05$ ] or the day of test [ $F_{(3,16)}=0.496$   $p>0.05$ ] with no overall interaction left/right\*group\*day [ $F_{(3,18)}=0.856$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(1,18)}=1.425$   $p>0.05$ ] but was however affected by the day of testing [ $F_{(3,16)}=47.998$   $p<0.001$ ]. It was significantly decreased from day 4 to day 14 [ $p<0.001$ ] but not on consecutive sessions [ $p>0.05$ ].

#### **Retention Phase:**

During the retention phase (Figure 23A-D), there was a significant difference of exploration between the familiar and novel object [ $F_{(1,16)}=89.046$   $p<0.001$ ] with an effect of group [ $F_{(1,16)}=50.249$   $p<0.001$ ]. The control (vehicle) group spent more time exploring the novel object on day 4 [ $t_{(9)}=-6.244$   $p<0.001$ ], day 14 [ $t_{(9)}=-4.923$   $p<0.001$ ], day 35 [ $t_{(9)}=-3.180$   $p<0.05$ ], and day 70 [ $t_{(9)}=-2.372$   $p<0.05$ ]. There was no difference in exploration for the A $\beta$ o group on any day: day 4 [ $t_{(8)}=-0.715$   $p>0.05$ ], day 14 [ $t_{(8)}=-0.890$   $p>0.05$ ], day 35 [ $t_{(9)}=-0.251$   $p>0.05$ ], and day 70 [ $t_{(9)}=-1.419$   $p>0.05$ ]. As in the acquisition phase, there was a significant decrease of the total exploration time as an effect of the day of testing [ $F_{(3,14)}=11.216$   $p<0.001$ ] but not the group [ $F_{(1,16)}=2.528$   $p>0.05$ ] with no group\*day interaction [ $F_{(3,14)}=0.278$   $p>0.05$ ]. Total exploration time was decreased from day 14 to day 35 [ $p<0.05$ ].

***Discrimination index:***

In the control group a DI significantly superior to zero (equivalent to equal exploration of the objects) was found on day 4 [ $t_{(9)}=9.562$   $p<0.001$ ], day 14 [ $t_{(9)}=5.175$   $p<0.001$ ], day 35 [ $t_{(9)}=3.320$   $p<0.01$ ], and day 70 [ $t_{(9)}=2.968$   $p<0.05$ ]. In contrast the DI in the A $\beta$ o group was not significantly different to zero on any day: day 4 [ $t_{(8)}=1.157$   $p>0.05$ ], day 14 [ $t_{(8)}=1.325$   $p>0.05$ ], day 35 [ $t_{(9)}=0.409$   $p>0.05$ ], and day 70 [ $t_{(8)}=1.646$   $p>0.05$ ] (Figure 23E). There was a significant difference in DI between groups [ $F_{(1,17)}=36.361$   $p<0.001$ ] independent of the day of testing [ $F_{(3,17)}=0.847$   $p>0.05$ ]. When analysing the results in relation to test day, DI of the A $\beta$ o group was significantly lower than control on day 4 [ $t_{(17)}=4.969$   $p<0.001$ ] and day 14 [ $t_{(18)}=4.087$   $p<0.001$ ] but not on day 35 [ $t_{(18)}=1.834$   $p>0.05$ ] or day 70 [ $t_{(18)}=1.155$   $p>0.05$ ].

In summary, independent of the day of testing, the Vehicle group significantly spent more time exploring the novel over the familiar object, an effect that was abolished in the group receiving ICV administration of A $\beta$ o<sub>1-42</sub>.

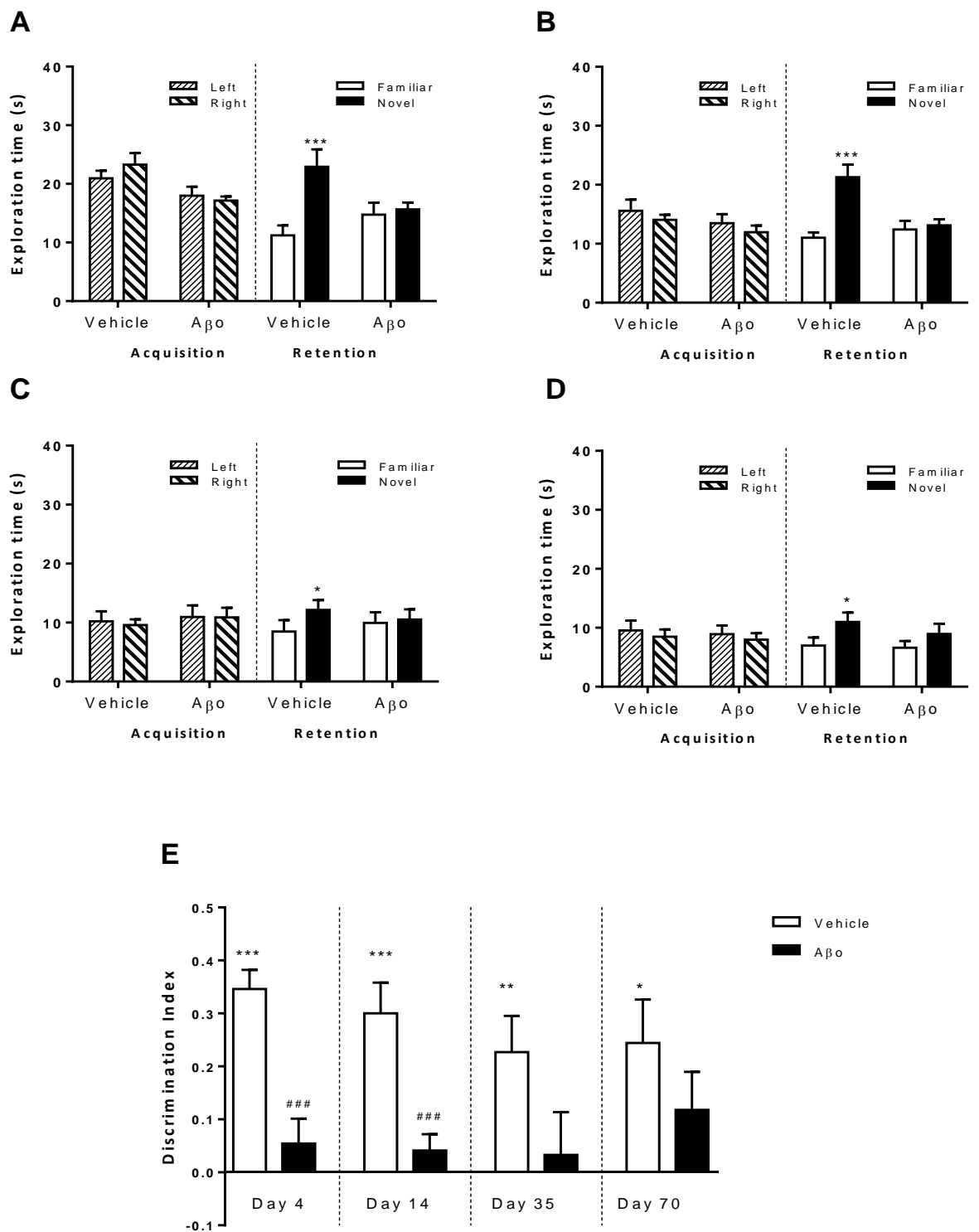


Figure 23: Experiment 1 – A $\beta$  administration induces a deficit in the NOR task from day 4 to day 70 after surgery. Exploration times on day 4 (A), 14 (B), 35 (C) and 70 (D) after ICV administration of Vehicle or A $\beta$ . DIs on each day of test (E). Data are presented as mean + SEM, (n=9-10 per group). (A), (B), (C), and (D): Paired two-way ANOVA and Student's t-test, \*p<0.05 \*\*\*p<0.001 Novel vs. Familiar. (E): Univariate Student's t-test \*p<0.05 \*\*p<0.01 \*\*\*p<0.001 vs. zero; two-way ANOVA and Bonferroni post-hoc, ###p<0.001 A $\beta$  vs. Vehicle.

### 4.3.2 Experiment 2: Dose-response study

#### **Acquisition Phase:**

During the acquisition phase, all groups explored both objects equally (Figure 24A). There was no difference in left/right object exploration [ $F_{(1,46)}=1.035$   $p>0.05$ ] regardless of treatment or dose administered [ $F_{(3,46)}=1.075$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(3,46)}=1.685$   $p>0.05$ ].

#### **Retention Phase:**

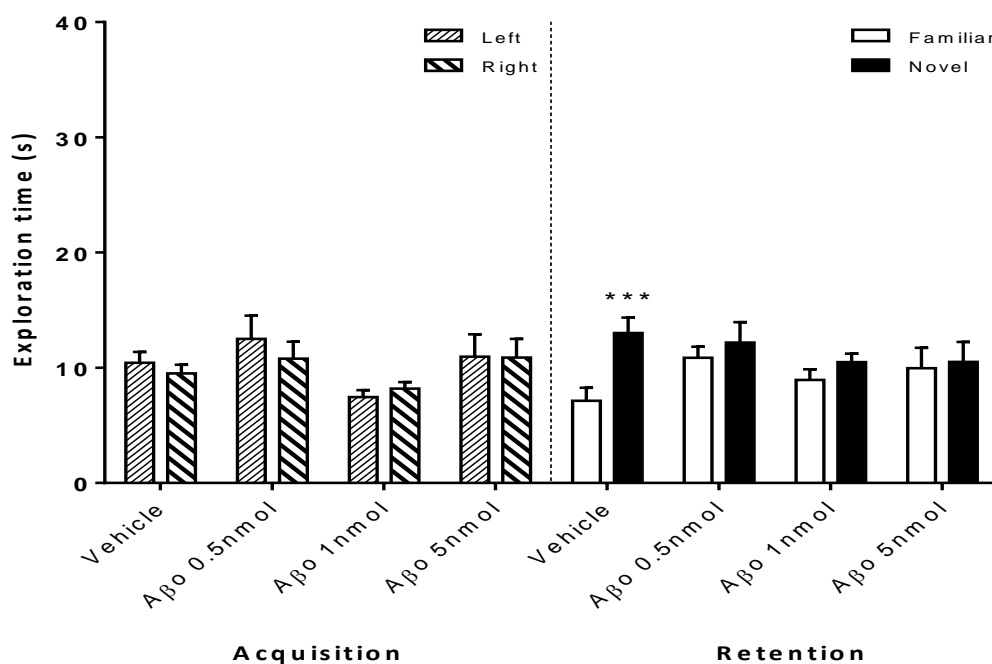
During the retention phase (Figure 24A), there was a significant difference of exploration between the familiar and novel object [ $F_{(1,46)}=7.449$   $p<0.01$ ] with an effect of dose administered [ $F_{(1,46)}=2.780$   $p=0.05$ ]. The control group spent significantly more time exploring the novel object [ $t_{(19)}=-4.627$   $p<0.001$ ]. There was no difference of exploration in any of the A $\beta$  groups: 5nmol [ $t_{(9)}=-0.251$   $p>0.05$ ], 1nmol [ $t_{(9)}=-1.251$   $p>0.05$ ], 0.5nmol [ $t_{(9)}=-0.684$   $p>0.05$ ]. As in the acquisition phase, total object exploration was not different between groups [ $F_{(3,46)}=0.418$   $p>0.05$ ].

#### **Discrimination index:**

In the control group a DI significantly superior to zero (equivalent to equal exploration of the objects) was found [ $t_{(19)}=5.416$   $p<0.001$ ]. In contrast there were no significant difference in any of the A $\beta$  treated groups; 5nmol [ $t_{(9)}=0.409$   $p>0.05$ ], 1nmol [ $t_{(9)}=1.350$   $p>0.05$ ], 0.5nmol [ $t_{(9)}=0.390$   $p>0.05$ ] (Figure 24B). There was a significant difference of DI between groups [ $F_{(3,46)}=4.701$   $p<0.01$ ]. A planned contrast test showed a significant difference in the DI of control and A $\beta$  groups [ $t_{(46)}=3.712$   $p<0.001$ ].

In summary, independent of the dose of A $\beta_{1-42}$  administered, the Vehicle group significantly spent more time exploring the novel over the familiar object, an effect that was abolished in the groups receiving A $\beta_{1-42}$ .

**A.**



**B.**

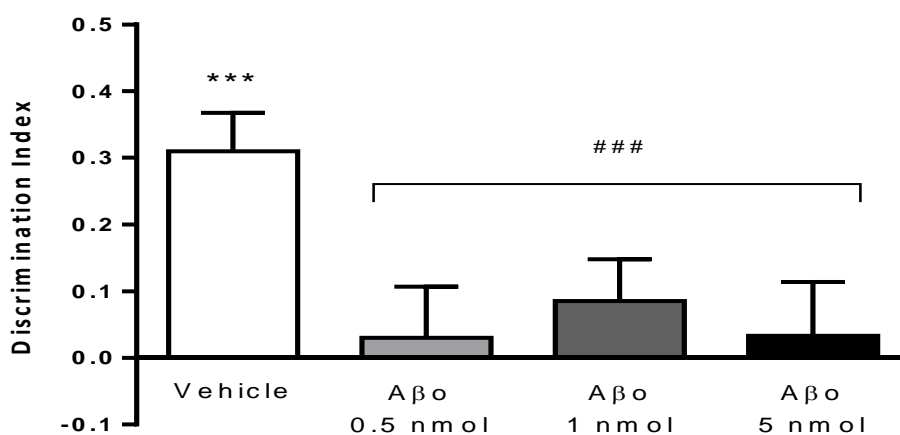


Figure 24: Experiment 2 – A $\beta$ o administration induces a deficit in the NOR task at all doses tested. Exploration times (A) and DIs (B) 35 days after ICV administration of Vehicle or A $\beta$ o. Data are presented as mean + SEM, (n=20 Vehicle, 10 in each A $\beta$ o group). (A): Paired one-way ANOVA and Student's t-test, \*\*\*p<0.001 Novel vs. Familiar. (B): Univariate Student's t-test \*\*\*p<0.001 vs. zero; one-way ANOVA and planned contrast, ###p<0.001 vs. Vehicle.



### 4.3.3 Experiment 3: Sex-difference study

#### **Acquisition Phase:**

During the acquisition phase, all groups explored both objects equally (Figure 25A). There was no difference in left/right object exploration [ $F_{(1,36)}=0.005$   $p>0.05$ ] regardless of group [ $F_{(1,36)}=1.959$   $p>0.05$ ] or sex [ $F_{(1,36)}=0.836$   $p>0.05$ ] with no overall interaction left/right\*group\*sex [ $F_{(1,36)}=0.467$   $p>0.05$ ]. Total object exploration was not different between Vehicle and A $\beta$  groups for each sex [ $F_{(1,36)}=0.663$   $p>0.05$ ]. However, female rats spent more time overall exploring the objects [ $F_{(1,36)}=21.342$   $p<0.001$ ].

#### **Retention Phase:**

During the retention phase (Figure 25A), there was a significant difference of exploration between the familiar and novel object [ $F_{(1,35)}=40.563$   $p<0.001$ ] with a significant effect of group [ $F_{(1,35)}=34.649$   $p<0.001$ ]. Both Vehicle groups spent significantly more time exploring the novel object, female [ $t_{(9)}=-6.244$   $p<0.001$ ] and male [ $t_{(9)}=-4.927$   $p<0.001$ ]. There was no difference of exploration in both A $\beta$  treated groups, female [ $t_{(9)}=-0.715$   $p>0.05$ ] and male [ $t_{(9)}=0.162$   $p>0.05$ ]. Total object exploration was not different between animals, regardless of group [ $F_{(1,35)}=0.051$   $p>0.05$ ] or sex [ $F_{(1,35)}=1.042$   $p>0.05$ ].

#### **Discrimination index:**

In the control group a DI significantly superior to zero (equivalent to equal exploration of the objects) was found in both sexes: female [ $t_{(9)}=9.562$   $p<0.001$ ], male [ $t_{(9)}=5.249$   $p<0.001$ ]. In contrast there was no significant difference in A $\beta$  treated groups: female [ $t_{(8)}=1.157$   $p>0.05$ ], male [ $t_{(9)}=0.158$   $p>0.05$ ] (Figure 25B). There was a significant difference in DI with an effect of group [ $F_{(1,35)}=35.935$   $p<0.001$ ], but not sex [ $F_{(1,35)}=3.626$   $p>0.05$ ]. Both A $\beta$  groups showed a lower DI than their respective Vehicle groups: female [ $t_{(17)}=4.969$   $p<0.001$ ] and male [ $t_{(18)}=3.576$   $p<0.01$ ].

In summary, independent of the sex of the rats, Vehicle groups spent significantly more time exploring the novel over the familiar object, an effect that was abolished in the groups receiving ICV administration of A $\beta$ <sub>1-42</sub>. As such both female and male rats were similarly affected by administration of A $\beta$ <sub>1-42</sub>.

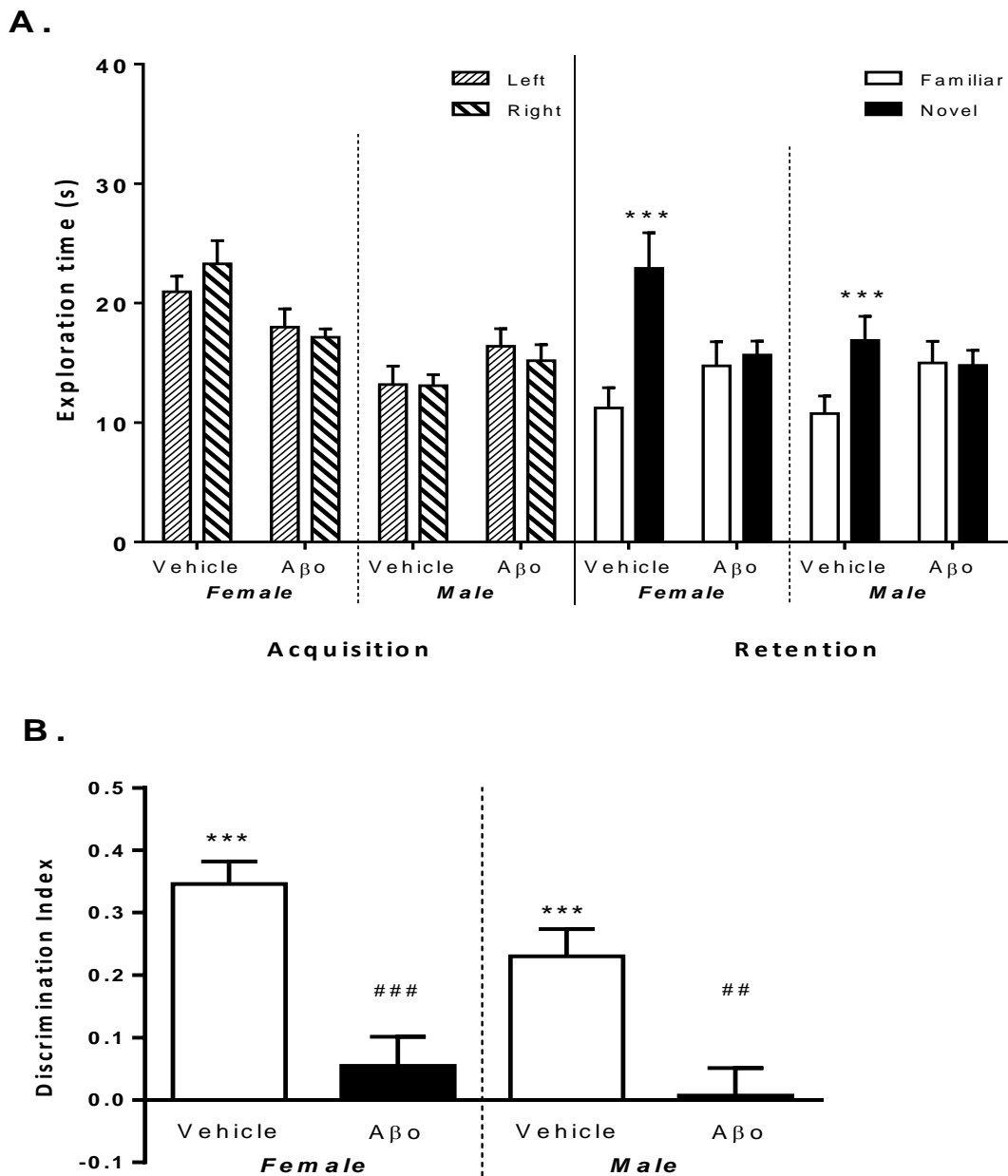


Figure 25: Experiment 3 – A $\beta$  administration induces a deficit in the NOR task in both female and male rats. Data are shown as Mean + SEM, n=9-10 per group. (A) Exploration time: Paired two-way ANOVA and Student t-test, \*\*\*p<0.001 Novel vs. Familiar. (B) DI: Two-way ANOVA and post-hoc Fischer PLSD test, \*\*\*p<0.001 vs. zero; ##p<0.01 ###p<0.001 vs. Vehicle

### 4.3.4 Experiment 4: Complementary behavioural data

#### **Y-maze**

There was an overall difference in the %Alternation index between groups [ $F_{(2,26)}=5.258$   $p<0.05$ ]. %Alternation was decreased in the Scopolamine group when compared to Vehicle and A $\beta$  groups, both [ $p<0.05$ ]. However, both the Vehicle and A $\beta$  groups performed equally [ $p>0.05$ ] (Figure 26A).

#### **NOR with no Inter-trial Interval (ITI)**

In this version of the NOR task, the objects are swapped while the rat is left undisturbed in the test arena, between the Acquisition and the Retention phase.

#### ***Acquisition Phase:***

During the acquisition phase (Figure 26C), all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,18)}=1.218$   $p>0.05$ ] regardless of group [ $F_{(1,18)}=0.035$   $p>0.05$ ]. Total object exploration was not different between Vehicle and A $\beta$  groups [ $F_{(1,18)}=0.062$   $p>0.05$ ].

#### ***Retention Phase:***

During the retention phase (Figure 26C), there was a significant difference of exploration between the familiar and novel object [ $F_{(1,18)}=43.554$   $p<0.001$ ] but no effect of group [ $F_{(1,18)}=0.315$   $p>0.05$ ]. Both groups spent more time exploring the novel object: Vehicle group [ $t_{(9)}=-4.525$   $p<0.001$ ] and A $\beta$  group [ $t_{(9)}=23.104$   $p<0.001$ ]. Total object exploration was not different between groups [ $F_{(1,18)}=0.416$   $p>0.05$ ].

### ***Discrimination index:***

A DI significantly superior to zero (equivalent to equal exploration of the objects) was found in both groups: Vehicle group [ $t_{(9)}=4.457$   $p<0.01$ ] and A $\beta$ o group [ $t_{(9)}=5.565$   $p<0.001$ ]. There was no difference of DI between the two groups [ $t_{(18)}=-0.720$   $p>0.05$ ] (Figure 26D). In summary, in the NOR task with no ITI, both groups spent significantly more time exploring the novel over the familiar object.

### **Bodyweight**

A regular increase of bodyweight was observed in both groups (Figure 26B), with a significant effect of day [ $F_{(3,54)}=87.817$   $p<0.001$ ] but not of group [ $F_{(1,54)}=0.527$   $p>0.05$ ], and no interaction [ $F_{(3,54)}=0.286$   $p>0.05$ ]. There was no significant increase from day 0 to day 4 [ $p>0.05$ ], but a significant increase from day 4 to day 14 [ $p<0.001$ ], and from day 14 to day 30 [ $p<0.001$ ] was observed in both groups.

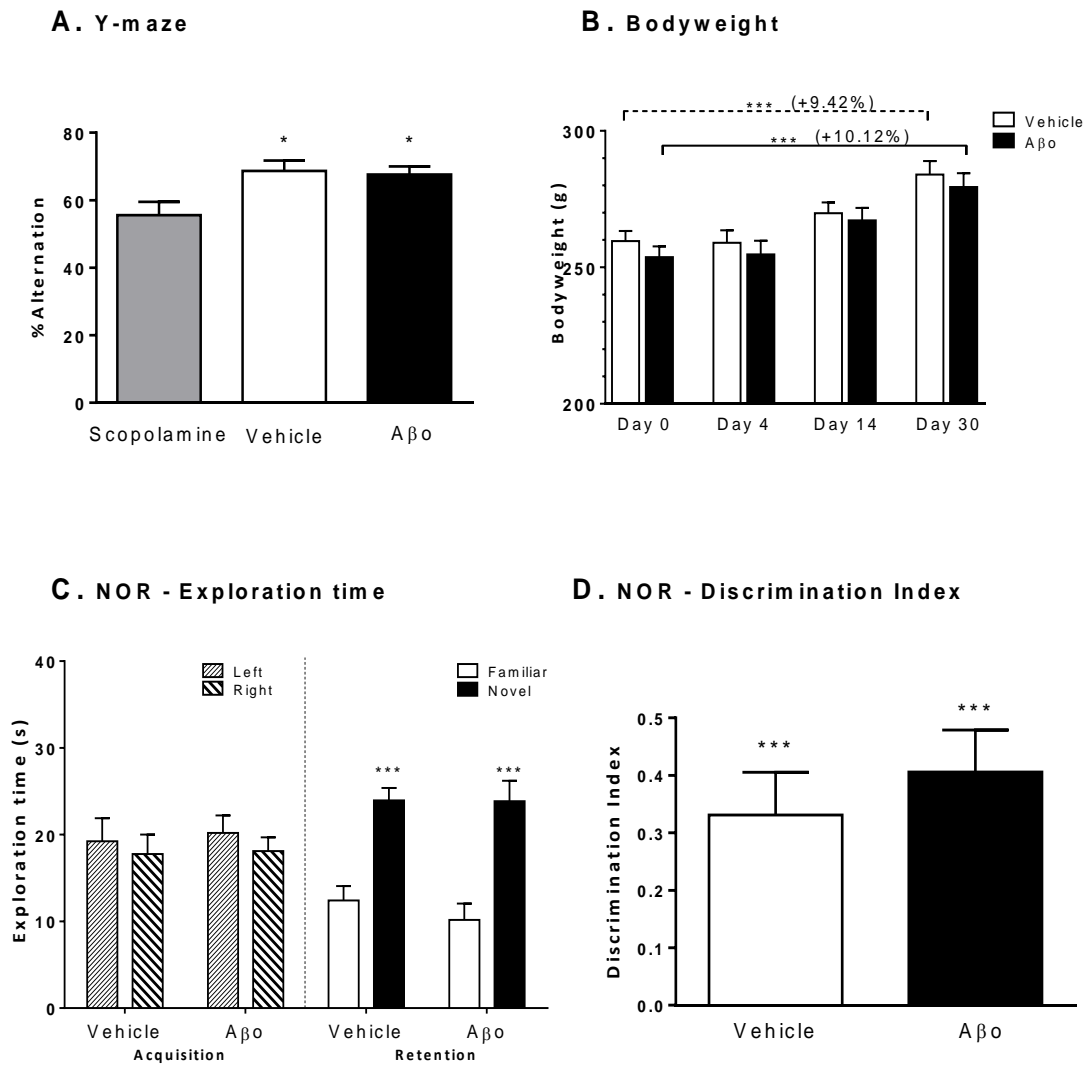


Figure 26: Experiment 4 – Complementary behavioural data. (A) Y-maze, day 35. (B) Bodyweight days 0 to 30. (C - D) NOR no ITI, day 4. Data are presented as Mean + SEM (n=10 per group). (A): One-way ANOVA and Bonferroni post-hoc, \* $p < 0.05$  vs. Scopolamine group. (B): Paired samples Student's  $t$ -tests, \*\*\* $p < 0.001$  from Day 0 to Day 30. (C) Exploration time: Paired samples Student's  $t$ -test, \*\*\* $p < 0.001$  Novel vs. Familiar. (D) DI: Independent samples Student's  $t$ -test, \*\*\* $p < 0.001$  vs. zero.

## 4.4 DISCUSSION

The current study demonstrated the appearance of a robust and lasting cognitive deficit as early as day 4 and up to day 70 following an acute ICV administration of 5 nmol of A $\beta$ <sub>01-42</sub>, in both female and male Lister Hooded rats, with no overall noticeable effect on the general health status.

In the time-course study, during the acquisition phase both treatment groups showed no difference in their explorative behaviour of the familiar objects (Figure 23A-D). In contrast, during the retention phase only the Vehicle group could perform the task, showing a clear preference for the novel object over the familiar one (Figure 23). It is however noteworthy to observe that in both groups there was an overall decrease in total exploration time in both the acquisition and the retention phases on later test days. The same apparatus has been used on each day of testing and, in line with previous reports, the novelty of the task appears to decrease over time for the rats; lowering their willingness to explore the environment and the objects (Akkerman et al., 2012). This habituation to the apparatus and decrease in total exploration time has been observed in both groups with no significant difference of total exploration time between the Vehicle and the A $\beta$  groups, hence not biasing the results on object exploration. However, it induces a higher variability between each rat's object exploration times, lowering the significance of the statistical tests. This could account for the lack of significance between the DIs of the Vehicle and the A $\beta$  groups on days 35 and day 70 (Figure 23). Nonetheless, only the Vehicle group's DI remains significantly different from chance and has a significant difference of exploration time between familiar and novel object, while the A $\beta$  group cannot perform the task. The NOR task is a qualitative but not quantitative task; the apparent decrease in significant difference between the two groups hence does not underline a potential recovery of the A $\beta$  group from the A $\beta$ -induced deficit.

In the dose-response study, the dose-range tested varied from 0.5 nmol of  $A\beta_{0_{1-42}}$ , as administrated in the mouse model from SynAging (Desbene et al., 2012, Youssef et al., 2008) to a 10-fold higher quantity (5 nmol). All the groups administrated with  $A\beta_0$  showed a deficit in the NOR task on day 35, with no measureable influence of the dose on the observed cognitive deficit (Figure 24). Current hypothesis suggests  $A\beta_0$  as a potential trigger rather than executioner in the early stages of the disease – see (Karran et al., 2011) for review. It is thus possible that even the lowest dose injected in the current study was higher than the required threshold needed to induce short-term recognition memory deficit.

The third study demonstrated a deficit in the NOR task in both female and male Lister Hooded rats following ICV administration of  $A\beta_{0_{1-42}}$ .

These results supplement and confirm behavioural data from SynAging mouse model using the same preparation of  $A\beta_0$  (Youssef et al., 2008, Garcia et al., 2010, Desbene et al., 2012, Bouter et al., 2013). The SynAging lab has shown that an ICV injection of  $A\beta_0$  in wild type C57BL/6 mouse induced an impairment in the NOR, a significant decrease of %alternation in the Y-maze, 4 days after surgery, and impaired performances in the MWM in both the learning and probe trials, 7 days after surgery.

These data support the hypothesis of short term recognition memory being one of the early cognitive domains affected in the current model. Results from the Y-maze and the NOR with no ITI might suggest a susceptibility of the cognitive impairment to distraction factors (Grayson et al., 2014). Both these tasks were equally performed by the Vehicle and  $A\beta_{0_{1-42}}$  groups. Unlike the traditional NOR task, where the animal is taken away from the test arena during the ITI, the Y-maze and NOR with no ITI involve no or little distraction for the animal. Similar results have been shown following  $A\beta_{1-40}$ -peptide administration

(1 nmol/ $\mu$ L), where these rats could perform the 8-ARM task up to the same criteria as vehicle treated animals. Distraction (i.e. 30 s delay imposed between the 4<sup>th</sup> and 5<sup>th</sup> arm entry) did not modify the control group's performances, however it impaired the ability of the A $\beta$ <sub>1-40</sub> treated animals to perform the task (McDonald et al., 1994, Sweeney et al., 1997). Alongside an involvement of distraction, the current data might suggest that the rats can encode the information (they can perform the NOR task with no ITI) but not retrieve it as they lose their ability to recognise the novel object after an ITI of 2 min.

The rat Y-maze results are in contrast to the deficit observed following A $\beta$ <sub>1-42</sub> administration in the mouse model at SynAging (Bouter et al., 2013), suggesting that A $\beta$  might affect differently mice and rats or underlying a dose-effect of the A $\beta$  toxicity. The NOR task is known to involve the perirhinal and prefrontal cortex (Norman and Eacott, 2004, Barker et al., 2007) with a limited role for the hippocampus in relation to object recognition (Dere et al., 2007, Barker and Warburton, 2011). The Y-maze task (spatial memory), on the other hand, is more heavily dependent on hippocampal function (Morris et al., 1982, Hughes, 2004, Hartley et al., 2014). It can thus be hypothesised that the current model shows recognition memory impairment but not altered hippocampal functions in the early stages of the pathology. It raises the question of the propagation of the injected A $\beta$ <sub>1-42</sub> in the current model. So far, attempts in tagging the A $\beta$  in order to follow their propagation in the brain have been unsuccessful. Research is made to study the spread of A $\beta$ <sub>1-42</sub> in order to better understand the mechanisms and brain area involved.



## 4.5 CONCLUSION

Following the establishment and behavioural characterisation of the model, the next step was to investigate relevant biomarkers that would lead to a better understanding of the underlying mechanisms as well as providing a platform for drug testing.

Early stages of AD are thought to involve neuroinflammation (Heneka et al., 2015, Heppner et al., 2015, Smith et al., 2000, Nunomura et al., 2001), and significant increase in the level of free radicals in the hippocampus and olfactory bulbs have been measured in SynAging mouse model, 2 days after ICV administration of A $\beta$ <sub>01-42</sub> (Youssef et al., 2008). In the current model, the first objective was hence to identify neuroinflammatory changes following ICV administration of A $\beta$ <sub>01-42</sub> in the rat. Alongside neuroinflammation, synaptic failure appears to be a concomitant phenomenon (Selkoe, 2002, Small et al., 2001, Lacor et al., 2004, Shankar et al., 2008, Townsend et al., 2006). Particularly in the frontal cortex and hippocampus where a significant decrease of the presynaptic vesicle protein synaptophysin has been shown to correlate with the progression of the disease (Sze et al., 1997, Dickson et al., 1995, Masliah et al., 2001). Deficits in synaptic proteins have thus been suggested as an early phenomenon in the illness and a marker of cognitive decline in AD. Colleagues at SynAging have demonstrated a significant decrease of the post-synaptic marker PSD-95 in the hippocampus of their C57/BL6 mouse model following ICV injection of A $\beta$ o (Garcia et al., 2010, Desbene et al., 2012).

I thus aimed to develop a well characterised model featuring early memory impairment, increased neuroinflammatory markers and synaptic deficit. Validating these three hallmarks of early A $\beta$ <sub>01-42</sub> induced pathology would provide a relevant rat model for AD research.

**Chapter 5 INVESTIGATING THE EFFECTS OF  
AMYLOID-B OLIGOMERS ON  
NEUROINFLAMMATORY, SYNAPTIC AND  
NEURONAL PATHWAYS IN THE RAT**

## 5.1 INTRODUCTION

This chapter focuses on better understanding the mechanisms underlying the lasting NOR deficit observed in the previous chapter. Investigation of the pathways has been made following three main directions.

First, I attempted to better define the cognitive deficit by establishing the What-where-which (WWhich) task, another spontaneous recognition task which is more complex than the NOR. Both tasks are qualitative rather than quantitative. However, the added difficulty of the WWhich can allow discrimination between rats which can successfully perform the NOR. Indeed, it has been shown previously that some strains of transgenic mice could perform the NOR task at a similar level to controls but failed in the WWhich task (Davis et al., 2013b). The WWhich, however, turned out to be too complex for the control Lister Hooded rats and its validity as a reliable cognitive task will be discussed.

I then focused on biochemical analysis of three types of markers: neuroinflammatory markers, synaptic markers, and neuronal markers. As discussed in the general introduction, neuroinflammation is believed to contribute to the early stages of AD, where A $\beta$  induce synaptic deficit which over time results in neuronal death and the overt clinical symptoms that characterise AD. These experiments were conducted to gain a better insight into the role of neuroinflammation in this model and the neuropathological changes following A $\beta$  administration.

The last set of experiments investigated the pharmacological reversal of the cognitive deficit in relation to acute administration of the potential cognitive enhancer rolipram.

## 5.2 WHAT-WHERE-WHICH TASK

### 5.2.1 Rationale

Episodic memory as first described in 1972, refers to the capacity of human beings to mentally “time travel” by recalling a personal past event or representing themselves in the future, and relies on the self – as opposed to semantic memory of general facts and knowledge (Tulving, 1972). In AD patients, episodic memory is affected during the early clinical phases of the disease progression and before semantic memory (Backman et al., 2001, Murphy et al., 2008, Leyhe et al., 2009). The spatial component of episodic memory has been shown to be particularly impaired in MCI and moderate AD stages (Plancher et al., 2012). This deficit is associated with medial-temporal alterations and localised hippocampal atrophy (Thomann et al., 2012, Westerberg et al., 2013), consistent with episodic memory relying on the hippocampus (Copara et al., 2014, Sidhu et al., 2013, Smith and Mizumori, 2006, Staresina and Davachi, 2009).

In preclinical behavioural experiments, the presence of episodic memory was first tested in scrub jays, by assessing how they memorise the location (where) of different types of food (what) and its decay state (when) (Clayton and Dickinson, 1998). Translatability of this episodic-like memory (what-where-when) to human episodic memory is still the subject of debate but has since been successfully adapted to rodent models – see (Crystal, 2009, Dere et al., 2006, Easton and Eacott, 2013) for review. To avoid possible bias from training and food reward, current tasks rely on rodent preference for novelty. Based on the NOR task (Ennaceur and Delacour, 1988), rodents have to explore between objects which vary in type (what), location (where) and recency (when) (Figure 27). Studies in Wistar rats (Kart-Teke et al., 2007, Kart-Teke et al., 2006) showed an identification of the “what” and “where” components but results on

the “when” criteria where sometimes contradictory. Limitations on correct investigation and interpretation of the temporal component (when) of rat memory in NOR-based tasks have been raised, as discussed by (Ennaceur, 2010).

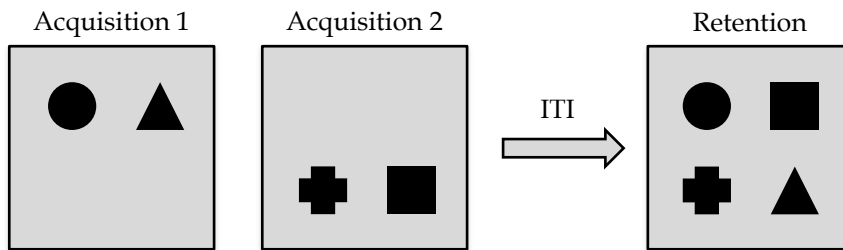


Figure 27: Example of What-where-when task. The objects in the Retention phase, by order of expected increasing novelty are as follow: cross (recent stationary), square (recent displaced) and round (old stationary), triangle (old displaced).

Alternatively, the use of the WWWhich task has been suggested to avoid misinterpretations on the “when” component (Eacott and Norman, 2004, Easton and Eacott, 2008, Easton et al., 2012). It relies on the context of appearance of the object (“which”) rather than its recency (“when”). The experiment is divided between two acquisition phases and a retention phase where the rat is presented with two configurations of object, place and context (Figure 28). For example:

- Acquisition 1: the rat is placed in a round box ● and presented with object A in location ① and object B in location ②. The two triads are (A;①;●) and (B;②;●)
- Acquisition 2: the rat is placed in a square box □ and presented with the same objects in reversed locations. The two new triads are (A;②;□) and (B;①;□)
- Retention: the rat is placed in the square box □ with two copies of the object A in both locations. There is thus a familiar triad from Acquisition 2 (A;②;□) and a new triad (A;①;□) never encountered before.

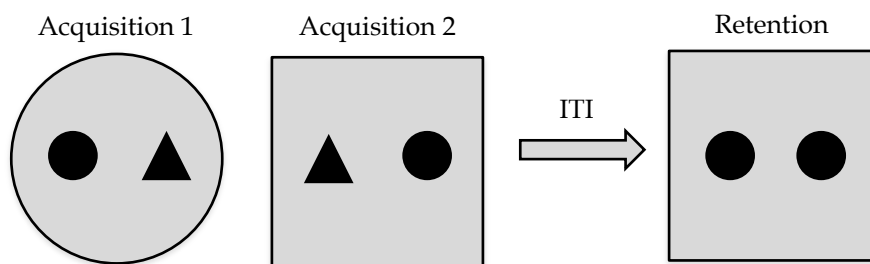


Figure 28: Example of What-where-which task. The left object in the Retention phase is the novel object. It has never been presented before in this context and this location simultaneously.

The WWWhich task has been successfully performed by control (Eacott and Norman, 2004), sham surgery, and cholinergic lesioned male Dark Agouti adult rats (Easton et al., 2011). Further studies in both rats and mice showed that their performances could be impaired by hippocampal lesion in adult male Lister Hooded rats (Langston and Wood, 2010), ageing processes in 129sv/c57bl6 mice (Davis et al., 2013a), and in the 3xTgAD transgenic model of AD (Davis et al., 2013a, Davis et al., 2013b), see Table 2. The main interest of this test in the current project, is to further classify rats that can perform the NOR test by challenging them with a more complex task relying on higher cognitive functions.

## 5.2.2 Materials and methods

Subjects were 30 female and 10 male Lister Hooded rats (Charles River, UK), weighing 210-293 g at the beginning of the experiments. Rats were housed in groups of 5 (females) or 2 to 4 (males), on a 12-hour light-dark cycle, with free access to food and water, in a controlled environment of temperature ( $21\pm 2^\circ\text{C}$ ) and humidity ( $55\pm 5\%$ ) in the Biological Services Facility at the University of Manchester. Experiments were conducted during the light cycle, in the morning. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) 1986 Act and University ethical guidelines. The WWWhich task was performed as describe previously (Chapter 3 Materials and methods. 3.3.2).

Results are expressed as mean  $\pm$  SEM. Only results from the retention phases are shown. For statistics analysis, object exploration was compared in each group by paired samples Student's t-test. DI was tested versus zero in each group by univariate Student's t-test. All the statistical analyses have been calculated using IBM SPSS (version 20).

The rats were used across 3 different experiments.

Experiment 1: 10 female Lister Hooded rats were tested on 3 different occasions in the same apparatus at day 1, day 7 and day 53.

Experiment 2: 10 female Lister Hooded rats, which had been previously tested in the NOR task (referred to as Non-naïve females), were tested in the WWWhich task 60 days after NOR testing.

Experiment 3: 10 male Lister Hooded rats were tested in the same apparatus.

### 5.2.3 Results

Experiment 1: Rats underwent the WWWhich task on 3 different occasions: day 1, day 7 and day 53. During the Acquisition 1 and Acquisition 2 phases on each day of test, the rats explored both configurations equally (data not shown). During the retention phase, the rats explored both configurations equally on each day of testing: day 1 [ $t_{(8)}=-1.142$   $p>0.05$ ], day 7 [ $t_{(8)}=-1.290$   $p>0.05$ ], day 53 [ $t_{(6)}=-1.611$   $p>0.05$ ] (Figure 29A). The DI was not significantly different to zero (equivalent to equal exploration of the objects) on day 1 [ $t_{(8)}=1.268$   $p>0.05$ ] and day 7 [ $t_{(8)}=2.004$   $p>0.05$ ]. A DI significantly superior to zero was found on day 53 [ $t_{(6)}=2.466$   $p<0.05$ ] (Figure 29B).

Experiment 2: Non-naïve females previously successfully performed a NOR task in the same apparatus (data not shown). It has thus been decided to investigate if they could perform the WWWhich task. During the Acquisition 1 and Acquisition 2 phases, the rats explored both configurations equally (data not shown). During the retention phase, the rats explored both configurations equally [ $t_{(8)}=0.581$   $p>0.05$ ] (Figure 29A). The DI was not significantly different to zero [ $t_{(8)}=0.462$   $p>0.05$ ] (Figure 29B).

Experiment 3: With the last experiment, effect of the gender was assessed by running the task with male adult Lister Hooded rats. During the Acquisition 1 and Acquisition 2 phases, the rats explored both configurations equally (data not shown). During the retention phase, the rats explored both configurations equally [ $t_{(8)}=0.28$   $p>0.05$ ] (Figure 29A). The DI was not significantly different to zero [ $t_{(8)}=0.345$   $p>0.05$ ] (Figure 29B).



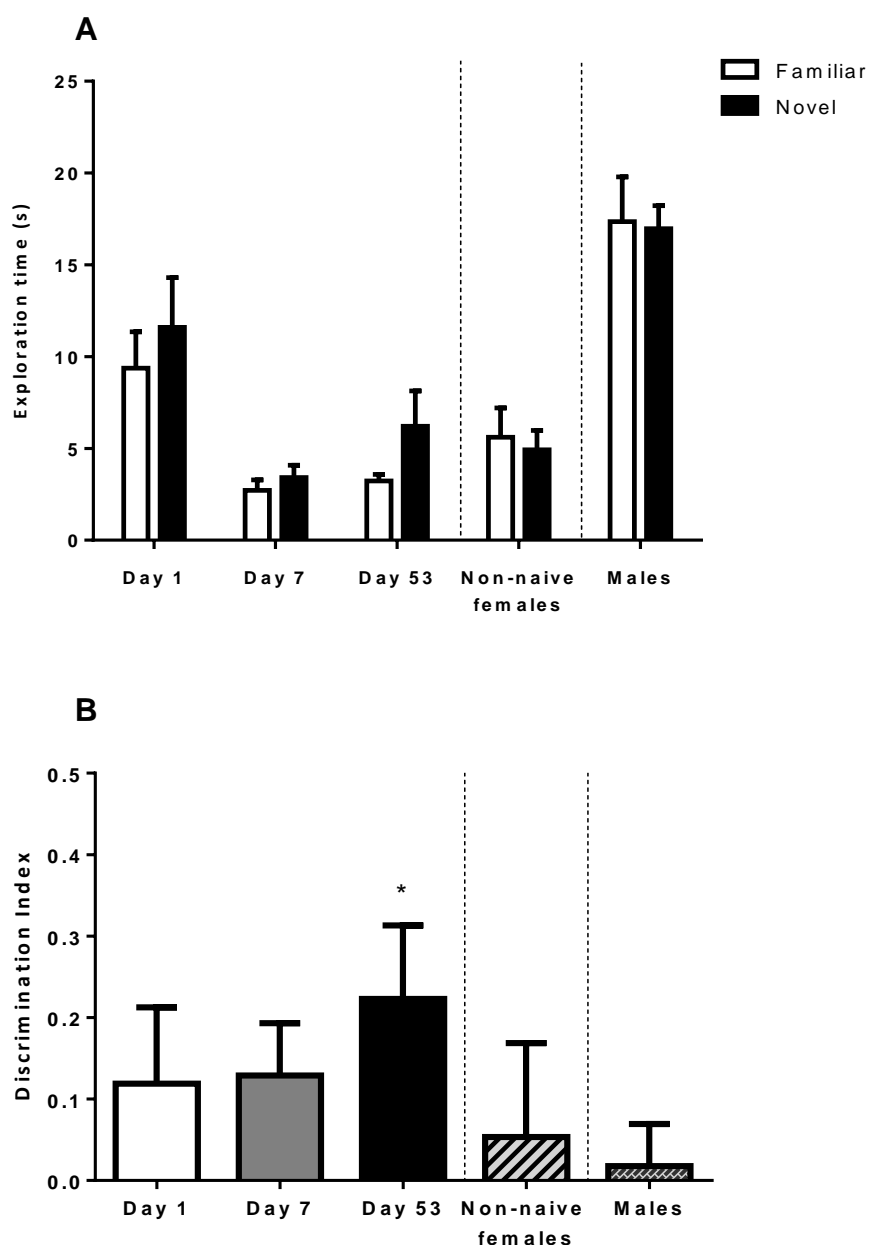


Figure 29: WWhich results. (A) No group spent more time exploring the novel compared to the familiar configuration during the Retention phase of the WWhich test. Data are presented as mean $\pm$ SEM ( $n=9-10$  per group), paired-sample Student's  $t$ -tests for each Familiar/Novel couple showed no significant difference. (B) Discrimination index in each group during the Retention phase. Data are presented as mean $\pm$ SEM ( $n=9-10$  per group), \* $p<0.05$ , univariate Student's  $t$ -test vs zero.

### 5.2.4 Discussion

In the current study, none of the groups of Lister Hooded rats managed to perform the WWWhich task. Although the DI of rats on day 53 is significantly different from 0 (Figure 29B), there is no difference in the exploration time of both configurations (Figure 29A). It is thus important to always look at both the exploration time and the DI, as the later does not take into account low exploration times. In this experiment, since the exploration time of both configurations is low (3.23 s Familiar, 6.21 s Novel), the difference in DI is actually negligible. It is interesting to note that the non-naïve female rats performed successfully in the NOR task previous to the WWWhich task, and that male rats performed successfully in the NOR task after the WWWhich task (data not shown). This demonstrates that these rats showed no impairment in recognition memory. The same apparatus was used for both the WWWhich and NOR tasks, and the experiments have been run and scored by the same experimenters; apparatus or human biases can thus be ruled out as a cause of low performance in the WWWhich task.

It is interesting to compare male rats' results in this study with those of Langston (Langston and Wood, 2010). The test sessions were 3min in both cases with the same scoring criteria. Exploration times and DI scores in the current study were much lower and did not prove significant. Main differences between the two studies' protocols that could explain divergent results are (1) feeding conditions: in Langston's study, rats were maintained at 90% of their free-feeding bodyweight in order to increase their exploration behaviour, (2) differences in the test environment which could modify the likeliness of the animals to explore the objects, (3) differences in the housing environment as it has previously been reported that rodents living in enriched environments spent less time exploring familiar and novel objects (Leger et al., 2012, Viola et al.,

2010). While rats in the current study are housed in cages of standard dimensions, they are provided with a tunnel and an elevated platform which might be considered as enriched environment. It is as well important to mention that rats in Langston's study underwent a sham surgical procedure. Overall, DI scores in the current study are lower than the ones reported in the literature (Table 2). Although some DI scores in the current study are comparable to the significant scores found in the literature, the statistical analysis showed no significance when compared to chance. However, inter-individual variance, difference in protocols, experiment conditions, experimenters, and species tested (especially mouse or rat) can make reproducibility and results harder to compare.

In the current experiments, it has been shown that adult Lister Hooded could not perform the WWWhich task, despite positive performances in the NOR. The task appeared to be too difficult for rats to either encode or remember which configuration was familiar as opposed to the novel one. These results raise awareness on the difficulty of reproducing this cognitive task in *in vivo* models. In light of the results the WWWhich task was discarded as a potential test for the A $\beta$  administration model presented in this project.

Author	Species	Treatment	DI significantly different from zero?
(Eacott and Norman, 2004)	Dark Agouti rats 4 months old	Control (n=10).	<b>Yes</b> , except at 120min ITI.
	Dark Agouti rats 8 months old	Sham surgery; Perirhinal cortex lesion; Postrhinal cortex lesion; Fornix lesion. (n total=46)	<b>Yes</b> for all groups but fornix lesion. Fornix lesion group significantly impaired compared to other groups. The other lesion groups do not differ from sham group.
(Langston and Wood, 2010)	Male Lister Hooded rats 260 to 310g	Sham surgery (n=13); Hippocampal lesion (n=15). Food restriction.	Sham surgery: <b>Yes</b> Hippocampal lesion: <b>No</b>
(Easton et al., 2011)	Male Dark Agouti rats 180 to 200g	Sham surgery (n=12); Cholinergic neurons lesions (n=10).	Both groups: <b>Yes</b>
(Davis et al., 2013a)	Female mice 3 months old	Control (n=12); 3TgxAD (n=18).	Control: <b>Yes</b> , except at 15min ITI. Overall both groups can perform the task with 3TgxAD group performing more poorly.
	Female mice 12 months old	Control (n=10); 3TgxAD (n=12).	Both: <b>No</b> (overall). 3TgxAD group can perform the task only at 5min ITI while Control group cannot.
(Davis et al., 2013b)	Female mice 6 months old	Control (n=10); 3xTgAD (n=12).	Control: <b>Yes</b> at 2; 5 and 10 min but not at 15 and 30 min ITI. 3xTgAD: <b>No</b> . Control group's DI is significantly different from 3xTgAD only at 5min ITI.

Table 2: Comparison of WWWhich studies involving rodents. Performances of the rodents for similar settings vary depending on the age and strain, as well as treatment used.

## 5.3 NEUROINFLAMMATORY, SYNAPTIC AND NEURONAL MARKERS

### 5.3.1 Rationale

As highlighted in Chapter 1 (General introduction), neuroinflammation is suggested to play a key role in A $\beta$  pathology. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have thus been measured in brain samples as 3 common markers of neuroinflammation in AD – see (Swardfager et al., 2010) for review. These 3 markers have been reported to be increased within 36 hours after administration of A $\beta$  (Rosales-Corral et al., 2004). In the current study, their levels have been measured on day 35 after surgery, after proving that the NOR deficit is still present.

Following indicators of neuroinflammation, levels of both pre-synaptic (Synaptosomal-associated protein 25 kDa (SNAP-25)) and post-synaptic (PSD-95) proteins have been measured to investigate synaptic deficit. Similarly to the neuroinflammatory markers, synaptic markers in both the frontal cortex and hippocampus have been investigated.

Finally, N-acetylaspartate (NAA) levels have been assessed as a general marker of neuronal function (Demougeot et al., 2001) and viability (Schuff et al., 2006). NAA levels have been found to be reduced in AD patients' grey matter (Adalsteinsson et al., 2000, Jessen et al., 2009, Moffett et al., 2007) and has been suggested as a marker of disease progression (Zhang et al., 2009). To further investigate neuronal viability in a more specific sub-population of neurons, density of parvalbumin(PV)-positive cells in the frontal cortex have been measured by immunostaining. PV is a marker of GABAergic interneurons found in the rat frontal cortex (Kawaguchi and Kondo, 2002) and has been shown to be reduced in AD patients (Arai et al., 1987, Satoh et al., 1991, Brady and Mufson, 1997).

### 5.3.2 Materials and methods

Neuroinflammatory markers in the frontal cortex and the hippocampus have been measured by Western Blot (IL-1 $\beta$ ) and ELISA (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ). For Western Blot, brains were taken on day 1, 4, 7 and 14; for ELISA, brains were taken and dissected on day 35 following ICV administration of either vehicle or A $\beta$ <sub>1-42</sub> (5 nmol). A NOR task was performed on the same day, prior to culling the rats, to confirm the presence of cognitive deficits in the A $\beta$  group.

Synaptic markers (SNAP-25 and PSD-95) were measured in synaptosomal preparations by ELISA, on day 35 following ICV administration of either vehicle or A $\beta$ .

NAA was measured by HPLC, in 6 brain regions (frontal cortex, prefrontal cortex, striatum, temporal cortex, dorsal hippocampus, ventral hippocampus). Brains were extracted and frozen on day 14 following ICV administration of either vehicle or A $\beta$ <sub>1-42</sub> (5 nmol). PV-positive cells were counted by immunostaining, on day 70 following ICV administration of either vehicle or A $\beta$ <sub>1-42</sub> (5 nmol).

All the methods used are described in Chapter 3 Materials and methods, (□, 3.4.4, 3.4.3, 3.4.6). For statistics analysis, scores were compared between Vehicle and A $\beta$  groups by independent samples Student's t-test, or independent samples Mann-Whitney test in the case of NAA levels. An outline of the different time points and markers investigated is presented in Table 3.

Day after A $\beta$ administration	1, 4 and 7	14	35	70
Markers measured	<b>IL-1<math>\beta</math></b>	<b>IL-1<math>\beta</math></b> , NAA	<b>IL-1<math>\beta</math>, IL-6, TNF-<math>\alpha</math>, SNAP-25, PSD-95</b>	Parvalbumin

Table 3: Time points of the markers measured. Neuroinflammatory markers in bold font, synaptic markers in italic font.

### 5.3.3 Results

#### 5.3.3.1 Neuroinflammatory markers

The first analysis of IL-1 $\beta$  levels has been run by Western Blot. Despite varying the sampling time point (1, 4, 7, or 14 days after surgery), protein loading quantity and blocking solution/antibody concentrations, no IL-1 $\beta$  could be detected in the samples. Figure 30 shows an example image of Western Blot membrane for IL-1 $\beta$ . This image represents the standard images obtained despite the different parameter tested. Samples were normalised by loading the same amount of total protein in each lane following a protein assay. The ChemiDoc™ MP System software performs as well an internal normalisation of the different lanes by measuring both the intensity of the total protein per lane and of the protein of interest as explained in the Materials and Methods chapter (see 3.4.5.5). The groups investigated were (1) following no surgery as a control group, (2) following sham surgery, which was expected to trigger some inflammation and (3) following surgery and ICV administration with A $\beta$ <sub>0</sub>. An IL-1 $\beta$  standard was used to check the band position and the ability of the test to detect the protein. The lack of positive results, i.e. no band at 17 kDa (size of IL-1 $\beta$ ) and the presence of a number of artefacts could be explained by (1) a poor sensitivity and specificity of this assay for IL-1 $\beta$ , (2) a quick degradation of IL-1 $\beta$  during the sampling and protein extraction steps, (3) the absence of IL-1 $\beta$  in the samples, or levels below the detection range. Because the assay could detect low levels of the IL-1 $\beta$  standard, attempts to refine the method have been made by: (1) dissecting the brain immediately following extraction, prior to snap-freezing them, in order to reduce the risks of protein degradation from freeze-thaw cycles; (2) running a more sensitive assay such as ELISA.

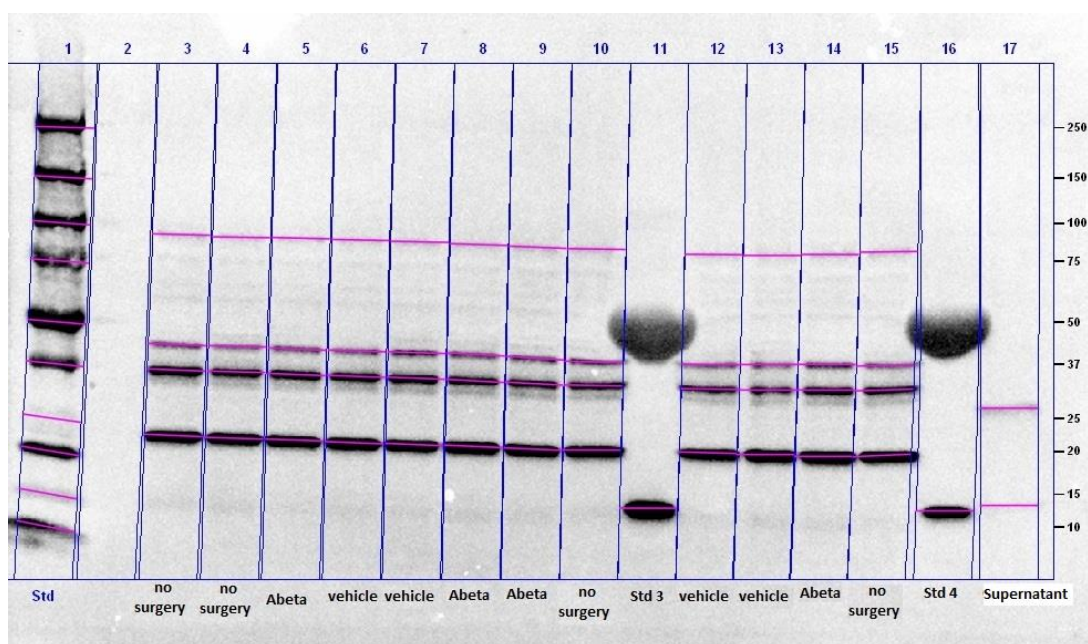


Figure 30: Example image of a Western Blot membrane. Experimental conditions: gel 8-16%, blocked in 1% BSA for 1 hour, primary antibody: 0.2  $\mu\text{g}/\text{mL}$  in 0.1% BSA for 24 hours at  $+4^{\circ}\text{C}$ , secondary antibody: 1:1000 in 3% milk for 1 hour, 30  $\mu\text{g}$  of total protein per lane. Lanes 11 and 16: IL-1 $\beta$  standard reconstituted in 1% BSA in PBS (R&D DuoSet ELISA rat IL-1 $\beta$  / IL-1F2) at 36.25 ng/mL and 18.13 ng/mL respectively). Samples from rats following no surgery, sham surgery (vehicle) or surgery with ICV administration of A $\beta_0$ , culled 24 hours after surgery. Lane 1: ladder.

The following assay on IL-1 $\beta$  levels was run by ELISA, on rats culled 35 days after ICV administration of either vehicle or A $\beta_{0-42}$ ; alongside ELISA for IL-6 and TNF- $\alpha$  levels (Figure 31). Two regions, frontal cortex and hippocampus, were investigated for their role in memory. There was no difference in the levels of IL-1 $\beta$  in the hippocampus [ $t_{(18)}=-0.451$   $p>0.05$ ]. In contrast, the level of IL-1 $\beta$  in the frontal cortex was significantly higher in the group administered with A $\beta_0$  [ $t_{(15)}=-2.694$   $p<0.05$ ]. There was no difference in the levels of IL-6 in the hippocampus [ $t_{(17)}=0.862$   $p>0.05$ ] or the frontal cortex [ $t_{(16)}=-1.264$   $p>0.05$ ]. There was no difference in levels of TNF- $\alpha$  in the hippocampus [ $t_{(17)}=0.752$   $p>0.05$ ]. In contrast, levels of TNF- $\alpha$  in the frontal cortex were significantly higher in the group administered with A $\beta_0$  [ $t_{(16)}=-2.299$   $p<0.05$ ].



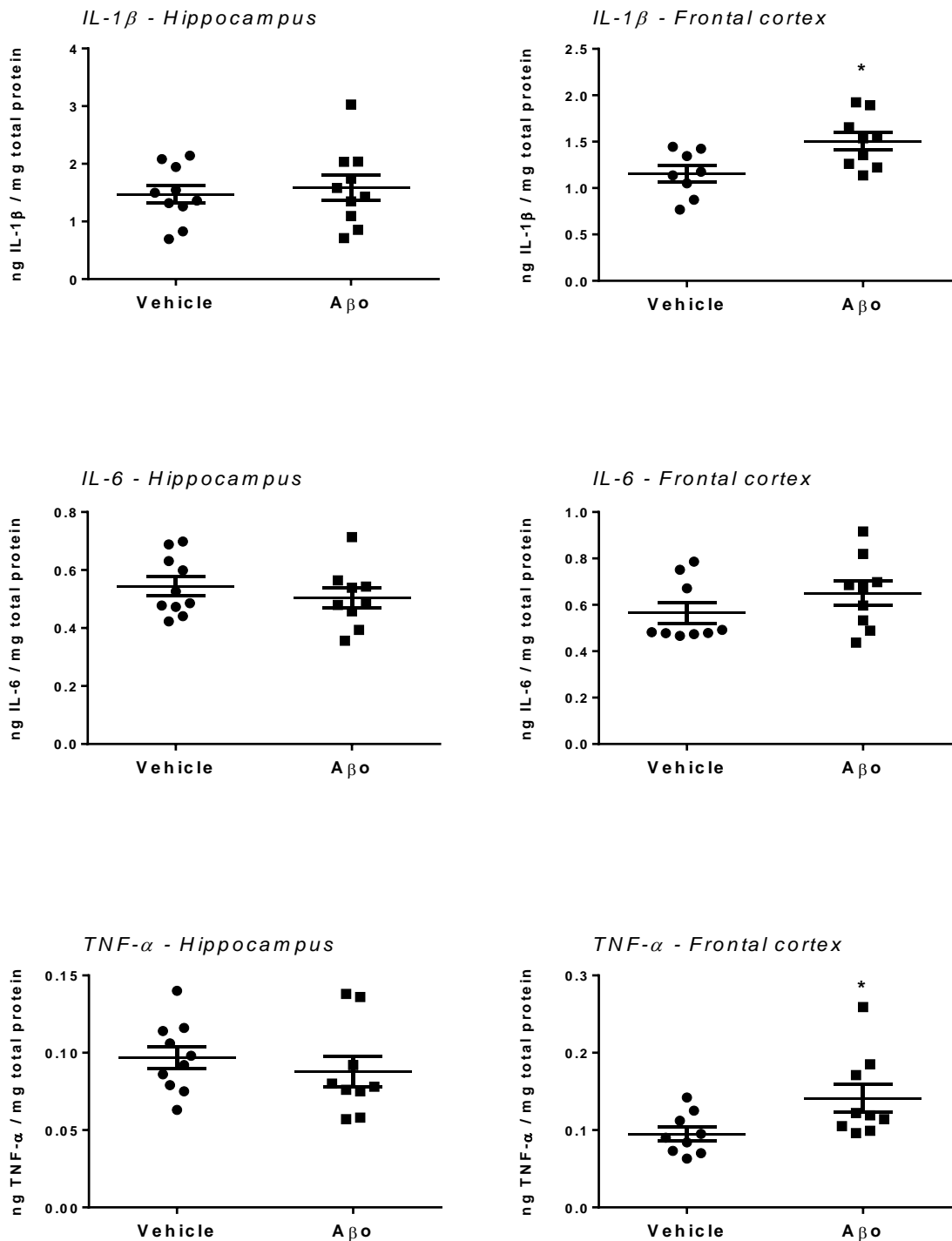


Figure 31: Results from the ELISA on neuroinflammatory markers in the hippocampus (left panel) and frontal cortex (right panel). Rats have been culled 35 days after ICV administration of either vehicle or A $\beta$ 0. Data are presented as individual plots, mean $\pm$ SEM (n=8-10 per group), \*  $p$ <0.05 vs. Vehicle, independent samples Student's t-test.

### 5.3.3.2 Synaptic markers

Levels of SNAP-25 and PSD-95 in the hippocampus and the frontal cortex were measured by ELISA in brains taken 35 days after ICV administration of either vehicle or A $\beta$ <sub>1-42</sub> (Figure 32). There was no difference in levels of SNAP-25 in the hippocampus [ $t_{(17)}=1.186$   $p>0.05$ ] or the frontal cortex [ $t_{(16)}=1.908$   $p>0.05$ ]. There was no difference in levels of PSD-95 in the hippocampus [ $t_{(14)}=0.752$   $p>0.05$ ]. In contrast, levels of PSD-95 in the frontal cortex were significantly lower in the group administrated with A $\beta$  [ $t_{(16)}=3.298$   $p<0.01$ ].

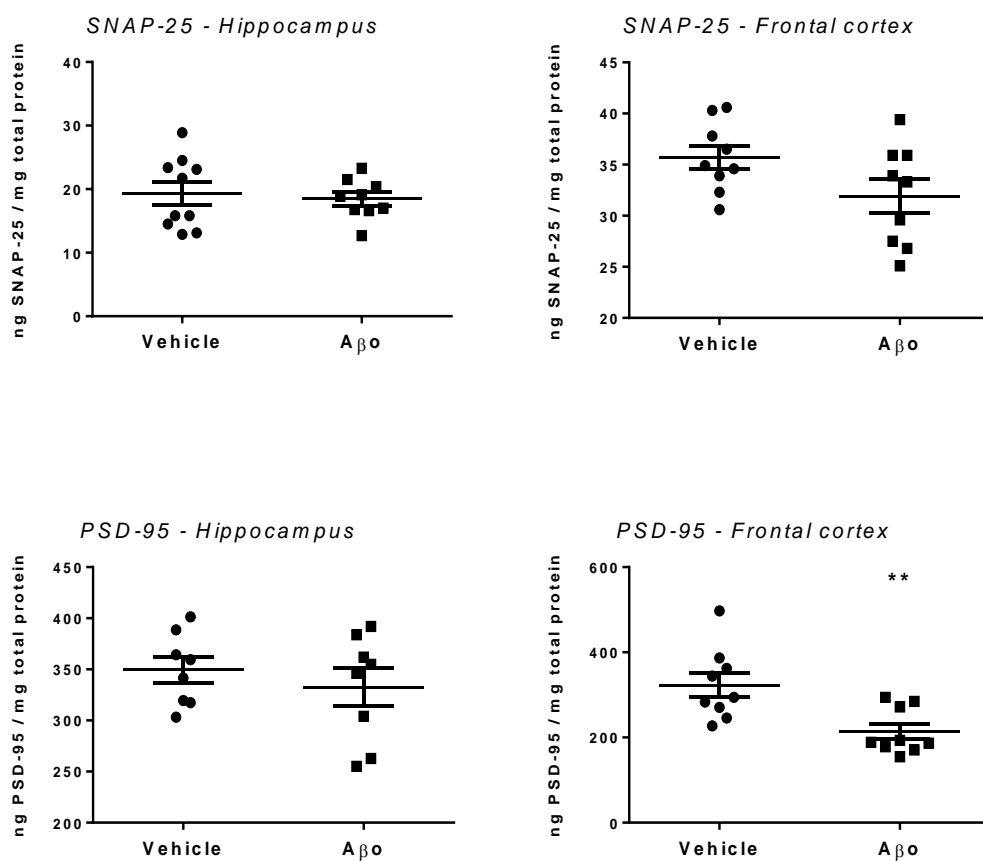


Figure 32: Results from the ELISA on synaptic markers in the hippocampus (left panel) and frontal cortex (right panel). Rats have been culled 35 days after ICV administration of either vehicle or A $\beta$ . Data are presented as individual plots, mean $\pm$ SEM ( $n=8-10$  per group), \*\*  $p<0.01$  vs. Vehicle, independent samples Student's  $t$ -test.

### 5.3.3.3 Neuronal markers

There was no difference in the levels of NAA between groups in each of the 6 brain regions investigated (Figure 33A). However, PV-positive cells density was significantly reduced in the frontal cortex of rats, 70 days following administration of A $\beta$ <sub>1-42</sub> [ $t_{(15)}=4.137$   $p<0.001$ ] (Figure 33B).

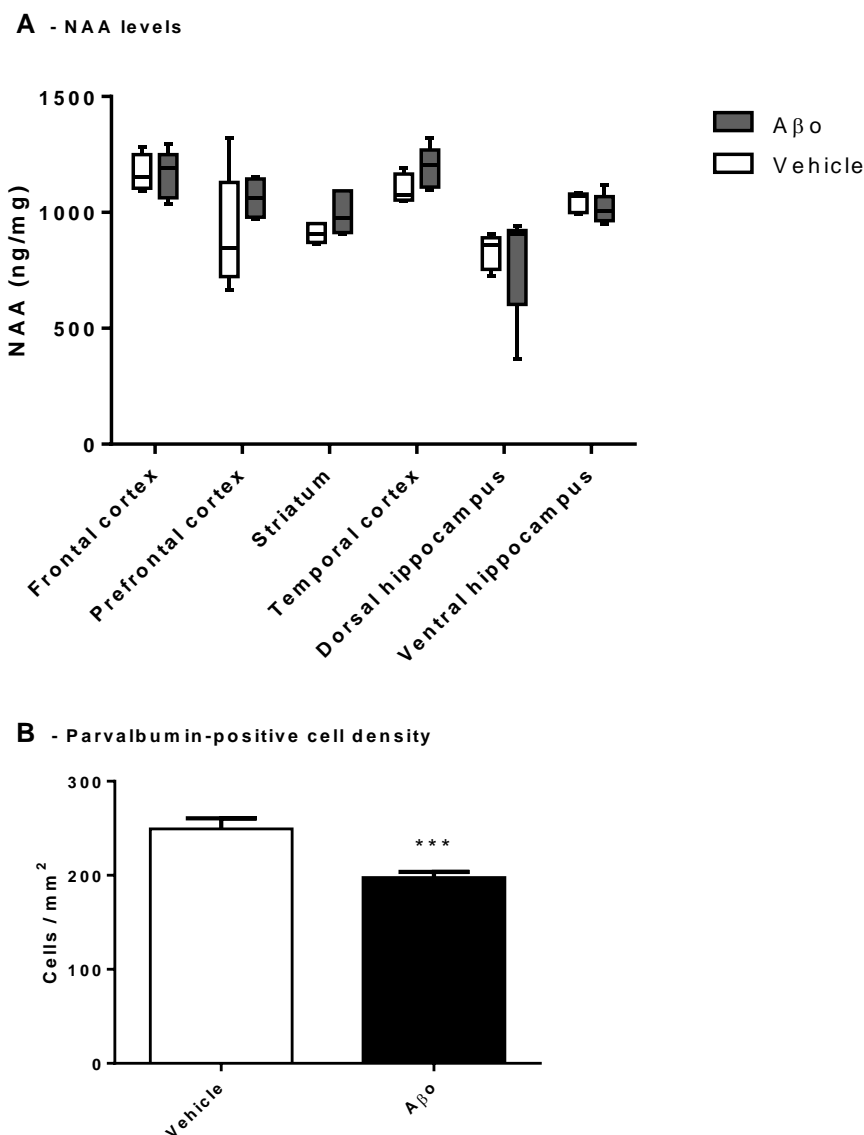


Figure 33: Neuronal markers (A) Results from the HPLC assay on NAA levels across 6 brain areas, 14 days following administration of either vehicle or A $\beta$ . Data are presented as median $\pm$ min/max ( $n=5$  per group), no significant difference between the two groups, Mann-Whitney test. (B) Density of PV-positive cells in the frontal cortex, 70 days following administration of either vehicle or A $\beta$ . Data are presented as mean $\pm$ SEM ( $n=8-9$  per group), \*\*\* $p<0.001$  independent samples Student's  $t$ -test.

### 5.3.4 Discussion

Results from the neuroinflammatory markers showed increased levels of IL-1 $\beta$  and TNF- $\alpha$  in the frontal cortex, 35 days after ICV administration of A $\beta$ <sub>1-42</sub>. IL-1 $\beta$  is known to be one of the earliest pro-inflammatory cytokines released following central nervous system insult (Griffin et al., 1998). Moreover, IL-1 $\beta$  is believed to be an initiator of inflammation, following acute injury (Shaftel et al., 2008), with IL-6 and TNF- $\alpha$  release being delayed in time (Rosales-Corral et al., 2004). However, kinetics data on the mid- and long term changes in rodent *in vivo* models of A $\beta$  administration remain an unmet need. In the current study, the presence of increased levels of IL-1 $\beta$  and TNF- $\alpha$  35 days after administration of A $\beta$  could highlight an underlying phenomenon of constant and lasting inflammation, contributing to the NOR deficit observed at this time point. The lack of increase in IL-6 levels remains puzzling. However, IL-6 shows both pro- and anti-inflammatory properties and interacts with IL-1 $\beta$  and TNF- $\alpha$  (Hunter and Jones, 2015). It is thus possible that in the present model, a lack of IL-6 increased levels is not in contraction with the inflammatory action of both IL-1 $\beta$  and TNF- $\alpha$ , but rather reveals complex inflammatory mechanisms. The absence of increased levels of pro-inflammatory cytokines in the hippocampus could suggest that this structure is yet undamaged by the A $\beta$  pathology at day 35. In line with these findings, spatial memory (which was assessed by the Y-maze and is dependent on hippocampal input) was preserved at day 35 in this model (see Chapter 4). It is thus hypothesised that hippocampal memory is preserved and that the neuroinflammatory phenomenon did not spread to the hippocampus, at least 35 days after ICV administration of A $\beta$ .

This hypothesis is further supported by levels of synaptic markers (SNAP-25 and PSD-95) which were similar to control in the hippocampus. However, PSD-95 levels were found to be reduced in the frontal cortex. A $\beta$  are known to

specifically bind to PSD-95 positive sites (Lacor et al., 2004). Decreased levels of PSD-95 are indicative of a disruption of synaptic activity in this area, supporting the inflammatory changes seen in this region. Taken together these results suggest decreased synaptic activity in the frontal cortex following ICV administration of A $\beta$ , which could partially explain the deficit observed in the NOR test in the same animals (Morici et al., 2015).

There were no changes in the levels of NAA in any brain region investigated between control and A $\beta_{1-42}$  treated groups 14 days following surgery, highlighting a lack of non-specific neuronal death due to toxic concentrations. These findings are also consistent with clinical findings where neuronal loss is seen at a later stage following amyloid deposition and other subsequent neuropathological changes. NAA is used as a non-specific neuronal marker with changes indicating general neuronal loss/dysfunction. Current findings may indicate a more subtle change in synaptic function rather than robust neuronal loss following administration of A $\beta_{1-42}$ . Investigation of specific subsets of GABAergic interneurons with PV staining showed reduced levels in A $\beta$  treated animals, 70 days after surgery. Deficits in PV-positive cell density have been found in a number of clinical and preclinical studies in relation to schizophrenia (Zhang et al., 2002) and AD (Arai et al., 1987, Satoh et al., 1991). In rats, decrease of PV-positive cell density is used as a marker of GABAergic interneurons (Kawaguchi and Kondo, 2002) and could result from neuronal damages in the frontal cortex, following neuroinflammation and synaptic deficit observed in the same area at day 35.

Taken together, these data demonstrate a number of changes in the frontal cortex, with no sign of hippocampal pathology. The next step to investigate the underlying mechanisms was to assess the predictive validity of the model by testing documented drugs.

## 5.4 TREATMENT WITH ROLIPRAM

### 5.4.1 Rationale

Phosphodiesterases (PDEs) are enzymes that regulate the hydrolysis of cAMP and cGMP, two second messengers involved in controlling levels of phospho-cAMP response element-binding (pCREB) in the brain and indirectly playing a role in modulation of LTP, synaptic plasticity and memory (Garcia-Osta et al., 2012). Inhibiting PDEs that hydrolyse cGMP could have beneficial effects on dementia (Domek-Lopacinska and Strosznajder, 2010) and opens new therapeutic possibilities. In animal models of dementia, the inhibitor of the PDE-4, rolipram, has shown promising results on restoring cognition (Gong et al., 2004, Cheng et al., 2010, Wang et al., 2012). In a model of APP/PS1 transgenic mouse, acute and chronic treatment by daily administration of 0.03 mg/kg of rolipram for 3 weeks, reversed the deficit in contextual fear conditioning, even after a 2-month washout of the drug (Gong et al., 2004). In rats receiving an acute administration of A $\beta$ o in the hippocampus, chronic treatment with daily IP injections of rolipram at 0.1, 0.25 and 0.5 mg/kg, 1H before testing, improved performances in the MWM after 2 and 3 weeks of treatment, in a dose dependant manner (Cheng et al., 2010, Wang et al., 2012). Both teams suggested rescued levels of pCREB in the hippocampus as a probable protective effect of rolipram on LTP and synaptic function.

In the following experiments, two chronic doses of rolipram have been tested, with or without washout period, for its potential capacity to reverse the NOR deficit in the current A $\beta$ o rat model.

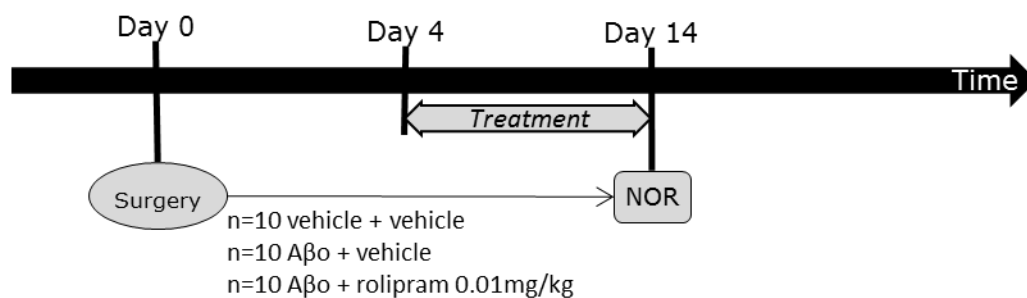
### 5.4.2 Materials and methods

Subjects were adult female Lister Hooded rats (Charles River, UK) aged 3-month old at the time of surgery. Rats were housed in groups of 5, on a 12-hour light-dark cycle, with free access to food and water, in a controlled environment of temperature ( $21\pm 2^\circ\text{C}$ ) and humidity ( $55\pm 5\%$ ) in Biological Services Facility at the University of Manchester. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) 1986 Act and University ethical guidelines. Surgery for administration of  $\text{A}\beta_{0_{1-42}}$  and behavioural testing (NOR) were performed as described previously (Chapter 3 Materials and methods, 3.2.1 and 3.3.1).

(Figure 34) Rats were ICV administered with 10  $\mu\text{L}$  of either vehicle or  $\text{A}\beta_{0_{1-42}}$  5 nmol, on day 0. Rats were treated with daily IP injection of either saline or rolipram 0.01 mg/kg (experiment A), or rolipram 0.1 mg/kg (experiments B & C). Treatment was given from day 4 to day 14 after surgery (A) or starting from the day before surgery and up to 14 days after surgery (B & C). NOR testing was performed on day 14, 1 hour after the last injection of rolipram (A & B) or day 35 (C) following ICV administration of either vehicle or  $\text{A}\beta_{0_{1-42}}$ .

Results are expressed as mean  $\pm$  SEM. NOR data were analysed by 1-way ANOVA. In cases of significance ( $p < 0.05$ ), individual paired samples Student's t-tests were run in each group, comparing the exploration time of both objects. Total exploration time and DI were analysed by 1-way ANOVA and Bonferroni post-hoc. DI were also analysed by univariate Student's t-test versus zero. All the statistical analyses have been calculated using IBM SPSS (version 20).

### Experiment A



### Experiment B (Day 14) and C (Day 35)

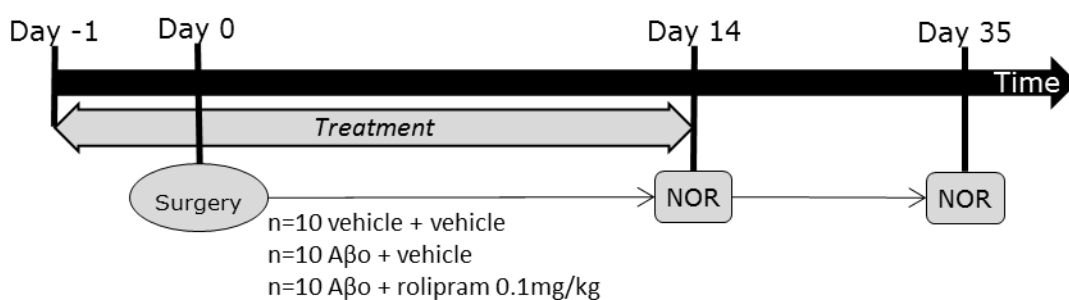


Figure 34: Outline of experiments A, B and C. Rats are treated with rolipram 0.01mg/kg (A) or 0.1mg/kg (B&C) and tested in the NOR task at day 14 (A & B) or 35 (C) following ICV administration of either vehicle or A $\beta$ o. Experiments B & C explore the performances of the same batch of rats.



### 5.4.3 Results

#### 5.4.3.1 Experiment A - Chronic treatment 0.01 mg/kg

##### **Acquisition Phase:** (Figure 35A)

On day 14 after surgery, during the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,27)}=0.461$   $p>0.05$ ], regardless of group [ $F_{(2,27)}=1.476$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(2,27)}=0.679$   $p>0.05$ ].

##### **Retention Phase:** (Figure 35A)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,26)}=19.763$   $p<0.001$ ] with an effect of group [ $F_{(2,26)}=7.572$   $p<0.01$ ]. Only the control group spent more time exploring the novel object: Vehicle+Vehicle group [ $t_{(9)}=-4.923$   $p<0.001$ ]. There was no difference in exploration for the A $\beta$ o administrated groups: A $\beta$ o+Vehicle group [ $t_{(8)}=-0.890$   $p>0.05$ ], A $\beta$ o+Rolipram group [ $t_{(9)}=-1.439$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(2,26)}=1.772$   $p>0.05$ ].

##### **Discrimination index:** (Figure 35A)

In the control group a DI significantly superior to zero (equivalent to equal exploration of the objects) was found: Vehicle+Vehicle [ $t_{(9)}=5.175$   $p<0.001$ ]. In contrast, DIs in the A $\beta$ o groups were not significantly different to zero: A $\beta$ o+Vehicle [ $t_{(8)}=1.331$   $p>0.05$ ], A $\beta$ o+Rolipram [ $t_{(9)}=1.550$   $p>0.05$ ]. There was a significant difference in DI between groups [ $F_{(2,26)}=5.082$   $p<0.05$ ]. A planned contrast test showed a significant difference between the DI of the Vehicle+Vehicle and both A $\beta$ o groups [ $t_{(26)}=3.088$   $p<0.01$ ].

In summary, the Vehicle+Vehicle group significantly spent more time exploring the novel over the familiar object, an effect that was abolished in the

group receiving ICV administration of A $\beta$ <sub>1-42</sub> and not rescued by chronic IP treatment with Rolipram 0.01 mg/kg from day 4 to day 14 after surgery.

#### **5.4.3.2 Experiment B - Chronic treatment 0.1 mg/kg**

Following the unsuccessful reversal of the A $\beta$ -induced NOR deficit, it has been decided to increase the dose of rolipram to 0.1 mg/kg. Treatment was administered on the day before surgery to assess potential preventive, rather than reversal, effect of rolipram. (Figure 34B).

##### **Acquisition Phase:** (Figure 35B)

On day 14 after surgery, during the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,27)}=0.031$   $p>0.05$ ], regardless of group [ $F_{(2,27)}=0.581$   $p>0.05$ ].

##### **Retention Phase:** (Figure 35B)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,23)}=39.951$   $p<0.001$ ] with an effect of group [ $F_{(2,23)}=7.622$   $p<0.01$ ]. Control and rolipram treated groups spent more time exploring the novel object: Vehicle+Vehicle group [ $t_{(9)}=-6.189$   $p<0.001$ ] and A $\beta$ +Rolipram group [ $t_{(7)}=-3.579$   $p<0.01$ ]. There was no difference in exploration for the A $\beta$ +Vehicle group [ $t_{(7)}=-0.497$   $p>0.05$ ].

##### **Discrimination index:** (Figure 35B)

DI significantly superior to zero was found in the control group (Vehicle+Vehicle [ $t_{(9)}=7.489$   $p<0.001$ ]) and the rolipram treated group (A $\beta$ +Rolipram [ $t_{(7)}=3.444$   $p<0.05$ ]). In contrast the DI in the A $\beta$ +Vehicle group was not significantly different to zero [ $t_{(7)}=0.354$   $p>0.05$ ]. There was a significant difference in DI between groups [ $F_{(2,23)}=9.723$   $p<0.001$ ]. DI of the A $\beta$ +Vehicle group was significantly lower than control (Vehicle+Vehicle group [ $p<0.001$ ]), and treated group (A $\beta$ +Rolipram [ $p<0.05$ ]).

In summary, the Vehicle+Vehicle group significantly spent more time exploring the novel over the familiar object, an effect that was abolished in the group receiving ICV administration of A $\beta$ <sub>1-42</sub>. This deficit was prevented by chronic IP treatment with rolipram 0.1 mg/kg.

#### **5.4.3.3 Experiment C - Chronic treatment 0.1 mg/kg, with 21-day washout**

Following the successful prevention of the A $\beta$ -induced NOR deficit, the IP treatment with rolipram has been stopped and the animals retested in the NOR task after a 21-day washout period (Figure 34C).

##### **Acquisition Phase:** (Figure 35C)

On day 35 after surgery, during the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,27)}=0.013$   $p>0.05$ ], regardless of group [ $F_{(2,27)}=0.222$   $p>0.05$ ].

##### **Retention Phase:** (Figure 35C)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,18)}=27.195$   $p<0.001$ ] with an effect of group [ $F_{(2,18)}=21.103$   $p<0.001$ ]. Only the control group spent more time exploring the novel object: Vehicle+Vehicle [ $t_{(4)}=-5.517$   $p<0.01$ ]. There was no difference in exploration for the A $\beta$  administrated groups: A $\beta$ +Vehicle group [ $t_{(6)}=0.343$   $p>0.05$ ], A $\beta$ +Rolipram group [ $t_{(8)}=-0.466$   $p>0.05$ ].

##### **Discrimination index:** (Figure 35C)

In the control group a DI significantly superior to zero was found: Vehicle+Vehicle [ $t_{(4)}=5.445$   $p<0.01$ ]. In contrast, DIs in the A $\beta$  groups were not significantly different to zero: A $\beta$ +Vehicle [ $t_{(6)}=-0.636$   $p>0.05$ ], A $\beta$ +Rolipram [ $t_{(8)}=0.380$   $p>0.05$ ]. There was a significant difference in DI between groups [ $F_{(2,18)}=21.014$   $p<0.001$ ]. DI of both the A $\beta$  groups were

significantly lower than control: A $\beta$ <sub>0</sub>+vehicle [ $p < 0.001$ ], A $\beta$ <sub>0</sub>+Rolipram [ $p < 0.001$ ].

In summary, the Vehicle+Vehicle group significantly spent more time exploring the novel over the familiar object, an effect that was abolished in the group receiving ICV administration of A $\beta$ <sub>01-42</sub>. This deficit was not reversed by preventive IP treatment with Rolipram 0.1 mg/kg, after a washout period of 21 days.

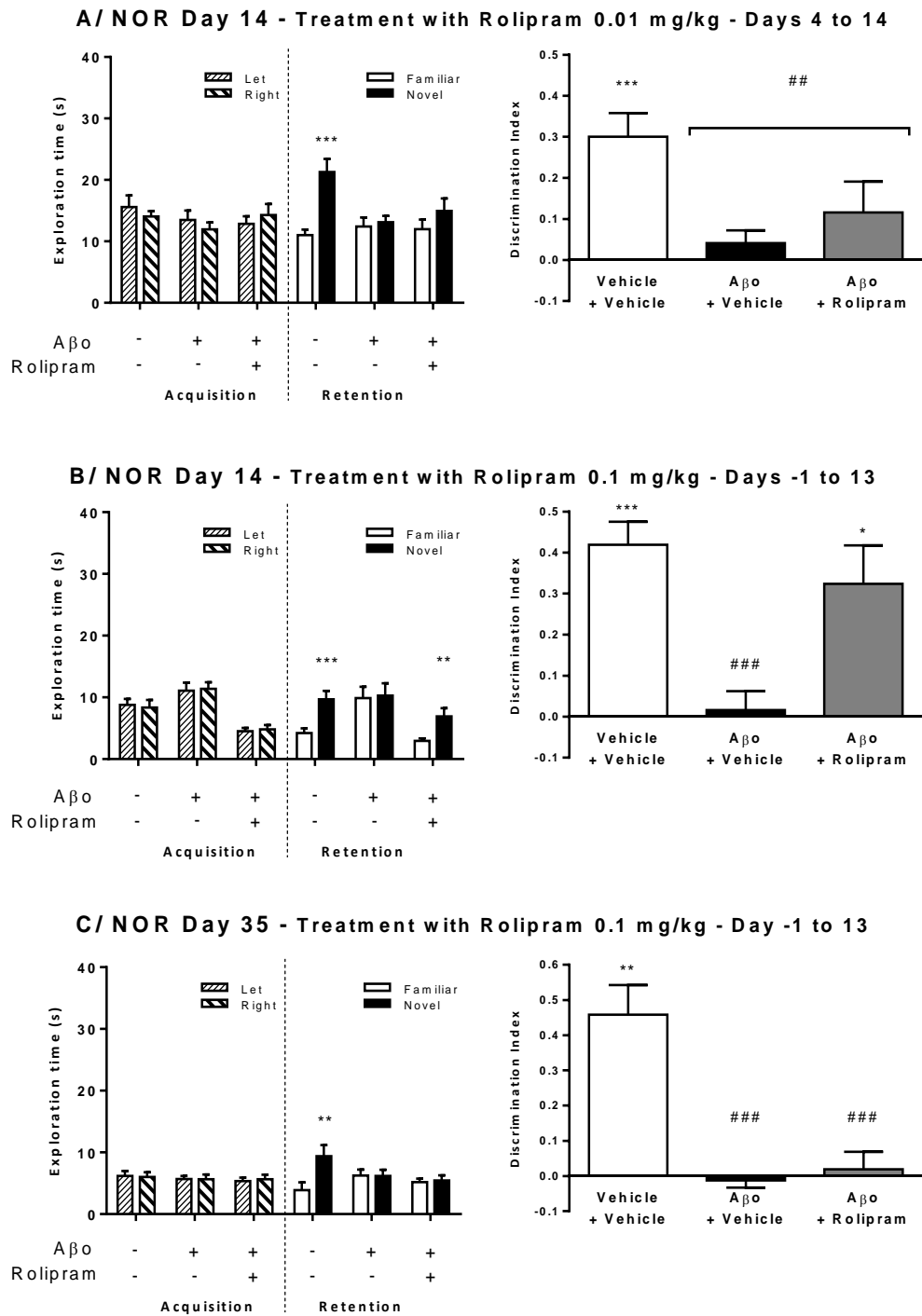


Figure 35: NOR task after IP treatment with Vehicle or Rolipram A/ 0.01mg/kg IP Day 4 to Day 14 after ICV administration of Vehicle or A $\beta$  5nmol; B/&C/ 0.1mg/kg IP Day -1 to Day 13 after ICV administration of Vehicle or A $\beta$  5nmol. Data are presented as mean+SEM (n=5-10 per group; 5 videos could not be scored following an error in the recording equipment). Left panel: Object exploration, \*\* $p$ <0.01 \*\*\* $p$ <0.001 Novel vs. Familiar, paired one-way ANOVA and Student  $t$ -test. Right panel: Discrimination Index, \* $p$ <0.05 \*\* $p$ <0.01 \*\*\* $p$ <0.001 vs. zero, ## $p$ <0.01 ### $p$ <0.001 vs. Vehicle+Vehicle group, one-way ANOVA and Bonferroni post-hoc.

### 5.4.4 Discussion

It has thus been shown that chronic treatment by rolipram 0.01 mg/kg had no effect on the NOR deficit in the current model. However, in this experiment (Figure 34A and Figure 35A), treatment was started 4 days after surgery, once A $\beta$  toxic mechanisms are already in place (Chapter 4, 4.3.1, Figure 23A). Treatment with rolipram at a dose of 0.01 mg/kg could thus not reverse the deficit. It then has been decided to start the treatment before the appearance of the NOR deficit and with a dose 10-fold higher and closer to the ones found in the literature. At a dose of 0.1 mg/kg, preventive and chronic treatment with rolipram (Figure 34B&C) could prevent the appearance of the NOR deficit (Figure 35B) but had no lasting effect after a 21-day washout period (Figure 35C). Rolipram crosses the BBB and has a half-life of a 1-3 hours in the rat (Krause and Kuhne, 1988). It was hence not present in the blood stream after the washout period.

Reversal of cognitive deficits in rat A $\beta$ -administrated rat models has been reported for similar and higher doses of rolipram (Cheng et al., 2010, Wang et al., 2012). However, the lasting effects after cessation of treatment observed in transgenic mice (Gong et al., 2004) could not be reproduced in the current model. One of the suggested beneficial mechanisms of action of rolipram is the restoration of pCREB levels in the hippocampus. Previous results showed that the current model had no deficit in hippocampal-dependant behavioural tasks (Y-maze – Chapter 4, 4.3.4, Figure 26A) or in hippocampal synaptic markers (Chapter 5, 5.3.3.2 Synaptic markers). It can thus be hypothesised that rolipram could have lasting effects on hippocampal function but not in the mechanisms involved in the current model, which showed injuries in the frontal cortex (neuroinflammatory markers increased, decrease in synaptic markers levels, and deficit in NOR). Rolipram has been discontinued in human because of

its narrow therapeutic window, and hence does not fit this project's objectives of drug-discovery.

## 5.5 CONCLUSION

Experiments presented investigated performances of control rats in the WWWhich task, which they were not able to perform in the current conditions of testing. Investigation of synaptic markers SNAP-25 and PSD-95, and inflammatory markers IL-1 $\beta$ , IL-6 and TNF- $\alpha$  showed no deficits in the hippocampus, 35 days after ICV administration of A $\beta$ <sub>01-42</sub>. While hippocampal functions appeared to be preserved, synaptic activity was impaired in the frontal cortex as shown by reduced levels of PSD-95. This impairment was associated with inflammatory phenomenon with increased levels of IL-1 $\beta$  and TNF- $\alpha$ . It thus appears that ICV administration of A $\beta$ <sub>01-42</sub> causes an inflammatory phenomenon in the frontal cortex, which is hypothesised to be associated with local synaptic deficit and could induce the observed cognitive deficits in the NOR task. The role of inflammation is supported by a treatment with rolipram, a PDE-4 inhibitor with antidepressant and anti-inflammatory properties, which can temporarily reverse the NOR deficit. Following the time-course progression of A $\beta$ -toxicity, there appears to be a reduction of PV-containing cells in the frontal cortex, 70 days after surgery, indicating later neuronal damages of particular relevance to cognition.

There is thus strong evidence supporting the appearance of early neuroinflammation in the current model. This phenomenon will be further investigated by treatment with the NSAID mefenamic acid.

**Chapter 6 INVESTIGATING THE EFFECTS OF  
MEFENAMIC ACID ON AMYLOID-B  
OLIGOMER-INDUCED COGNITIVE AND  
NEUROPATHOLOGICAL DEFICITS IN THE  
RAT**



## 6.1 INTRODUCTION

To further study the role of IL-1 $\beta$  in the early inflammatory stages of the model, the NSAID mefenamic acid was assessed as a potential inhibitor of IL-1 $\beta$  production. In the case of sterile inflammatory processes, IL-1 $\beta$  production is initiated in glial cells. In response to damage-associated molecular patterns (DAMPs), a large multi-protein complex called the inflammasome is assembled (Latz et al., 2013). Inflammasomes, and particularly the NLRP3 inflammasome (Nucleotide-binding oligomerisation domain-Like Receptors family, Pyrin Domain containing 3), are known to activate the protease caspase-1 which will in turn cleave inactive pro-IL-1 $\beta$  into the active mature IL-1 $\beta$  form (Lu et al., 2014, Luheshi et al., 2012). The NLRP3 inflammasome has been recently linked to AD and a transgenic mouse model of AD (Heneka et al., 2013, Tan et al., 2013). A $\beta$ o have been recognised as potential DAMPs that can activate the NLRP3 inflammasome and initiate the production and secretion of IL-1 $\beta$  (Masters and O'Neill, 2011, Murphy et al., 2014, Halle et al., 2008). These findings have orientated the drug discovery research to molecules that are inhibitors of the NLRP3 inflammasome (Coll et al., 2015). NSAIDs have been widely suggested as an adjuvant to AD treatment because of their role as COX inhibitors (Gasparini et al., 2004). However, beneficial effects of NSAIDs have been observed as well through a COX-independent pathway (Weggen et al., 2001). Following promising *in vitro* data, the NSAID mefenamic acid is suggested as a strong inhibitor of IL-1 $\beta$  production by targeting NLRP3-dependent inflammation (Daniels et al., 2015 - submitted). Further *in vivo* testing in the current rat model is presented below to assess the preventive effect of mefenamic acid on the A $\beta$ o-induced NOR deficit, neuroinflammatory phenomenon and synaptic markers decrease.

## 6.2 MATERIALS AND METHODS

### 6.2.1 *Animals*

Subjects were 80 adult female Lister Hooded rats (Charles River, UK), divided in two experiments of  $n=40$ , aged 3-month old at the time of surgery. Rats were housed in groups of 5, on a 12-hour light-dark cycle, with free access to food and water, in a controlled environment of temperature ( $21\pm 2^{\circ}\text{C}$ ) and humidity ( $55\pm 5\%$ ) in the Biological Services Facility at the University of Manchester. NOR tasks were conducted during the light cycle, in the morning. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) 1986 Act and University ethical guidelines.

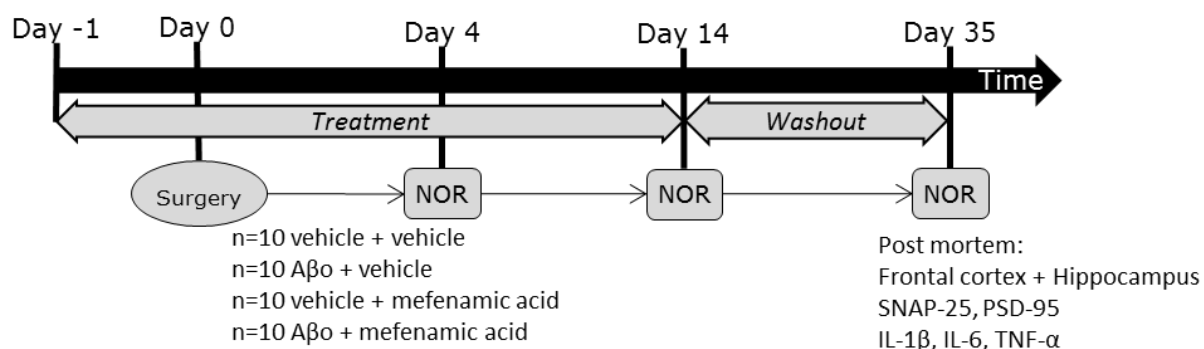
### 6.2.2 *Surgery, NOR task, and biochemical analysis*

Rats were ICV administrated with 10  $\mu\text{L}$  of either vehicle or  $\text{A}\beta_{0_{1-42}}$  5 nmol, on day 0 (Chapter 3 Materials and methods, 3.2.1). Rats were treated with daily IP injection of either saline or mefenamic acid 5 mg/kg, starting from the day before surgery and up to 13 days after surgery. Rats were divided in 4 groups ( $n=10$  per group), for each experiment: (1) Vehicle+Vehicle (control group), (2) Vehicle+Mefenamic acid, (3)  $\text{A}\beta_0$ +Vehicle, (4)  $\text{A}\beta_0$ +Mefenamic acid. In the first experiment, NOR testing (Chapter 3 Materials and methods, 3.3.1) was performed on day 4, day 14 (24H after the last IP injection) and day 35 (after a 21-day washout period) after surgery. In the second experiment, NOR testing was performed only on day 14 after surgery. Brains were then taken on the last day of NOR testing and analysed by ELISA for synaptic (SNAP-25, PSD-95) and inflammatory (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) markers, as described previously (Chapter 3 Materials and methods, 3.4.3 and 3.4.4).

Both experiments (Figure 36) were conducted to investigate neuropathological markers after a 21-day treatment washout period (experiment

A), or while the treatment was still given (Experiment B). The studies have not been run at the same time and behavioural and biochemical results could hence not be pooled together. They will instead be presented separately. The second experiment (without washout period) was conducted after analysis of the results from the first experiment, which suggested that an earlier time point was necessary to better understand the significance of the biochemical results. Only relevant biochemical measures have been repeated.

### Experiment A



### Experiment B

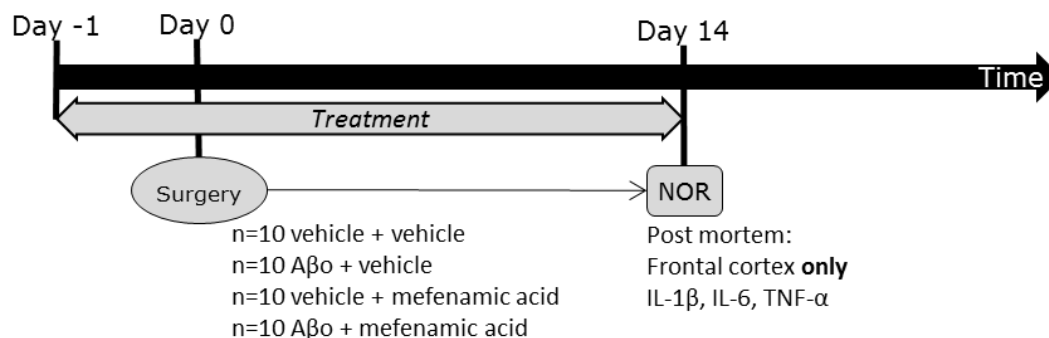


Figure 36: Outline of experiments A and B. Rats are treated with mefenamic acid and tested in the NOR task at day 4, 14, 35 (A) or 14 only (B) after surgery. Synaptic markers and inflammatory markers (A) or inflammatory markers only (B) are investigated on the last day of NOR.

### 6.2.3 Data analysis

Results are expressed as mean  $\pm$  SEM. NOR data were analysed by 1-way ANOVA on repeated measures with object as within-subject factor and group as in-between subject factor. In cases of significance ( $p < 0.05$ ), individual paired samples Student's t-tests were run in each group, comparing the exploration time of both objects. Total exploration time and DI were analysed by 1-way ANOVA and Bonferroni post-hoc. DI were also analysed by univariate Student's t-test versus zero. Levels of synaptic markers (SNAP-25 and PSD-95), as well as inflammatory markers (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ), were analysed by 1-way ANOVA followed by Bonferroni post-hoc. All the statistical analyses have been calculated using IBM SPSS (version 20).

## 6.3 RESULTS

### 6.3.1 Mefenamic acid prevents A $\beta$ -induced deficits in cognition

#### 6.3.1.1 Day 4 after surgery (Experiment A)

##### **Acquisition Phase:** (Figure 37 Top)

During the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,35)}=1.935$   $p>0.05$ ], regardless of group [ $F_{(3,35)}=0.310$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(3,35)}=1.047$   $p>0.05$ ].

##### **Retention Phase:** (Figure 37 Top)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,34)}=30.747$   $p<0.001$ ] with an effect of group [ $F_{(3,34)}=5.784$   $p<0.01$ ]. The control and mefenamic acid treated groups spent more time exploring the novel object: Vehicle+Vehicle [ $t_{(9)}=-4.025$   $p<0.01$ ], Vehicle+Mefenamic acid [ $t_{(8)}=-3.823$   $p<0.01$ ], and A $\beta$ +Mefenamic acid group [ $t_{(9)}=-2.723$   $p<0.05$ ]. There was no difference in exploration for the A $\beta$ +Vehicle group [ $t_{(8)}=-0.961$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(3,34)}=1.259$   $p>0.05$ ].

##### **Discrimination index:** (Figure 37 Top)

A DI significantly superior to zero (equivalent to equal exploration of the objects) was found in the control and mefenamic acid treated groups: Vehicle+Vehicle [ $t_{(9)}=7.109$   $p<0.001$ ], Vehicle+Mefenamic acid [ $t_{(8)}=3.554$   $p<0.01$ ], A $\beta$ +Mefenamic acid [ $t_{(9)}=2.932$   $p<0.05$ ]. In contrast the DI in the A $\beta$ +Vehicle group was not significantly different to zero: [ $t_{(8)}=0.737$   $p>0.05$ ]. There was also a significant difference in DI between groups [ $F_{(3,34)}=7.081$   $p<0.001$ ]. DI of the A $\beta$ +Vehicle group was significantly lower than the Vehicle+Vehicle group

[ $p < 0.001$ ]. Although the A $\beta$ 0+Mefenamic acid group could perform the test, its DI was lower than the Vehicle+Vehicle group [ $p < 0.05$ ].

In summary, IP treatment with mefenamic acid prevented the early A $\beta$ 0-induced deficit in the NOR task at day 4 after surgery.

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### 6.3.1.2 Day 14 after surgery (Experiment A)

**Acquisition Phase:** (Figure 37 Middle)

During the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,36)} = 0.018$   $p > 0.05$ ], regardless of group [ $F_{(3,36)} = 0.656$   $p > 0.05$ ]. Total object exploration was not different between groups [ $F_{(3,36)} = 3.396$   $p > 0.05$ ].

**Retention Phase:** (Figure 37 Middle)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,33)} = 70.220$   $p < 0.001$ ] with an effect of group [ $F_{(3,33)} = 6.446$   $p < 0.001$ ]. The control and mefenamic acid treated groups spent more time exploring the novel object: Vehicle+Vehicle [ $t_{(9)} = -6.182$   $p < 0.001$ ], Vehicle+Mefenamic acid [ $t_{(9)} = -6.578$   $p < 0.001$ ], and A $\beta$ 0+Mefenamic acid group [ $t_{(8)} = -4.406$   $p < 0.01$ ]. There was no difference of exploration for the A $\beta$ 0+Vehicle group [ $t_{(7)} = -0.497$   $p > 0.05$ ]. Total object exploration was not different between groups [ $F_{(3,33)} = 1.586$   $p > 0.05$ ].

**Discrimination index:** (Figure 37 Middle)

A DI significantly superior to zero was found in the control and mefenamic acid treated groups: Vehicle+Vehicle [ $t_{(9)} = 7.439$   $p < 0.001$ ], Vehicle+Mefenamic acid [ $t_{(9)} = 8.183$   $p < 0.001$ ], A $\beta$ 0+Mefenamic acid [ $t_{(8)} = 5.720$   $p < 0.001$ ]. In contrast the DI in the A $\beta$ 0+Vehicle group was not significantly different to zero: [ $t_{(7)} = 0.354$   $p > 0.05$ ]. There was a significant difference in DI between groups [ $F_{(3,36)} = 10.929$   $p < 0.001$ ]. DI of the A $\beta$ 0+Vehicle group was significantly lower

than all the other groups: Vehicle+Vehicle [ $p < 0.001$ ], Vehicle+Mefenamic acid [ $p < 0.001$ ], and A $\beta$ 0+Mefenamic acid [ $p < 0.001$ ].

In summary, IP treatment with mefenamic acid prevented the early A $\beta$ 0-induced deficit in the NOR task at day 14 after surgery.

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### 6.3.1.3 Day 35 after surgery (Experiment A)

**Acquisition Phase:** (Figure 37 Bottom)

During the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,35)} = 0.090$   $p > 0.05$ ], regardless of group [ $F_{(3,35)} = 0.060$   $p > 0.05$ ]. Total object exploration was not different between groups [ $F_{(3,35)} = 1.327$   $p > 0.05$ ].

**Retention Phase:** (Figure 37 Bottom)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,27)} = 102.622$   $p < 0.001$ ] with an effect of group [ $F_{(3,27)} = 11.899$   $p < 0.001$ ]. The control and mefenamic acid treated groups spent more time exploring the novel object: Vehicle+Vehicle [ $t_{(4)} = -5.517$   $p < 0.01$ ], Vehicle+Mefenamic acid [ $t_{(8)} = -5.817$   $p < 0.001$ ], and A $\beta$ 0+Mefenamic acid group [ $t_{(9)} = -7.334$   $p < 0.001$ ]. There was no difference of exploration for the A $\beta$ 0+Vehicle group [ $t_{(6)} = 0.343$   $p > 0.05$ ]. Total object exploration was not different between groups [ $F_{(3,27)} = 0.299$   $p > 0.05$ ].

**Discrimination index:** (Figure 37 Bottom)

A DI significantly superior to zero was found in the control and mefenamic acid treated groups: Vehicle+Vehicle [ $t_{(4)} = 5.445$   $p < 0.01$ ], Vehicle+Mefenamic acid [ $t_{(8)} = 15.459$   $p < 0.001$ ], A $\beta$ 0+Mefenamic acid [ $t_{(9)} = 8.324$   $p < 0.001$ ]. In contrast the DI in the A $\beta$ 0+Vehicle group was not significantly different to zero: A $\beta$ 0+Vehicle group [ $t_{(6)} = -0.636$   $p > 0.05$ ]. There was a significant difference in DI between groups [ $F_{(3,27)} = 21.106$   $p < 0.001$ ]. DI of the A $\beta$ 0+Vehicle group was

significantly lower than all the other groups: Vehicle+Vehicle [ $p<0.001$ ], Vehicle+Mefenamic acid [ $p<0.001$ ], and A $\beta$ o+Mefenamic acid [ $p<0.001$ ].

In summary, IP treatment with mefenamic acid prevented A $\beta$ o-induced deficits in the NOR task at day 35 after surgery. In this study the beneficial effects of mefenamic acid were still observed 21 days after the last IP injection.



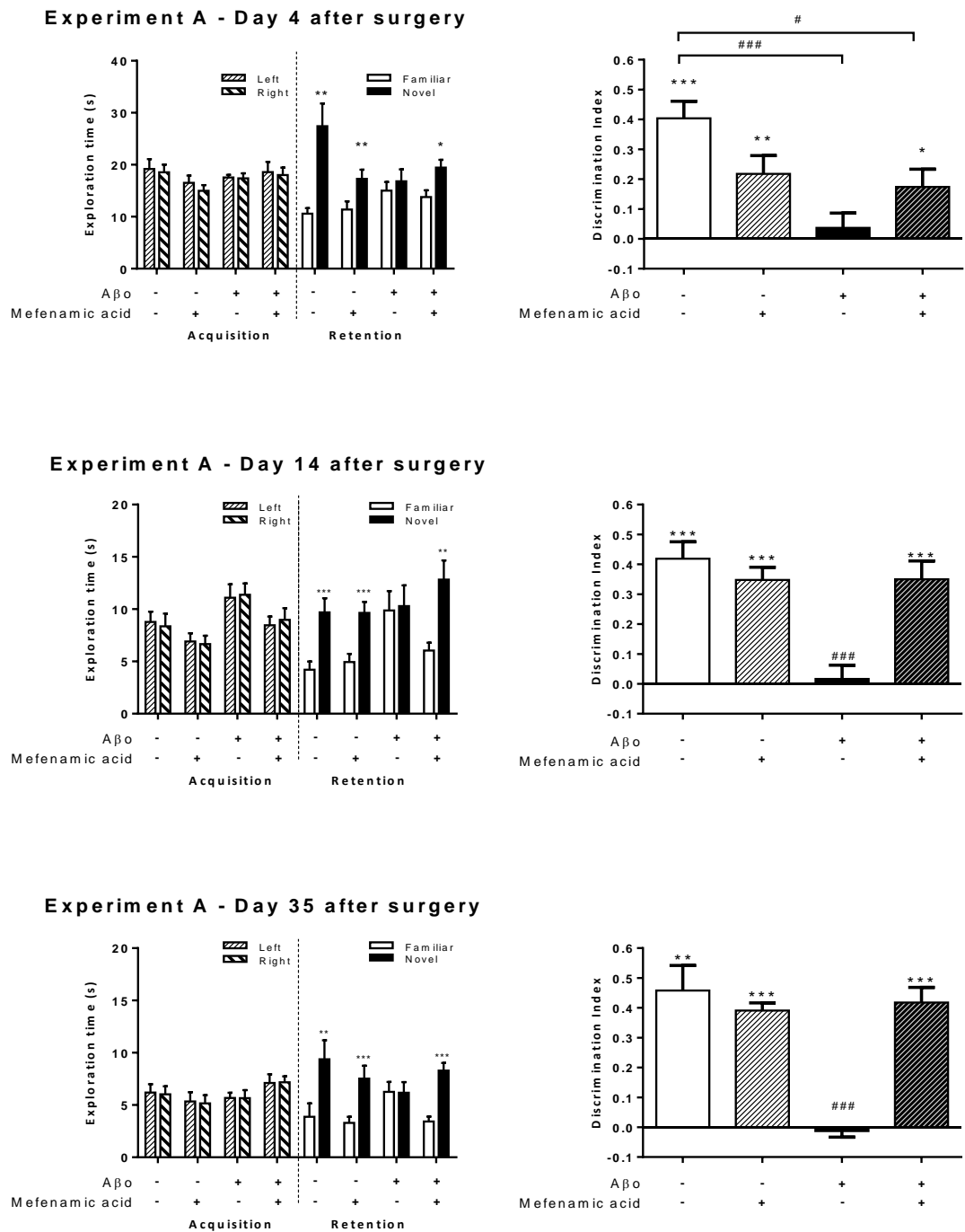


Figure 37: Mefenamic acid treatment reverses A $\beta$ 0-induced NOR deficit. Levels of exploration of the objects were tested on day 4 (top), day 14 (middle), and day 35 (bottom) after surgery. Exploration of the objects (left panel) is presented as mean exploration time + SEM (n=5-10 per group, 5 videos could not be scored following an error in the recording equipment). \*p<0.05 \*\*p<0.01 \*\*\*p<0.001 Novel versus Familiar, paired samples one-way ANOVA followed by Student's t-test. Discrimination index (right panel) is presented as mean DI + SEM (n=5-10 per group). \*p<0.05 \*\*p<0.01 \*\*\*p<0.001 DI versus zero, one-way ANOVA followed by univariate Student's t-test; #p<0.05 ###p<0.001 versus control, one-way ANOVA followed by Bonferroni post-hoc.

### 6.3.1.4 Re-run of Day 14 after surgery (Experiment B)

These results were confirmed by the second cognitive experiment where the NOR performances were investigated on day 14.

#### **Acquisition Phase:** (Figure 38 Left)

During the acquisition phase, all groups explored both objects equally. There was no difference in left/right object exploration [ $F_{(1,33)}=1.162$   $p>0.05$ ], regardless of group [ $F_{(3,33)}=1.081$   $p>0.05$ ].

#### **Retention Phase:** (Figure 38 Left)

During the retention phase, there was a significant difference of exploration between the familiar and novel object [ $F_{(1,33)}=42.961$   $p<0.001$ ] however, the group effect was unclear [ $F_{(3,33)}=2.698$   $p>0.05$ ]. It can be assumed that 3 of the groups had similar preference for the novel object, which reduced the significance of the test. It appeared that the control and mefenamic acid treated groups spent more time exploring the novel object: Vehicle+Vehicle [ $t_{(8)}=-3.534$   $p<0.01$ ], Vehicle+Mefenamic acid [ $t_{(9)}=-4.689$   $p<0.01$ ], and A $\beta$ o+Mefenamic acid group [ $t_{(8)}=-3.289$   $p<0.05$ ]. There was no difference of exploration for the A $\beta$ o+Vehicle group [ $t_{(8)}=-1.241$   $p>0.05$ ]. Total object exploration was not different between groups [ $F_{(3,33)}=1.287$   $p>0.05$ ].

#### **Discrimination index:** (Figure 38 Right)

A DI significantly superior to zero was found in the control and mefenamic acid treated groups: Vehicle+Vehicle [ $t_{(8)}=3.995$   $p<0.01$ ], Vehicle+Mefenamic acid [ $t_{(9)}=6.127$   $p<0.001$ ], A $\beta$ o+Mefenamic acid [ $t_{(8)}=4.221$   $p<0.01$ ]. In contrast the DI in the A $\beta$ o+Vehicle group was not significantly different to zero: [ $t_{(8)}=0.636$   $p>0.05$ ]. There was a significant difference in DI between groups [ $F_{(3,33)}=3.452$   $p<0.05$ ]. Planned comparison vs. DI of the A $\beta$ o+Vehicle group showed that it was significantly lower than Vehicle+Vehicle [ $p<0.05$ ] and A $\beta$ o+Mefenamic acid [ $p<0.05$ ].

In summary, IP treatment with mefenamic acid prevented the early A $\beta$ -induced deficit in the NOR task at day 14 after surgery.

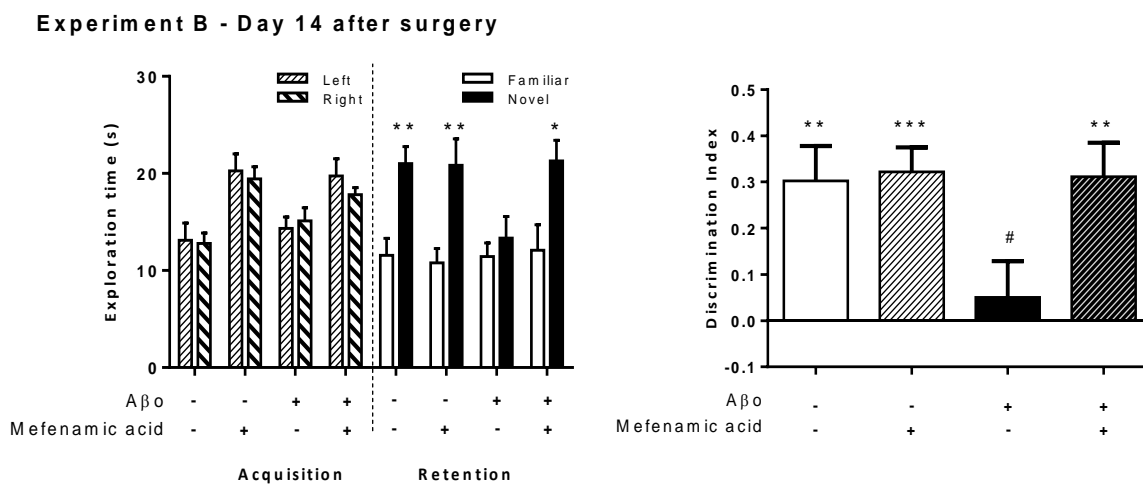


Figure 38: Confirmation that mefenamic acid treatment reverses A $\beta$ -induced NOR deficit. Levels of exploration of the objects were tested on day 14 after surgery. Exploration of the objects (left panel) is presented as mean exploration time + SEM (n=9-10 per group). \* $p < 0.05$  \*\* $p < 0.01$  Novel versus Familiar, paired samples one-way ANOVA followed by Student's *t*-test. Discrimination index (right panel) is presented as mean DI + SEM (n=9-10 per group). \*\* $p < 0.01$  \*\*\* $p < 0.001$  DI versus zero, one-way ANOVA followed by univariate Student's *t*-test; # $p < 0.05$  versus control, one-way ANOVA followed by planned comparison.

### 6.3.2 Mefenamic acid does not prevent neuropathological changes

#### 6.3.2.1 Biochemical analysis, Experiment A, hippocampus, day 35

##### **Synaptic Markers:** (Figure 39 Top)

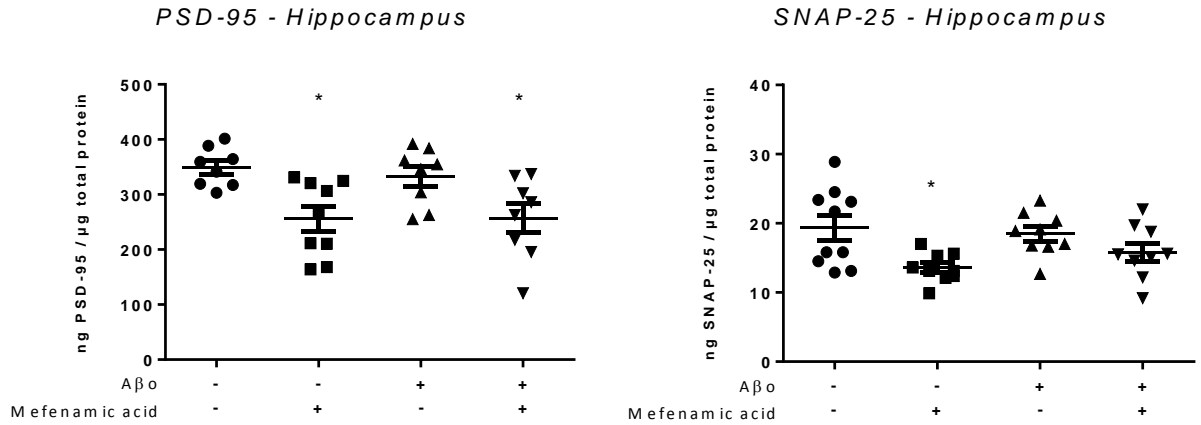
There was a significant difference in PSD-95 levels between groups [ $F_{(3,29)}=5.555$   $p<0.01$ ]. Concentrations in the Vehicle+Mefenamic acid and A $\beta$ o+Mefenamic acid groups were lower than control, both [ $p<0.05$ ]. For statistical analysis, levels of SNAP-25 have been transformed in Log10 of their respective values to obtain homogeneity of variance between groups. There was a significant difference in SNAP-25 levels between groups [ $F_{(3,33)}=4.107$   $p<0.01$ ]. Concentration of SNAP-25 in the Vehicle+Mefenamic acid group were lower than control [ $p<0.05$ ].

##### **Neuroinflammatory Markers:** (Figure 39 Bottom)

Levels of IL-6 and TNF- $\alpha$  were not significantly different between groups, respectively [ $F_{(3,33)}=1.113$   $p>0.05$ ] and [ $F_{(3,33)}=0.797$   $p>0.05$ ].

In summary, there was no change in synaptic or inflammatory markers in the hippocampus on day 35 after surgery in A $\beta$ o treated animals. Interestingly levels of the post-synaptic marker PSD-95 were reduced in groups following treatment with mefenamic acid.

Synaptic markers



Neuroinflammatory markers

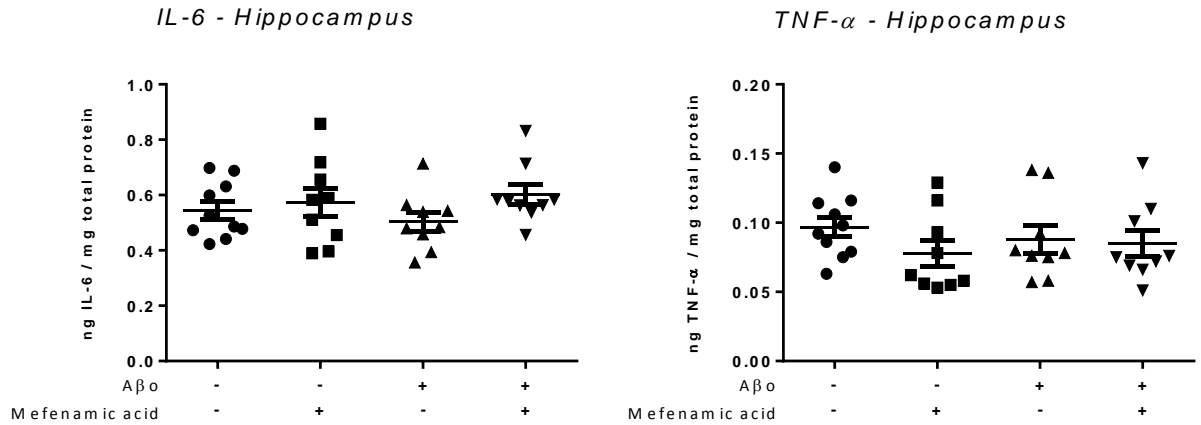


Figure 39: Levels of synaptic (top) and neuroinflammatory (bottom) markers in the hippocampus, 35 days after surgery. Data are presented as individual plots and mean $\pm$ SEM ( $n=8-10$  per group), \* $p<0.05$  versus control, one way ANOVA and Bonferroni post-hoc.

### 6.3.2.2 Biochemical analysis, Experiment A, frontal cortex, day 35

#### **Synaptic Markers:** (Figure 40A)

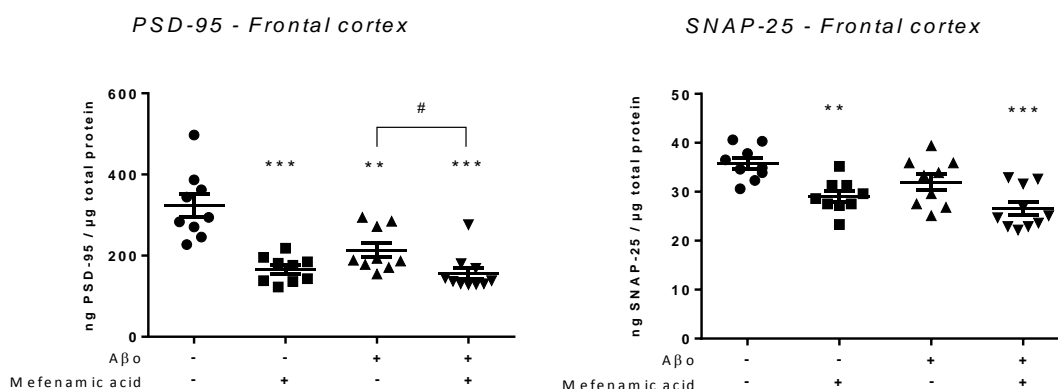
For statistical analysis, levels of PSD-95 have been transformed in Log10 of their respective values to obtain homogeneity of variance between groups. There was a significant difference in PSD-95 levels between groups [ $F_{(3,33)}=18.461$   $p<0.001$ ]. All groups had reduced levels of PSD-95 when compared to control: Vehicle+Mefenamic acid [ $p<0.001$ ], A $\beta$ o+Vehicle [ $p<0.01$ ] and A $\beta$ o+Mefenamic acid [ $p<0.001$ ]. Moreover, there was a significant reduction of PSD-95 levels in both mefenamic acid treated groups when compared to A $\beta$ o+Vehicle [ $p<0.05$ ]. There was a significant difference in SNAP-25 levels between groups [ $F_{(3,33)}=9.008$   $p<0.001$ ]. Only groups treated with mefenamic acid showed significantly reduced levels when compared to control: Vehicle+Mefenamic acid [ $p<0.01$ ], and A $\beta$ o+Mefenamic acid [ $p<0.001$ ].

#### **Neuroinflammatory Markers:** (Figure 40B)

Levels of IL-6 and TNF- $\alpha$  were not significantly different between groups, [ $F_{(3,33)}=2.514$   $p>0.05$ ] and [ $F_{(3,33)}=1.546$   $p>0.05$ ] respectively. In contrast, there was a significant difference in levels of IL-1 $\beta$  between groups [ $F_{(3,28)}=10.201$   $p<0.001$ ]. The level of IL-1 $\beta$  was significantly higher in the group administrated with A $\beta$ o+Vehicle [ $p<0.01$ ]. This effect was not reversed by treatment with mefenamic acid (Vehicle+Mefenamic acid [ $p<0.01$ ]) when compared to control.

In summary, A $\beta$ o administration induced a deficit in the post-synaptic marker PSD-95 and an increase in IL-1  $\beta$  levels in the frontal cortex 35 days after surgery. These neuropathological markers were not rescued by treatment with mefenamic acid, after a 21-day washout period.

**Synaptic markers**



**Neuroinflammatory markers**

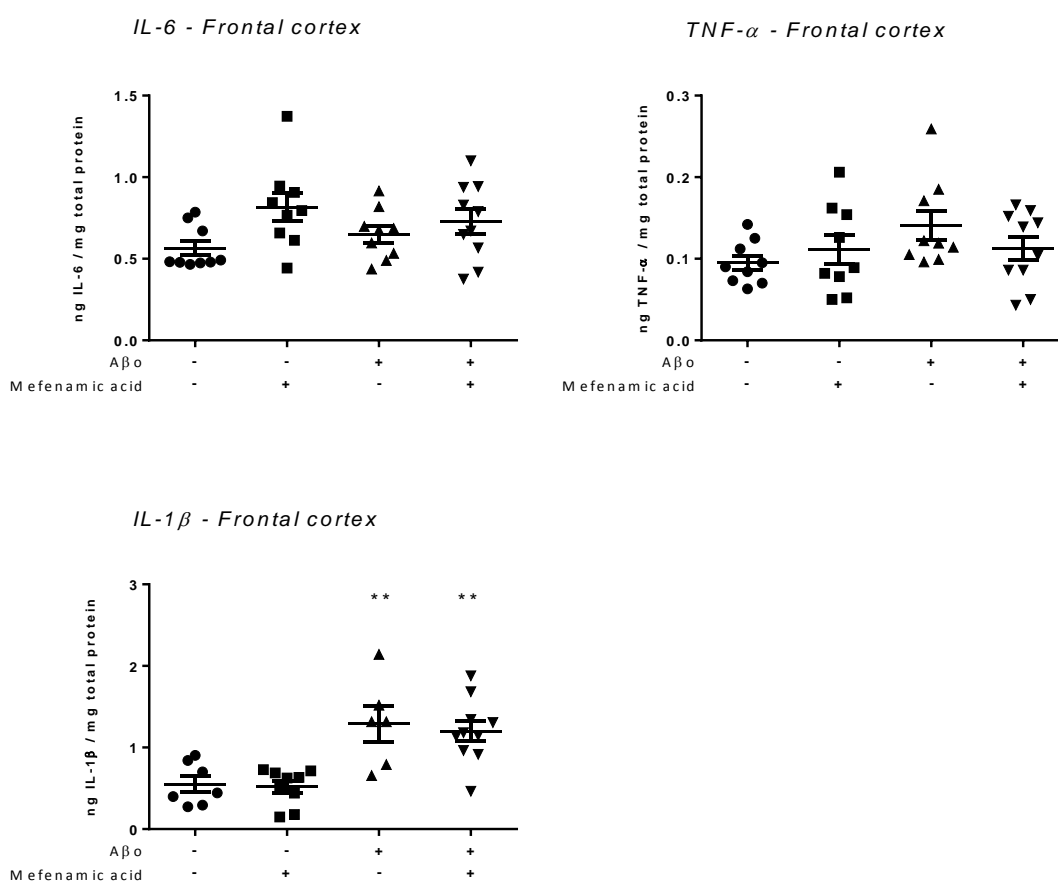


Figure 40: Levels of synaptic (top) and neuroinflammatory (bottom) markers in the frontal cortex, 35 days after surgery. Data are presented as individual plots and mean  $\pm$  SEM ( $n=8-10$  per group), \*\* $p<0.01$ , \*\*\* $p<0.001$  versus control, #  $p<0.05$  between groups, one way ANOVA and Bonferroni post-hoc.

### **6.3.2.3 Biochemical analysis, Experiment B, frontal cortex, day 14**

To confirm whether the high levels of IL-1 $\beta$  in the A $\beta$ o+Mefenamic acid group in the previous study (Figure 40B) where due to an inability of the mefenamic acid to prevent the inflammation or due to the washout period, inflammatory markers (but not synaptic markers) have been measured in the frontal cortex in a second experiment, 14 days after surgery and chronic treatment (Figure 36 Experiment B).

#### **Neuroinflammatory Markers:** (Figure 41)

Levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were not significantly different between groups, respectively [ $F_{(3,33)}=1.464$   $p>0.05$ ], [ $F_{(3,33)}=0.474$   $p>0.05$ ], [ $F_{(3,33)}=1.249$   $p>0.05$ ].

In summary, A $\beta$ o administration had no effect on neuroinflammatory markers levels in the frontal cortex 14 days after surgery.



**Neuroinflammatory markers - Experiment B**

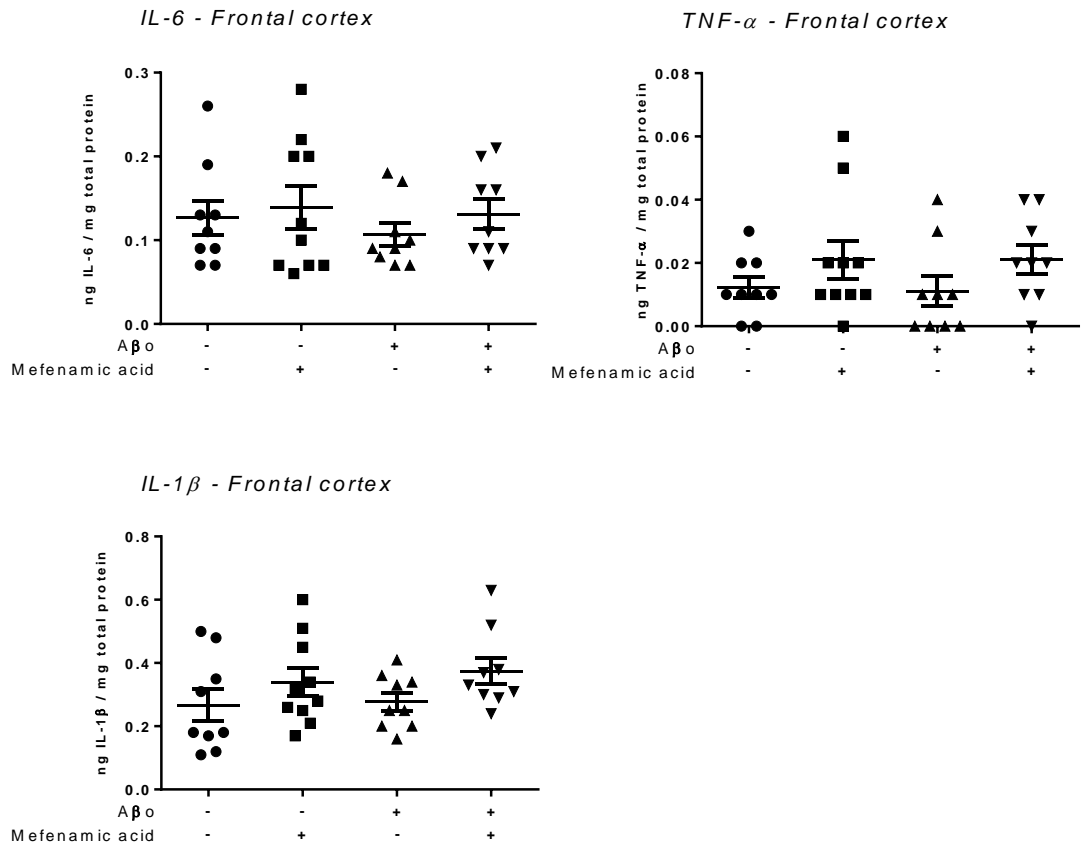


Figure 41: Levels of neuroinflammatory markers in the frontal cortex, 14 days after surgery. Data are presented as individual plots and mean $\pm$ SEM (n=9-10 per group), no significant difference between groups, one way ANOVA.

## 6.4 DISCUSSION

These data show that mefenamic acid, a fenamate demonstrated to inhibit neuroinflammation by blocking NLRP3 inflammasome induced release of IL-1 $\beta$ , prevented A $\beta$ -induced deficits in the NOR task (Figure 38, Figure 37A,B). This protective effect, following a 14-day chronic treatment, lasted even after a washout period of 21 days (Figure 37C). From previous results with chronic treatment (rolipram), where the NOR deficit reappeared after cessation of treatment (Chapter 5, 5.4.3 Results), it can be hypothesised that (1) A $\beta$ <sub>1-42</sub> might still be present and able to inhibit recognition memory after cessation of the treatment, or that (2) the oligomers are eventually cleared out but the rolipram treatment had a symptomatic effect. In the latter case, the treatment could have provided a temporary prevention of the inflammatory phenomenon which is later overcome by other detrimental mechanisms (such as synaptic deficit or LTP impairment) when the treatment is stopped. In the current study it appears that the protective effect of mefenamic acid lasts at least for 21 days after cessation of the treatment, suggestive a stronger action with interesting therapeutic applications.

Investigation of synaptic markers showed no deficit in PSD-95 and SNAP-25 levels in the hippocampus in the A $\beta$ +Vehicle animals (Figure 39A). However, levels of PSD-95 in the A $\beta$ +Vehicle were significantly lower than control in the frontal cortex, while levels of SNAP-25 were unchanged (Figure 40A). This deficit in PSD-95 levels was not rescued by treatment with mefenamic acid. Moreover, levels were decreased in mefenamic acid-treated groups, whether they had been administered with A $\beta$  or not. This could be due to the treatment itself or and will need to be repeated for further investigation.

Levels of inflammatory markers (IL-6, TNF- $\alpha$ , and IL-1 $\beta$ ) were not modified in the hippocampus (Figure 39B), supporting the hypothesis that this structure

remains unaffected at this time-point. In the frontal cortex, levels of IL-6 and TNF- $\alpha$  were not modified in any of the groups. Neuroinflammation was confirmed by increased levels of IL-1 $\beta$  in the A $\beta$ <sub>0</sub>+Vehicle treated animals, an effect that was surprisingly not rescued by treatment with mefenamic acid. (Figure 40B). When assessing the effect of mefenamic acid on inflammatory markers in the current study, it is important to consider the time point at which the biochemical analysis was conducted. Analyses were carried out 35 days after administration of A $\beta$ <sub>01-42</sub> and 21 days after cessation of the mefenamic acid treatment. Levels of IL-6 and TNF- $\alpha$  are known to peak within a few days following A $\beta$  administration (Rosales-Corral et al., 2004). It is thus possible that the time point of this study is past this primary inflammatory response. The dual role of IL-6 as a pro- and anti-inflammatory cytokine (Hunter and Jones, 2015) could present a possible explanation of the control-like level observed, as discussed in the previous chapter (Chapter 5, 5.3 Neuroinflammatory, synaptic and neuronal markers). However, increased levels of IL-1 $\beta$  at day 35 after surgery suggest that A $\beta$  induce a sustained inflammatory response. Raised IL-1 $\beta$  levels further supports the involvement of the NLRP3 inflammasome in the current model. Despite a clear inhibition of IL-1 $\beta$  production by mefenamic acid *in vitro* (Daniels et al., 2015 - submitted), levels *in vivo* remained higher than control and similar to non-treated animals (Figure 40B). Discontinuation of the treatment for 21 days could explain raised levels of IL-1 $\beta$ . It can be hypothesised that after 21 days, the inflammatory process initiated by A $\beta$  arises again. The lack of lasting effect with the mefenamic acid treatment could also highlight additional inflammatory mechanisms independent from NLRP3 activation. This suggest that either the NOR deficit could reappear after a further period without treatment or that mefenamic acid initiated compensative mechanisms that maintain recognition memory performances at a control level without preventing the whole neuroinflammatory phenomenon.

I then tried to elucidate the concern raised by the washout period by conducting neuroinflammatory markers analysis on day 14 after surgery, while the treatment is still on board. Results were puzzling as the increase in IL-1 $\beta$  levels in the frontal cortex could not be repeated (Figure 41). This raises new questions regarding the inflammatory phenomenon in the current model and did not allow us to investigate the efficacy of the mefenamic acid treatment as an NLRP-3 inflammasome inhibitor. It appears as well that the concentrations of all the neuroinflammatory markers vary from a 5 to 10-fold between the two experiments (Figure 40B and Figure 41). This is probably due to an issue in repeatability during the brain extraction, sample preparation and ELISA. It is noteworthy to highlight that in the literature, ELISA results are sometimes presented in percentage or ratio to control rather than absolute value, in order to avoid this variability. In the context of this experiment, it has been decided to present the results in their absolute levels.

Future work thus includes repeating this experiment (1) to confirm a potential effect of mefenamic acid itself on synaptic markers, (2) conducting biochemical analysis at an earlier time point, i.e. within 36 hours after surgery, to confirm the apparition of an inflammatory phenomenon (Rosales-Corral et al., 2004), (3) investigating a more lasting inflammatory marker, such as microglia, to confirm the role of neuroinflammation in the model. This last step will be run on the right hemisphere of the brains from this study.

In conclusion, mefenamic acid presents potential therapeutic effects in the current rat model. Meta-analyses have shown a potential beneficial effect of NSAIDs in AD (in t' Veld et al., 2001). *In vitro*, it has been found that NSAIDs could reduce the production of A $\beta$ <sub>1-42</sub>, via a mechanism independent from COX inhibition (Weggen et al., 2001). Supporting data showed the role of the NLRP3 inflammasome in AD and transgenic mouse model, providing an explanation to

the efficacy of NSAID independently of COX inhibition (Heneka et al., 2013). These results suggest mefenamic acid as a potential treatment for the inflammatory phenomenon observed in AD, supported by *in vitro* evidence of its role as an NLRP3 inflammasome inhibitor (Daniels et al., 2015 - submitted). Moreover, mefenamic acid is already used in clinic for the treatment of menstrual pain, allowing a documented study of its safety. Retargeting a drug presents interesting advantages in terms of treatment availability and safety, allowing the drug to move more rapidly to clinical trials. Additional epidemiological data on the benefits of targeting the NLRP3 inflammasome and inhibiting IL-1 $\beta$  in AD patients would facilitate its future use.

## Chapter 7 CONCLUSIONS

## 7.1 MAIN RESULTS

With this project, I have established a rat model that produces cognitive (recognition memory deficits) and biochemical deficits (deficits in synaptic markers) following acute ICV administration of 5 nmol of small stable A $\beta$ <sub>1-42</sub>. The recognition memory deficit presented early (4 days after surgery), was found to be long-lasting (up to 70 days after surgery) and reproducible over the different experiments run. The deficit was found in both female and male rats, and was reproducible with doses of A $\beta$  as low as 0.5 nmol. Working memory in the Y-maze was not affected 35 days after surgery. When assessing performances in the NOR task, it appeared that attention played a role in the memory deficit with performances similar to control when no ITI was present.

Biochemical analyses showed that, in the hippocampus, there was no changes in the levels of synaptic (SNAP-25 and PSD-95) or inflammatory markers (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ), 35 days after surgery. However, in the frontal cortex, levels of the post-synaptic markers, PSD-95, were decreased 35 days after surgery. Levels of IL-1 $\beta$  and TNF- $\alpha$  were increased 35 days after surgery, but this change was not reproduced when assessing the same markers 14 days after surgery. When looking at neuronal markers, levels of the general neuronal marker NAA, were unchanged across the brain, 14 days after surgery. However investigation of the GABAergic system, in particular the PV subset of GABAergic interneurons demonstrated a significant deficit in PV-positive cell density in the frontal cortex, 70 days after surgery.

Drug intervention studies found that chronic treatment with rolipram (0.1 mg/kg daily for two weeks, starting the day before surgery), could prevent the appearance of the cognitive deficit in the NOR task. This effect was not sustained following a 3-week washout period. Treatment with the non-steroidal anti-inflammatory mefenamic acid (5 mg/kg), following the same treatment-regime

could not only prevent the appearance of the cognitive deficit in the NOR task but its effect was sustained, even after a 3-week washout period. However in this study the levels of synaptic and inflammatory markers were not rescued by treatment with mefenamic acid.

A summary of the main findings are presented below in Table 4.

Day after surgery	4	14	35	70
<b>Cognition</b>	NOR deficit in both female and male rats	NOR deficit no NOR deficit when no ITI (male rats)	NOR deficit at all doses tested (0.5, 1 and 5 nmol) no Y-maze deficit	NOR deficit
<b>Synaptic markers</b>	<i>Frontal cortex:</i> n/a	n/a	$\leftrightarrow$ SNAP-25 $\downarrow$ PSD-95	n/a
	<i>Hippocampus:</i> n/a	n/a	$\leftrightarrow$ SNAP-25 $\leftrightarrow$ PSD-95	n/a
<b>Inflammatory markers</b>	<i>Frontal cortex:</i> n/a	$\leftrightarrow$ IL-1 $\beta$ $\leftrightarrow$ IL-6 $\leftrightarrow$ TNF- $\alpha$	$\uparrow$ IL-1 $\beta$ $\leftrightarrow$ IL-6 $\uparrow$ TNF- $\alpha$	n/a
	<i>Hippocampus:</i> n/a	n/a	$\leftrightarrow$ IL-1 $\beta$ $\leftrightarrow$ IL-6 $\leftrightarrow$ TNF- $\alpha$	n/a
<b>Neuronal marker</b>	n/a	No changes in NAA levels	n/a	<b>Frontal cortex:</b> $\downarrow$ PV levels
<b>Rolipram (2 weeks)</b>	n/a	Reverses NOR deficit	NOR deficit back	n/a
<b>Mef. acid (2 weeks)</b>	Reverses NOR deficit	Reverses NOR deficit	Reverses NOR deficit  Does not reverse neuropathological markers	n/a

Table 4: Summary of the main results from the experiments presented. Unless otherwise specified, tests are run in female Lister hooded rats; dose of A $\beta$ <sub>1-42</sub> is 5 nmol in 10  $\mu$ L. Mef. Acid: Mefenamic acid,  $\leftrightarrow$ : no change,  $\downarrow$ : decreased levels,  $\uparrow$  increased levels, n/a: not assessed



## 7.2 VALIDITY OF THE MODEL

When compared to the models discussed in Chapter 2 (Systematic review of rat models based on the administration of amyloid- $\beta$  peptides), the current model presents a similar rapid and lasting memory deficit. While recognition memory was the main cognitive task investigated, investigating other cognitive tests, e.g. MWM, would bring an added asset by assessing learning and spatial memory in the model. The Y-maze has shown inconsistent deficits in other A $\beta$ -peptide administration models, so the lack of deficit in the current model was not surprising and in line with our biochemical data, probably highlights a preservation of the hippocampal functions at the time points tested. The NOR deficit has been reproducible over the experiments (Chapter 3) and hence presents a reliable, ethologic task. The current model presents good face validity when compared to similar A $\beta$ -peptide administration rat models.

This particular model is based around the administration of very stable low-n soluble A $\beta$  (Chapter 3). Similar to other models of this type, the main caveat remains the construct validity. Contrary to AD and genetic rodent models, the pathology in the current model does not result from a slow build-up of amyloid over time, but rather an acute A $\beta$ -induced pathology. It is thus important to stress that the model presented is a model of A $\beta$  pathology of relevance for AD research, in relation to investigating early mechanisms of relevance to the synaptic and cognitive deficits observed, but not a model of that incorporates the entire pathology itself. Hence, the translatability remains the main unmet need in this model. However, the appearance of a synaptic deficit and the indications of neuroinflammatory changes (Chapter 5) are consistent with features observed in AD and other A $\beta$ -peptide administration rat models. Moreover, the current model is supported by *in vitro* and *in vivo* data in mouse, that demonstrates the stability and toxicity of the A $\beta$  (reviewed in Chapter 4, 4.1 Introduction).

The predictive validity of the current model has not been thoroughly investigated. First, current treatments mainly focus on the cholinergic deficit and end stages of the disease while the current model investigates the early stages. However, results from the rolipram study (Chapter 5) and the mefenamic acid study (Chapter 6), are promising and support the neuroinflammatory hypothesis.

### 7.3 FUTURE WORK

There are two main axes that this project will now follow. It is first important to find reliable biochemical markers and investigate how these markers change over time.

Following on from the studies looking at levels of IL-1 $\beta$  and TNF- $\alpha$  in the frontal cortex, changes in other inflammatory markers and more importantly their time-course kinetics could be investigated. It is also planned to look at complimentary markers of neuroinflammatory changes, rather than the cytokines alone, whose levels might only be increased following the first days after surgery (Rosales-Corral et al., 2004). We will use immunohistochemistry for assessing microglia activation in the frontal cortex, from brain sections of the animals used in Chapter 6 (Experiment B, Day 14).

To further investigate synaptic markers, measuring synaptophysin might prove a more relevant and documented technique. Measurement of the neuropathological markers could also prove more sensitive and reliable by measuring more defined, smaller areas of the brains. For example by focusing on the CA1, CA3 areas rather than the whole hippocampus, by focusing on the prefrontal cortex rather than whole frontal cortex and by measuring levels of these markers in the perirhinal cortex which is known to be involved in the NOR task (Barker et al., 2007, Norman and Eacott, 2004). This could be achieved by

modifying the protocol of brain extraction and utilising a more accurate technique such as cryostat slicing.

The second main development of the project will be drug screening. By following a protocol similar to treatment with mefenamic acid (Chapter 6), other drugs can be investigated in that model, for both their effect on the cognitive deficit and their effect on biochemical markers. Grant applications with collaborators from other labs at the University of Manchester are currently being discussed and submitted.

Altogether, this project succeeded in validating a model of relevance for AD research, with a clear cognitive deficit and changes in biochemical markers following ICV administration of well characterised, soluble low-n A $\beta$ <sub>01-42</sub>. This work remains an ongoing project with the ultimate aim of providing a successful model to aid AD research that will help selecting new drugs or repurposing current drugs for AD treatment.

## APPENDIX

### I. CONFERENCES ATTENDED

- Alzheimer Research UK conference – Manchester (UK) – March 2016
- The Little Event – Birmingham (UK) – January 2016
- LGBTSTEM – Sheffield (UK) – January 2016
- Alzheimer's Association International Conference – Washington D.C. (USA) – July 2015
- Dementia Research Day / Manchester Institute for Collaborative Research on Ageing (MICRA) – Manchester (UK) – June 2015
- Alzheimer Research UK conference – London (UK) – March 2015
- British Association of Psychopharmacology summer meeting – Cambridge (UK) – July 2014
- Dementia Research Day / Manchester Institute for Collaborative Research on Ageing (MICRA) – Manchester (UK) – June 2014

### II. PRIZES AND AWARDS

- The Little Event. Bursary to attend the Little Event workshop on public engagement, and membership toward annual BIG STEM communication network – January 2016
- 1<sup>st</sup> prize for best final oral presentation – Manchester Pharmacy School Postgraduate Research day – Manchester (UK) – April 2015
- Bursaries for BAP Training Members – Bursary to attend the British Association of Psychopharmacology summer meeting – 2014

### III. PUBLIC ENGAGEMENT AND TEACHING

- Volunteer for the science series "Genius" by Prof. Stephen Hawking – Bigger Bang production (<http://biggerbang.tv/>), aired on PBS and National Geographic – 2015/2016
- Tutor (Engineering, Biology) for KS2 and KS4 level pupils for the charity The Brilliant Club (<http://www.thebrilliantclub.org/>) – 2015/2016
- STEM Ambassador (<http://www.stemnet.org.uk/>) – 2015/2016
- Teaching assistant – Manchester Pharmacy School, The University of Manchester – 2015/2016
- Invited speaker at Swansea University – 2016
- MOOC forum moderator for the University of Manchester – 2013
- Member of the British Association of Psychopharmacology, Dementia Friends, BIG STEM communication network

#### IV. POSTERS

All poster realised during this PhD are presented on the following pages.

- As first author:

William Watremez, JC Neill, L Abdi, C McKee, R Rusli, B Grayson, N Fisher, T Lefebvre, V Koziel, T Pillot, MK Harte. Investigating cognitive deficits and neuronal markers in an animal model of relevance to the early stages of Alzheimer's disease: ICV administration of soluble amyloid- $\beta$  oligomers in the rat. - Presented at the British Association of Psychopharmacology summer conference - Cambridge (UK) - 2014

William Watremez, JC Neill, V Koziel, T Pillot, MK Harte. Acute ICV administration of soluble amyloid- $\beta$  oligomers induces robust cognitive deficits in rat. - Presented at the Alzheimer Research UK conference - London (UK) - 2015

William Watremez, V Fasolino, M Daniels, C Lawrence, J Jackson, G Galea, JC 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. - Presented at the University of Manchester Postgraduate Research Summer Showcase - Manchester (UK) - 2015

William Watremez, V Fasolino, J Jackson, S McLean, JC Neill, Thierry Pillot, MK Harte. Investigating cognitive deficit and pharmacological treatment in a rat model of amyloid- $\beta$  administration. - Presented at the Alzheimer Research UK conference - Manchester (UK) - 2016

- As co-author:

T. Pillot, W. Watremez, A. Allouche, J. Neill, B. Grayson, M. Harte. Stabilized low number amyloid- $\beta$  oligomers induce cognitive deficits associated with synaptic dysfunction in rats. - Presented at the Alzheimer Disease & Parkinson Disease conference - Nice (France) - 2015

Michael JD Daniels, Jack Rivers-Auty, Victoria Fasolino, William Watremez, Alex Baldwin, James Bagnall, James Galea, Hervé Boutin, Pawel Paszek, Thierry Pillot, Michael Harte, Catherine Lawrence, Sally Freeman, David Brough. Non-steroidal anti-inflammatory drugs (NSAIDs) as inhibitors of the NLRP3 inflammasome. - Presented at the Innate Immune Memory conference - Cambridge (UK) - 2015

Joshua D. Jackson, Heledd H. Griffiths, William JB. Watremez, Nigel M. Hooper, Michael K. Harte. Blockade of the cellular prion protein (PrP<sup>C</sup>) prevents oligomeric amyloid- $\beta$ -induced cognitive deficits in vivo. - Manchester Pharmacy School Postgraduate Research day - Manchester (UK) - 2015

## Investigating cognitive deficits and neuronal markers in an animal model of relevance to the early stages of Alzheimer's disease: ICV administration of soluble amyloid- $\beta$ oligomers in the rat



William Watremez<sup>1</sup>, JC Neill<sup>1</sup>, L Abdi<sup>1</sup>, C McKee<sup>1</sup>, R Rusli<sup>1</sup>, B Grayson<sup>1</sup>, N Fisher<sup>2</sup>, T Lefebvre<sup>2</sup>, V Koziel<sup>2</sup>, T Pilot<sup>2</sup>, MK Harte<sup>1</sup>,  
<sup>1</sup> Manchester Pharmacy School, University of Manchester, Manchester, M13 9PT, UK  
<sup>2</sup> SynAging, Institut Polytechnique National de Lorraine, Vandœuvre-lès-Nancy, FRANCE



### Introduction

With current treatments for Alzheimer's disease (AD) only providing temporary symptomatic benefits and an ageing population, disease modifying drugs are urgently required. This approach relies on improved understanding of the early pathophysiology of AD. A new hypothesis has emerged, in which early memory loss is considered a synapse failure caused by soluble Amyloid- $\beta$  ( $A\beta$ ) oligomers. These small soluble  $A\beta$  oligomers, which precede the formation of larger fibrillar assemblies, may be the main cause of early AD pathologies.

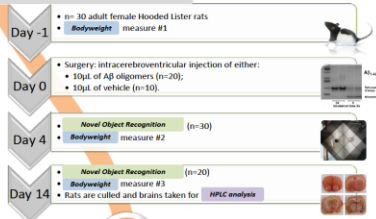
first symptomatic stages of the disease vary between physiological and low levels. Thus NAA has been suggested as a potential diagnostic marker for the conversion from mild cognitive impairment to dementia.

The aim of the current study was to determine whether the cognitive deficits observed following acute intracerebroventricular (ICV) administration of soluble  $A\beta$  oligomers in the rat was concomitant with changes in the levels of NAA in the brain.

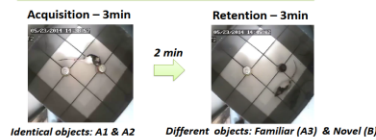
Previous studies have reported decreased N-acetylaspartate (NAA), a marker of neuronal loss, in AD patients while studies in patients during the

### Methods

#### Overview

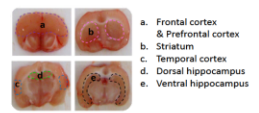


#### Novel Object Recognition (n=10-20/group)



#### HPLC analysis (n=5 per group)

Samples from the following brain areas:

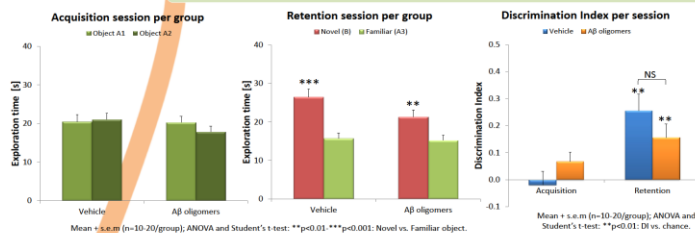


Comparison of NAA concentration between vehicle and  $A\beta$  oligomers group for each brain area

Short term memory indexes  
 \*Time spent exploring: novel ( $t_{nov}$ ) vs. familiar ( $t_{fam}$ ) object;  
 \*Discrimination Index =  $\frac{t_{nov} - t_{fam}}{t_{nov} + t_{fam}}$

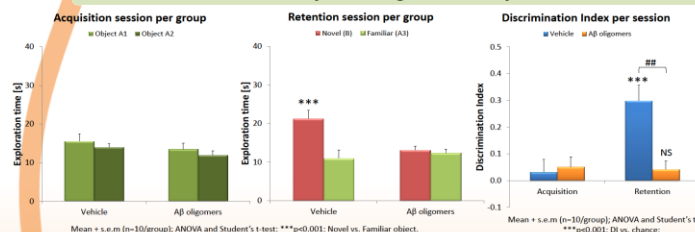
### Results

#### Novel Object Recognition – Day 4



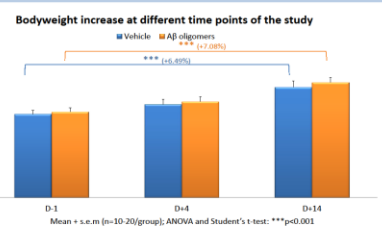
Acquisition: Both groups equally explored the two identical objects. Retention: the Vehicle group could clearly identify the new object while the  $A\beta$  oligomers group tended to perform less.

#### Novel Object Recognition – Day 14



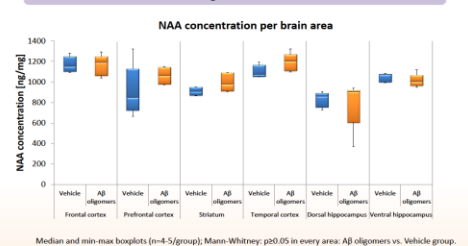
Retention: 14 days after surgery, vehicle treated rats spent significantly more time exploring the Novel compared to the Familiar object, an effect that was abolished in  $A\beta$  treated animals.

#### Bodyweight



ICV administration of  $A\beta$  oligomers did not alter the bodyweight increase

#### HPLC analysis : NAA levels



ICV administration of  $A\beta$  oligomers did not significantly modify the brain levels of NAA, compared to Vehicle

### Conclusions

- ICV administration of  $A\beta$  oligomers did not alter the bodyweight of the rat or their general health and behaviour.
- Short term memory is slightly impaired 4 days after  $A\beta$  administration, with a clear and significant deficit on day 14 compared with vehicle.
- The cognitive deficit observed in the NOR is not caused by an impaired neuronal activity as the NAA levels follow physiological values.

Hence, findings from the present study suggest that acute ICV administration of soluble  $A\beta$  oligomers causes robust cognitive deficits without causing neuronal loss. It is hypothesised that the early cognitive deficit could be linked to a decrease in synaptic activity and a neuroinflammatory phenomenon, which will both be further investigated.

Figure 42: Poster 1 – Presented at the British Association of Psychopharmacology summer conference – Cambridge (UK) - 2014

## Acute ICV administration of soluble amyloid- $\beta$ oligomers induces robust cognitive deficits in rat.



William Watremez<sup>1</sup>, JC Neill<sup>1</sup>,  
V Koziel<sup>2</sup>, T Pilot<sup>2</sup>, MK Harte<sup>1</sup>,

<sup>1</sup> Manchester Pharmacy School, University of Manchester, Manchester, M13 9PT, UK  
<sup>2</sup> SynAging, Institut Polytechnique National de Lorraine, Vandœuvre-lès-Nancy, FRANCE



### Introduction

With current treatments for Alzheimer's disease (AD) only providing temporary symptomatic benefits and an ageing population, disease modifying drugs are urgently required. This approach relies on improved understanding of the early pathophysiology of AD. A new hypothesis has emerged, in which early memory loss is considered a synapse failure caused by soluble amyloid- $\beta$  (A $\beta$ ) oligomers.

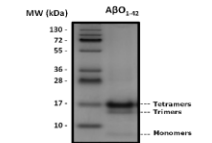
Our aim was to investigate cognitive function following an acute intracerebroventricular (ICV) injection of stabilized low-n A $\beta$  peptide oligomers in the rat. We studied the effect of different doses of oligomers, sex differences and the lasting effect of the cognitive deficit.

### Methods

#### Overview

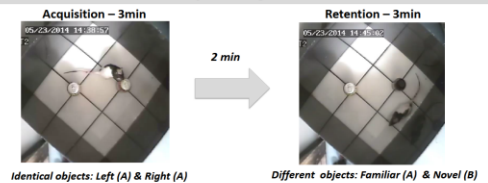
- Day 0**
    - Surgery: Intracerebroventricular (ICV) injection of either:
      - 10 $\mu$ l of vehicle (n=20 F, n=10 M);
      - 10 $\mu$ l of A $\beta$  oligomers: 0.5nmol (n=10 F), 1nmol (n=10 F, n=10 M).
  - Day 4**
    - Novel Object Recognition - Female vs Male
      - Female Vehicle or A $\beta$  oligomers 0.5nmol;
      - Male Vehicle or A $\beta$  oligomers 1nmol.
  - Day 35**
    - Novel Object Recognition - Dose/Response
      - Females only;
      - Vehicle; A $\beta$  oligomers 0.5nmol; 1nmol or 5nmol.
  - Day 70**
    - Novel Object Recognition - Time course study
      - Females only; Vehicle or A $\beta$  oligomers 0.5nmol;
      - Days 4, 14, 35 or 70 after surgery.
- Adult Female (n=60 F) and Male (n=20 M) Lister Hooded rats

#### A $\beta$ oligomers



Chemically synthesized full size amyloid- $\beta$  (aa 1-42) is incubated in various undissolved steps. After 24h of incubation, mostly soluble tetramers are present in the preparation. The preparation is stable for multiple months in frozen batches and can be easily reproduced by SynAging. The preparation is analyzed by boiling in reducing SDS sample buffer for 5 min and separation on SDS-PAGE.

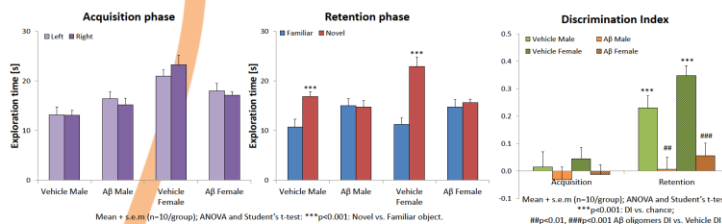
#### Novel Object Recognition (n=10/group)



Short term memory indexes: \*Time spent exploring: novel ( $t_{nov}$ ) vs. familiar ( $t_{fam}$ ) object; \*Discrimination Index =  $(t_{nov} - t_{fam}) / (t_{nov} + t_{fam})$

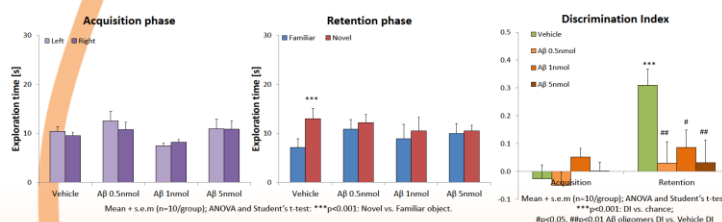
### Results

#### NOR - Female vs. Male - Day 4



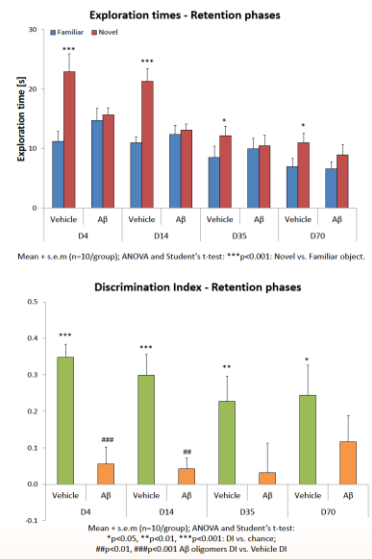
Acquisition: Each group equally explored the two identical objects. Retention: Vehicle groups (both M and F) could clearly identify the new object while A $\beta$  groups (both M and F) had deficits in NOR. No difference between the Vehicle Male and the Vehicle Female groups nor between the A $\beta$  Male and A $\beta$  Female groups were observed.

#### NOR - Dose/Response - Day 35



Acquisition: Each group equally explored the two identical objects. Retention: Vehicle treated rats spent significantly more time exploring the Novel compared to the Familiar object, an effect that was abolished in A $\beta$  treated animals. There is no significant difference between the different doses of A $\beta$  oligomers injected.

#### NOR - Time course - Days 4 to 70



Acute ICV administration of A $\beta$  oligomers induce a deficit in the NOR task as early as Day 4 after surgery and that lasts up to Day 70 after surgery.

### Conclusions

Findings from the present study suggest that acute ICV administration of soluble A $\beta$  oligomers causes robust long lasting cognitive deficits in rat.

Taken together the results suggest that acute ICV administration of stabilized low-n A $\beta$  oligomers may be a useful model to study the early mechanisms involved in Alzheimer's disease and may provide us with a platform for testing novel therapeutic approaches targeting the early underlying pathology.

It is hypothesised that the early cognitive deficit could be linked to a decrease in synaptic activity and a neuroinflammatory phenomenon, which will be further investigated.

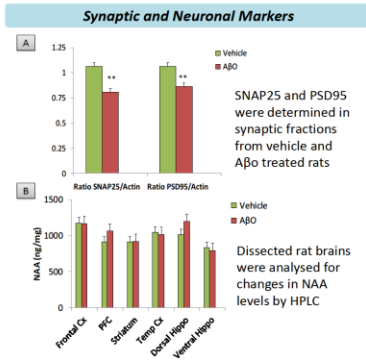
Figure 43: Poster 2 - Presented at the Alzheimer Research UK conference - London (UK) - 2015

## Establishment of a rat model of relevance to sporadic Alzheimer's Disease research: birth and development of a collaborative project.

William Watremez<sup>1</sup>, V Fasolino<sup>2</sup>, M Daniels<sup>2</sup>, C Lawrence<sup>2</sup>, J Jackson<sup>1</sup>, J Galea<sup>4</sup>  
 JC Neill<sup>1</sup>, T Pillot<sup>3</sup>, D Brough<sup>2</sup>, MK Harte<sup>1</sup>



<sup>1</sup> Manchester Pharmacy School, University of Manchester, Manchester, M13 9PT, UK  
<sup>2</sup> School of Life Science, University of Manchester, Manchester, M13 9PT, UK  
<sup>3</sup> SynAging, Institut Polytechnique National de Lorraine, Vandœuvre-lès-Nancy, FRANCE  
<sup>4</sup> Ninewells Hospital and Medical School, Dundee, DD1 9SY, UK  
 Contact: william.watremez@postgrad.manchester.ac.uk



Taken together the results suggest that acute ICV administration of stabilized low-n A $\beta$ O may be a useful model to study the early mechanisms involved in AD and may provide us with a platform for testing novel therapeutic approaches targeting the inflammatory processes that contribute to AD.

This project is only one item among a multitude of other interesting projects at the University of Manchester that aims to understand the early underlying pathology and eventually come together to defeat Alzheimer's disease.

### Alzheimer's disease research

### Future work

### Rodent Model of relevance to sporadic AD

Investigate cognitive and pathological deficits following intracerebroventricular (ICV) administration of well-defined soluble A $\beta$ O (synthesised by our collaborator "SynAging").

Post-mortem analysis revealed significant deficits in (A) synaptic (SNAP25 and PSD 95) but not (B) neuronal (n-acetylaspartate) markers in A $\beta$ O treated animals

### Post-mortem Analysis

Following behavioural testing, rats are culled and post-mortem analyses are conducted to link deficits in cognition to pathophysiological changes in the brain

### Rationale

The project is based on the current hypothesis which implicates soluble amyloid- $\beta$  oligomers (A $\beta$ O) as the root cause of Alzheimer Disease (AD) pathologies, notably on normal synaptic functions and memory

### Additional studies

- \*in vitro studies
- \*toxicity studies
- \*mouse in vivo studies

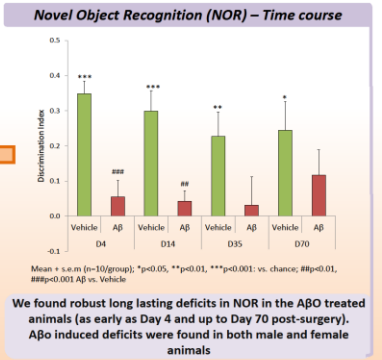
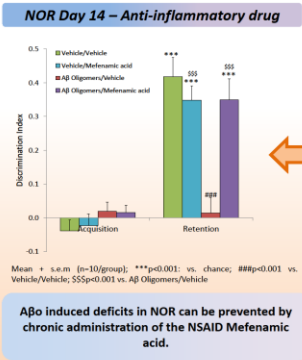
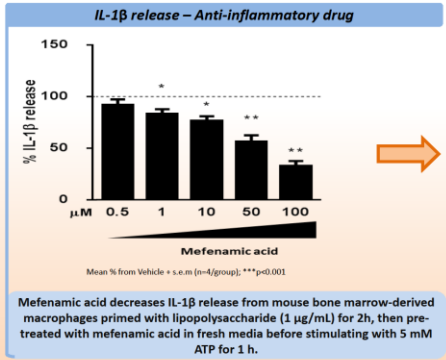
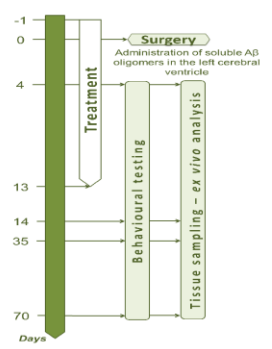
### Neuroinflammation

A $\beta$ O have been shown to activate microglia and induce neuroinflammation through activation of a multiprotein complex called the NLRP3 inflammasome and the subsequent processing of the pro-inflammatory cytokine IL-1 $\beta$ . A collaborating team at the University of Manchester (Daniels, Lawrence, Brough) have demonstrated that a specific subset of non-steroidal anti-inflammatory drugs (NSAIDs) appear to inhibit formation of the NLRP3 inflammasome and processing of IL-1 $\beta$ .

### Behavioural Analysis

The aim of the current study was to determine whether the cognitive deficits (as assessed using the NOR task) observed following acute ICV administration of soluble A $\beta$ O in the rat were prevented by chronic treatment with the NSAID Mefenamic acid.

**Novel Object Recognition (NOR)** in the rat is conducted by first exposing a rat to two identical objects (acquisition phase) and then replacing one object with a new object (retention phase). A rat with an intact memory will spend more time exploring the novel than the familiar object, as scored by the discrimination index (DI).  
 DI = novel – familiar / novel + familiar



Data presented in this poster, along with the complementary data from collaborative teams have been presented at several conferences, including:  
 \*Investigating cognitive deficits and neuronal markers in an animal model of relevance to the early stages of Alzheimer's disease: ICV administration of soluble amyloid- $\beta$  oligomers in the rat. William Watremez, JC. Neill, L. Abdi, C. McKee, R. Ruhl, B. Grayson, N. Fisher, T. Lefebvre, V. Kozel, T. Pillot, MK. Harte. Presented at the British Association of Psychopharmacology conference 2014.  
 \*Acute ICV administration of soluble amyloid- $\beta$  oligomers induces robust cognitive deficits in rat. William Watremez, JC. Neill, V. Kozel, T. Pillot, MK. Harte. Presented at Alzheimer Research UK conference 2015.  
 \*Stabilized Low Number Amyloid- $\beta$  Oligomers Induce Cognitive Deficits Associated with Synaptic Dysfunction in Rat. T. Pillot, W. Watremez, N. Fischer, A. Alouche, J. Neill, B. Grayson, M. Harte. Presented at Alzheimer Disease / Parkinson Disease conference 2015.  
 \*Non-steroidal anti-inflammatory drugs (NSAIDs) as inhibitors of the NLRP3 inflammasome. Michael JD. Daniels, J. Rivers-Auty, V. Fasolino, W. Watremez, A. Baldwin, J. Bagnall, J. Galea, H. Boutin, P. Paszek, T. Pillot, M. Harte, C. Lawrence, S. Freeman, D. Brough. Presented at Innate Immune Memory conference 2015.

Figure 44: Poster 3 - Presented at the University of Manchester Postgraduate Research Summer Showcase - Manchester (UK) - 2015





*Au cœur de la mémoire.*



## Stabilized Low Number Amyloid- $\beta$ Oligomers Induce Cognitive Deficits Associated with Synaptic Dysfunction in Rat

T. Pillot<sup>1</sup>, W. Watremez<sup>2</sup>, N. Fischer<sup>1</sup>, A. Allouche<sup>1</sup>, J. Neill<sup>2</sup>, B. Grayson<sup>2</sup>, M. Harte<sup>2</sup>

<sup>1</sup>SynAging, Institut Polytechnique National de Lorraine, Vandœuvre-lès-Nancy, France  
<sup>2</sup>Manchester Pharmacy School, University of Manchester, M13 9PL, United Kingdom

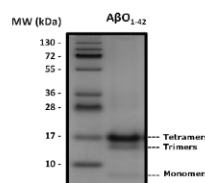
SynAging has established highly reproducible *in vitro* and *in vivo* Alzheimer's Disease models, based on the acute neurodegenerative effects of proprietary amyloid- $\beta$  oligomer (A $\beta$ O) preparations. A $\beta$ O are widely accepted as the initial cause for neurodegeneration in AD, followed by neuroinflammation and other complications. Unlike transgenic models, SynAging's acute models imitate sporadic AD and are used for fast and cost effective phenotypic validation of pharmaceutical candidates as a service for drug development.

Our aim was to investigate cognitive function & synaptic markers following intracerebroventricular injection of low-number A $\beta$ O in the rat. The following results establish that rats and mice can be used in acute A $\beta$ O models. Using rats can be an advantage as it is the premium toxicity testing species.

### Soluble, Low Number Oligomers

SynAging has industrialized the production of soluble amyloid beta peptide oligomers. Creating preparations of highest reproducibility in structure and toxicity from various natural amyloid beta peptides.

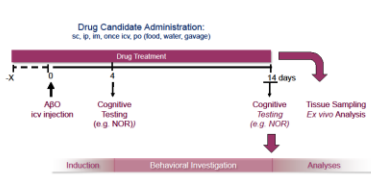
Chemically synthesized full size amyloid- $\beta$  (aa 1-42) is incubated in various undisclosed steps. After 24h of incubation, mostly soluble tetramers are present in the preparation. The preparation is stable for multiple months in frozen batches and can be easily reproduced by SynAging. The preparation is analyzed by boiling in reducing SDS sample buffer for 5 min and separation on SDA-PAGE.



### SynAging's Acute AD Rat Model

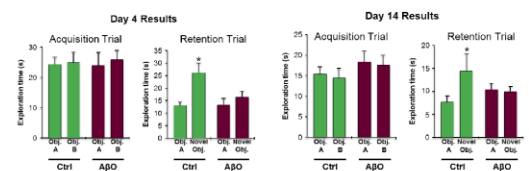
SynAging uses the same A $\beta$ O preparations for its *in vitro* and *in vivo* model. In rats (males and females), cognitive impairment is induced by a single icv injection of A $\beta$ O (500 pmol in 10 $\mu$ L) at day 0 and fully established at day +4. Without treatment, cognitive impairment is robust and long-lasting.

**Experimental scheme for testing compounds in rats:**  
Compound dosing (po, sc, ip, iv) can start before or after disease induction at day 0 by icv injection of A $\beta$ O. Cognitive testing is performed from day 4. Rats are sacrificed and brain tissue is collected for pathological and immunological investigation.

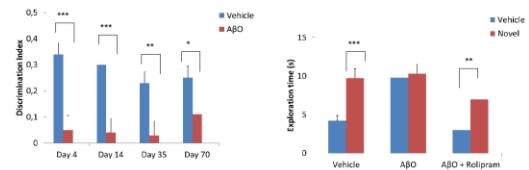


### Behavioural Analysis

Novel Object Recognition (NOR) in the rat is conducted by first exposing a rat to two identical objects (acquisition phase) and then replacing one object with a new object (retention phase). Rat exploration of the novel vs. familiar object is used as a measure for recognition memory. Behaviour in all trials is recorded on video for subsequent blind scoring.



- We found robust long lasting deficits in NOR in the A $\beta$ O treated animals
- The A $\beta$ O induced deficit was found in both male and female animals

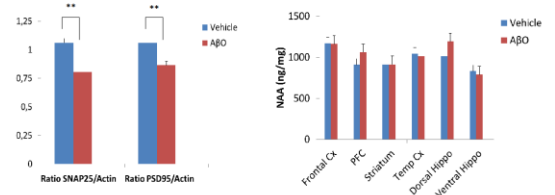


The A $\beta$ O induced deficit was present at day 4 and up to 70 days after single icv injection

Rolipram (0.1mg/kg ip), was able to prevent the A $\beta$ O induced cognitive deficit

### Post-Mortem Analysis

Post-mortem analysis revealed significant deficits in synaptic (SNAP25 and PSD 95) but not neuronal (n-acetylaspartate) markers in A $\beta$ O treated animals



SNAP25 and PSD95 were determined in synaptic fractions from vehicle and AO treated rats

Dissected rat brains were analysed for changes in NAA levels by HPLC

### Summary:

Taken together the results suggest that acute ICV administration of soluble A $\beta$ O may be utilised to model cognitive decline in early Alzheimer's Disease and provides a platform for testing novel therapeutic approaches that target symptomatic improvements or the underlying synaptic pathology.

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Figure 45: Poster 4 – Presented at the Alzheimer Disease & Parkinson Disease conference – Nice (France) - 2015

## Non-steroidal anti-inflammatory drugs (NSAIDs) as inhibitors of the NLRP3 inflammasome

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### Introduction

- Dysregulated sterile inflammation is a key factor in numerous diseases including Alzheimer's disease (AD), gout and stroke.
- The key driving factor in this inflammatory damage is IL-1 $\beta$ , secretion of which is dependent primarily on the inflammasome.
- Non-steroidal anti-inflammatory drugs (NSAIDs) are indicated in the majority of inflammatory disorders, recent epidemiological evidence has suggested NSAIDs may slow the progression of AD.
- We hypothesised that NSAID-mediated inflammatory suppression may occur through a mechanism dependent on inflammasome inhibition and aimed to test this.

### Results

#### A subset of NSAIDs inhibit IL-1 $\beta$ processing *in vitro*

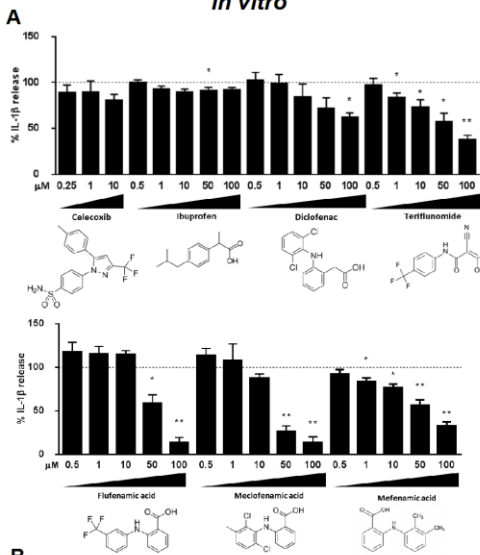


Figure 1: A) A specific subset of NSAIDs (generic name and molecular structure shown) inhibit IL-1 $\beta$  release from mouse bone marrow-derived macrophages (BMDMs). BMDMs were primed for 2 h with 1  $\mu$ g/ml lipopolysaccharide (LPS) then pre-treated with NSAID in fresh media before stimulating with 5 nM ATP for 1 h. Supernatants were taken and IL-1 $\beta$  measured by sandwich ELISA. Data are presented as mean  $\pm$  S.E.M. (n=4). B) Western blot analysis confirmed attenuated processing of pro (31 kDa) to mature (17 kDa) IL-1 $\beta$  by 100  $\mu$ M flufenamic and meclofenamic acid but not by diclofenac. \*P<0.05, \*\*P<0.01 determined by one-sample t-test vs. hypothetical value of 100%.

#### Inhibition of processing occurs upstream of ASC speck oligomerisation

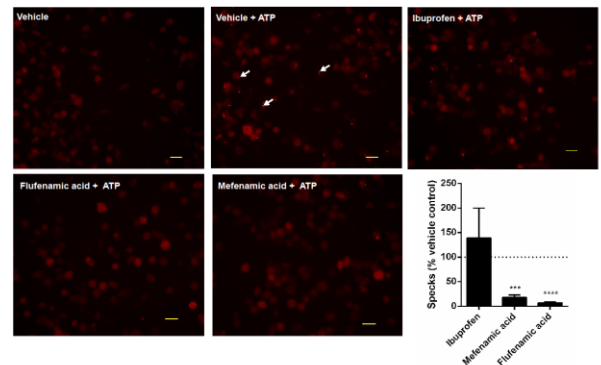


Figure 2: Flufenamic and mefenamic acid, but not ibuprofen prevent ATP-induced formation of ASC specks (examples indicated by white arrows). A mouse BMDM cell line was derived stably expressing the ASC protein conjugated to an mCherry fluorophore. Cells were primed with LPS as before then stimulated with 5 nM ATP for 30 minutes following 15 minute pre-incubation with selected drug or DMSO vehicle under live microscopy. Lower right, quantification of specks is presented as mean  $\pm$  S.E.M. (n=4). \*\*P<0.001, \*\*\*\*P<0.0001 determined by one-sample t-test vs. hypothetical value of 100%. Scale bars are 20  $\mu$ m.

#### Mefenamic acid protects against amyloid beta-induced memory deficits *in vivo*

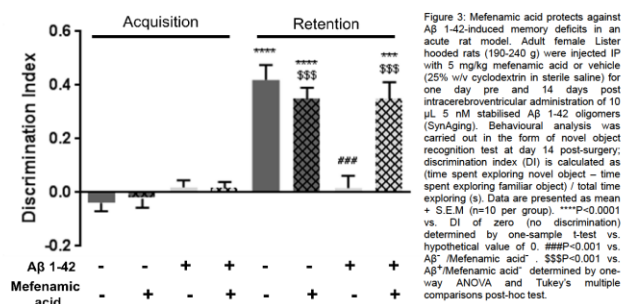


Figure 3: Mefenamic acid protects against A $\beta$  1-42-induced memory deficits in an acute rat model. Adult female Lister hooded rats (180-240 g) were injected IP with 5 mg/kg mefenamic acid or vehicle (25% w/v cyclodextrin in sterile saline) for one day pre and 14 days post intracerebroventricular administration of 10  $\mu$ L 5 nM stabilised A $\beta$  1-42 oligomers (SynAging). Behavioural analysis was carried out in the form of novel object recognition test at day 14 post-surgery; discrimination index (DI) is calculated as (time spent exploring novel object) / total time exploring (s). Data are presented as mean  $\pm$  S.E.M. (n=10 per group). \*\*\*\*P<0.0001 determined by one-sample t-test vs. hypothetical value of 0. ###P<0.001 vs. A $\beta$ \*/Mefenamic acid; \$\$\$P<0.001 vs. A $\beta$ \*/Mefenamic acid; determined by one-way ANOVA and Tukey's multiple comparisons post-hoc test.

### Conclusions

- A specific subset of NSAIDs inhibited formation of the NLRP3 inflammasome and processing of IL-1 $\beta$ .
- Mefenamic acid, a drug used regularly in the clinic, is able to protect against memory deficits induced by injection of stabilised A $\beta$  1-42 oligomers in rats.
- We therefore believe that these newly characterised inflammasome-inhibiting NSAIDs may provide the opportunity to repurpose ready-approved drugs for AD patients thus offering a rapidly translatable, novel clinical approach to the disease. The drugs may also be re-indicated for other inflammasome dependent diseases such as Cryopyrin Associated Periodic Syndromes (CAPS) or haemorrhagic stroke.

Figure 46: Poster 5 – Presented at the Innate Immune Memory conference – Cambridge (UK) - 2015



## Investigating cognitive deficit and pharmacological treatment in a rat model of amyloid- $\beta$ administration.

MANCHESTER  
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### Introduction

With current treatments for Alzheimer's disease (AD) only providing temporary symptomatic benefits and an ageing population, disease modifying drugs are urgently required. This approach relies on improved understanding of the early pathophysiology of AD. Ongoing research suggests that soluble amyloid- $\beta$  oligomers (A $\beta$ ) and inflammatory processes are among the early factors that contribute to the pathogenesis and progression of AD.

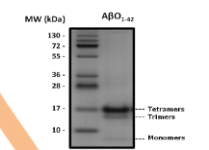
Our aim was to investigate cognitive function, following an acute intracerebroventricular (ICV) injection of stabilized low-n A $\beta$ <sub>1-42</sub> in the rat and subsequent treatment by donepezil, rolipram and risperidone (acute or chronic).

### Methods

A $\beta$  administered (ICV)

Novel Object Recognition

Studies outline



Chemically synthesized full size amyloid- $\beta$  (1-42) is incubated in various undisclosed steps. After 24h of incubation, mostly soluble tetramers are present in the preparation. The preparation is analyzed by boiling in reducing SDS sample buffer for 5 min and separation on SDS-PAGE.

Acquisition – 3min



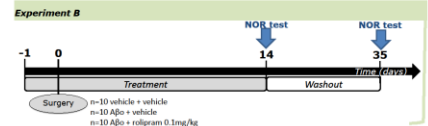
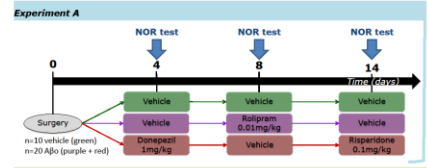
Identical objects: Left & Right

Retention – 3min



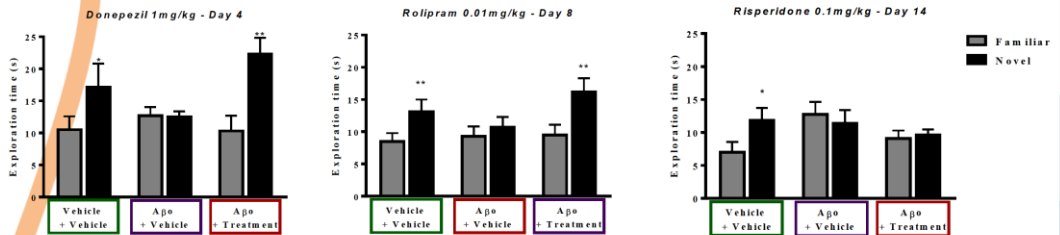
Different objects: Familiar & Novel

Time spent exploring: Novel vs. Familiar object



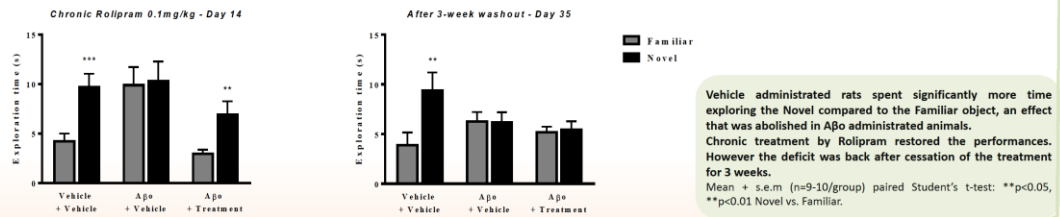
### Results

#### Experiment A – Acute treatments – NOR on day 4, 8 and 14



Vehicle administered rats spent significantly more time exploring the Novel compared to the Familiar object, an effect that was abolished in A $\beta$  administered animals. Acute treatment by Donepezil and Rolipram but not Risperidone restored the performances. However the deficit was back after cessation of the treatment, as Donepezil treated animals on day 4 received no treatment on day 8 and day 14 (red group) and Rolipram treated animals on day 8 received no treatment on day 14 (purple group). Mean + s.e.m (n=8-10/group) paired Student's t-test: \*p<0.05, \*\*p<0.01 Novel vs. Familiar.

#### Experiment B – Chronic treatment Rolipram 0.1mg/kg – NOR on day 14 and 35



Vehicle administered rats spent significantly more time exploring the Novel compared to the Familiar object, an effect that was abolished in A $\beta$  administered animals. Chronic treatment by Rolipram restored the performances. However the deficit was back after cessation of the treatment for 3 weeks. Mean + s.e.m (n=9-10/group) paired Student's t-test: \*\*p<0.05, \*\*\*p<0.01 Novel vs. Familiar.

### Conclusions

Findings from the present study suggest that acute ICV administration of soluble A $\beta$  oligomers causes robust long lasting cognitive deficits in rat. The deficits in NOR were reversed in the presence of the acetylcholinesterase inhibitor donepezil and the phosphodiesterase IV inhibitor rolipram, but not the atypical antipsychotic risperidone. Further studies are required to elucidate the mechanism and highlight potential pharmacological targets.

Taken together the results suggest that acute ICV administration of stabilized low-n A $\beta$  may be a useful model to study the early mechanisms involved in Alzheimer's disease and may provide us with a platform for testing novel therapeutic approaches targeting the early underlying pathology.

It is hypothesised that the early cognitive deficit could be linked to a decrease in synaptic activity and a neuroinflammatory phenomenon, which is currently further investigated by treatment with the non-steroidal anti-inflammatory drug mefenamic acid (see poster MJ DANIELS et al. Fenamate non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of the NLRP3 inflammasome and could be repurposed to treat Alzheimer's Disease).

Figure 48: Poster 7 – Presented at Alzheimer Research UK conference - Manchester (UK) - 2016

## V. AB-PEPTIDE ADMINISTRATION MODELS CLASSIFIED BY RAT STRAIN TESTED

The literature regarding A $\beta$ -peptide administration rat models reviewed in Chapter 2 (Systematic review of rat models based on the administration of amyloid- $\beta$  peptides) has been classified by strain of rat and alphabetically.

**Male Fisher:** (Chambers et al., 2000, Nakamura et al., 2001, Sigurdsson et al., 1996, Sigurdsson et al., 1997b, Sigurdsson et al., 1997a, Sigurdsson et al., 2000, Winkler et al., 1994)

**Male F344/DuCrj:** (Nakamura et al., 2006)

**Male Long-Evans:** (Netland et al., 1998, Lecanu et al., 2006, Lecanu et al., 2010)

**Male Sprague-Dawley:** (Ahmed et al., 2010, Badshah et al., 2015, Bergin et al., 2015, Cai et al., 2014, Campolongo et al., 2013, Chen et al., 1996, Chen et al., 2014a, Chen et al., 2014b, Chen et al., 2015, Cleary et al., 2005, Cui et al., 2012, Feng et al., 2012, Frautschy et al., 1991, Frautschy et al., 1992, Games et al., 1992, Giordano et al., 1994, Harkany et al., 1995b, Harkany et al., 1998, He et al., 2011, He et al., 2013, Jin et al., 2005, Kang et al., 2015, Kasa et al., 2000, Kowall et al., 1991, Liang et al., 2008, Li et al., 2004, Li et al., 2010b, Li et al., 2010a, Li et al., 2011, Li et al., 2015, Liu et al., 2011, Lu et al., 2015, Malin et al., 2001, McDonald et al., 1994, McDonald et al., 1996, McLarnon et al., 2006, Morimoto et al., 1998, Morimoto and Oda, 2003, Nag et al., 2001, Nag and Tang, 2001, O'Hare et al., 2010, O'Hare et al., 2014, Poling et al., 2008, Raymond et al., 2003, Reyes et al., 2004, Ryu et al., 2004, Ryu and McLarnon, 2006, Ryu et al., 2015, Shi et al., 2011, Snow et al., 1994, Stephan et al., 2001, Stephan et al., 2003, Sweeney et al., 1997, Tazangi et al., 2015, Tong et al., 2015, Turunc Bayrakdar et al., 2014, Villette et al., 2010, Wang et al., 2001, Wang et al., 2011, Wang et al., 2014, Weldon et al., 1998, Wu et al., 2014, Wu

et al., 2015, Xu et al., 2014, Yamaguchi and Kawashima, 2001, Yamaguchi et al., 2002, Yu et al., 2015, Zand et al., 2005, Zhang et al., 2013a, Zhang et al., 2015c, Zhang et al., 2015b, Zussy et al., 2013)

**Female Sprague-Dawley:** (Cui et al., 2012, Frautschy et al., 2001, Shin et al., 1997)

**Male Wistar:** (Abe et al., 1994, Alkadhi et al., 2011, Altobelli et al., 2015, Ammassari-Teule et al., 2002, Arias et al., 2002, Aرسالنده et al., 2015, Ashabi et al., 2013, Bagheri et al., 2011, Boyd-Kimball et al., 2005, Burgos-Ramos et al., 2008, Burgos-Ramos et al., 2009, Carrillo-Mora et al., 2010, Cetin and Dincer, 2007, Cetin et al., 2013, Chen et al., 2006, Cheng et al., 2006, Cioanca et al., 2013, Cioanca et al., 2014, Cioanca et al., 2015, Clarke et al., 2007, Cullen et al., 1996, Cullen et al., 1997, Ding et al., 2015, Di Stefano et al., 2010, Fedotova et al., 2012, Freir et al., 2001, Frozza et al., 2013, Fukuta et al., 2001, Giovannelli et al., 1995, Giovannini et al., 2002, Gonzalo-Ruiz et al., 2005, Gonzalo-Ruiz et al., 2006, Guo et al., 2010, Guo et al., 2013, Haghani et al., 2012, Haghani et al., 2015, Han et al., 2011, Harkany et al., 1995a, Harkany et al., 1999a, Harkany et al., 2000, Harkany et al., 2001, Hervas-Aguilar et al., 2005, Hritcu et al., 2014, Hritcu et al., 2015, Hu et al., 2014, Ierusalimsky et al., 2013, Itoh et al., 1996, Itoh et al., 1999, Jafari et al., 2015, Jing et al., 2009, Kaminsky and Kosenko, 2008, Kim et al., 2001, Klyubin et al., 2014a, Klyubin et al., 2014b, Kreutz et al., 2013, Li et al., 2013, Liang et al., 2015, Liu et al., 2008, Liu et al., 2015, Majlessi et al., 2012, Mohammadpour et al., 2015, Mura et al., 2012, Nabeshima and Nitta, 1994, Nabeshima and Itoh, 1998, Nakagawa et al., 2004, Nakagawa et al., 2005, Nell et al., 2015, Nitta et al., 1994, Nitta et al., 1997, Oka et al., 1999, Olariu et al., 2001, Olariu et al., 2002, Ozdemir et al., 2013, Rendakov et al., 2015, Rosales-Corral et al., 2003, Rosales-Corral et al., 2004, Saez-Valero et al., 2002, Salamone et al., 2014, Salgado-Puga et al., 2015, Schmid et al., 2009, Shankar et al., 2008, Shi et al.,

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**Female Wistar:** (Aguado-Llera et al., 2004, Arevalo-Serrano et al., 2008, Carrero et al., 2012, Gonzalez et al., 2007, Guo et al., 2013, Perez et al., 2010, Rendakov et al., 2015, Yamada et al., 1999c)

## VI. VALIDATION OF NOR OBJECTS

In the NOR task (Chapter 3 - Materials and methods, 3.3.1), in order to check that rats do not have a natural preference for any of the objects, or the left or right side of the test arena, each couple of objects are first tested in a single session. Naïve rats are presented with both objects and exploration times are measured for each object. Data for the validation of objects M and P (Figure 15) are presented below. Left or right location of the objects in the test arena was evenly balanced between rats. 20 female Lister Hooded rats receiving no prior surgery or treatment were tested in a single session.

There was no difference of exploration between objects or left/right side of the test arena (Figure 49). Paired samples Student's t-tests showed that there was no preference for the type of object [ $t_{(19)}=1.488$   $p>0.05$ ] or its left/right location [ $t_{(19)}=0.330$   $p>0.05$ ]. These results were confirmed by univariate Student's t-tests versus a value of zero which showed that the DI for object [ $t_{(19)}=1.229$   $p>0.05$ ] and location [ $t_{(19)}=0.255$   $p>0.05$ ] were not different from chance.

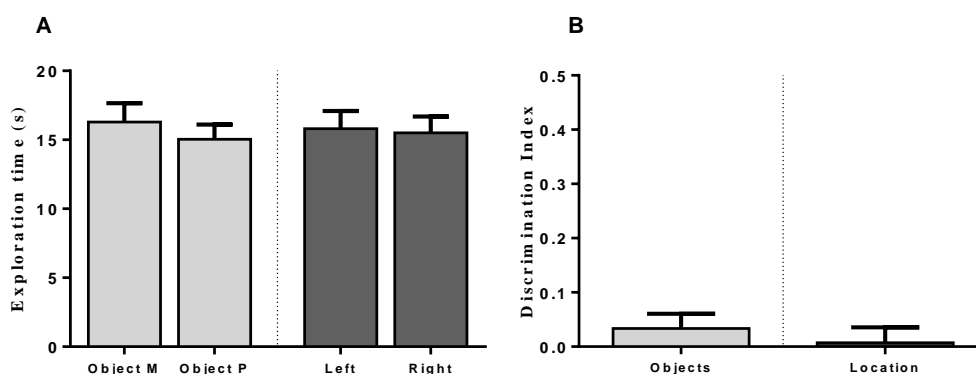


Figure 49: Validation of objects M and P in the NOR task. (A) There was no difference of exploration time between objects or location. Data are presented as mean+SEM ( $n=10$  per group), paired-sample Student's t-tests. (B) Discrimination index for Objects or Location. Data are presented as mean+SEM ( $n=10$  per group), univariate Student's t-test vs. zero.



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