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Abstract

ANTIBODIES PREVENT THE ACUTE INHIBITION OF LONG TERM POTENTIATION BY AMYLOID BETA IN THE RAT HIPPOCAMPUS

Andrew Barry MSc, PgDip Statistics, BSc

The present study investigated the mechanisms underlying the inhibition of long-term potentiation (LTP) of excitatory synaptic transmission by amyloid beta (Aβ) in the anaesthetized rat hippocampus. The role of soluble Aβ assemblies was investigated. Furthermore, we explored the ability of anti-oligomer and anti-fibril antibodies to prevent the inhibition of LTP by different peptides. Finally, we studied the role of the cellular prion protein (PrPc) in Aβ-mediated inhibition of LTP.

In the first strand of research, we compared the effects of intra-cerebroventricular injection of natural Aβ oligomers purified by different methods from conditioned medium of cultured cells over-expressing amyloid precursor protein. Natural Aβ oligomers purified with a one-step or a two-step method completely and potently inhibited LTP at 3 h without affecting baseline synaptic transmission. Aβ monomers purified with the two different methods had no effect on LTP. Aβ dimers purified with a 4-step protocol, to remove all other detectable proteins, similarly inhibited LTP. Moreover, the anti- Aβ antibody 6E10 prevented the inhibition of LTP by the highly purified dimers. These results indicate that Aβ dimers are the smallest assembly of Aβ that can potently inhibit LTP.

The primary objective of the second strand of research was to investigate the ability of a pan-oligomer antibody (A11) to abrogate the LTP-inhibiting effects of different peptide preparations. Co-injection of a dose of A11 that had no effect on LTP when injected on its own, prevented inhibition of LTP by purified cell-derived Aβ oligomers, soluble synthetic $A\beta_{1-42}$ and an amyloidogenic fragment ($PrP_{106-126}$ peptide) of PrPc. In contrast, an isotype control polyclonal antibody did not prevent the inhibition of LTP by $PrP_{106-126}$. Furthermore, the ability of pre-aggregated synthetic Aβ to inhibit LTP was only partially prevented by co-injection of A11. These results indicate that A11-sensitive soluble assemblies of Aβ mediate the inhibition of LTP by soluble cell-derived and synthetic Aβ.

The primary objective of the third strand of research was to investigate the ability of pan-(proto)fibril antibodies (WO1 and WO2) to prevent the inhibition of LTP by different Aβ preparations. Whereas co-injection of WO1 did not significantly affect the inhibition of LTP by purified cell-derived Aβ oligomers, both WO1 and WO2 partially prevented the inhibition of LTP by soluble synthetic Aβ. These results indicate that, in contrast to the cell-derived oligomers, soluble synthetic Aβ includes large Aβ assemblies recognized by the (proto)fibril antibodies, and that these larger soluble species contribute to the inhibition of LTP. Importantly, WO2 completely abrogated the inhibition of LTP by pre-aggregated Aβ, whereas an isotype control antibody (IgMκ) did not prevent the inhibition of LTP. Moreover, we found that repeatedly washed Aβ fibrils (i.e. containing no detectable soluble Aβ) had no effect on LTP. These results imply that soluble protofibril assemblies in our pre-aggregated Aβ preparation contribute to the inhibition of LTP.

The role of PrPc in Aβ–mediated inhibition of LTP was also studied. A pre-injection of the antibody fragment D13, that targets the amino acid sequence 95-105 on PrPc, prevented the inhibition of LTP by soluble synthetic $A\beta_{1-42}$. D13 also prevented the inhibition of LTP by the $PrP_{106-126}$ peptide. Remarkably, D13 completely prevented the inhibition of LTP by soluble Aβ dimer-containing human brain extract, and a control antibody fragment, R1, that targets the amino acid sequence 225-231 on PrPc, failed to prevent this inhibition of LTP. These results are consistent with an involvement of PrPc in synaptic plasticity disruption by Aβ and $PrP_{106-126}$ *in vivo*.

ANTIBODIES PREVENT THE ACUTE INHIBITION OF LONG TERM POTENTIATION BY AMYLOID BETA IN THE RAT HIPPOCAMPUS

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A dissertation submitted to the University of Dublin, Trinity College for the degree of Doctorate of Philosophy

September 2010

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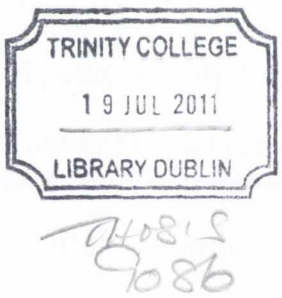

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Abbreviations

Aβ – amyloid-beta

Ab – antibody(s)

AC – associational commissural pathway

ACh – acetylcholine

AD - Alzheimer's disease

AMPA – a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

APP – amyloid precursor protein

BACE - beta-secretase

CA – cornu ammonis

CaMKII – calcium/calmodulin-dependent protein kinase II

CHO – Chinese hamster ovary

CM – cultured medium

CTF – c-terminal fragment

DG – dentate gyrus

EC – Entorhinal cortex

EEG – electroencephalogram

E-LTP – early phase long-term potentiation

ERK – extracellular signal-regulated kinase

EPSP – excitatory postsynaptic potentials

FAD – familial Alzheimer's disease

GABA – gamma-aminobutyric acid

HFS – high-frequency stimulation

i.c.v – intracerebroventricular

IE – ion exchange

iGluRs – metabotropic glutamate receptors

i.p. – intraperitoneal

LPP – lateral perforant pathway

LC – locus coeruleus

LTD – long-term depression

LTP – long-term potentiation

L-LTP – late phase long-term potentiation

mAbs – monoclonal antibodies

MAPK– mitogen-activated protein kinase

MF – mossy fibres

MPP – medial perforant pathway

NDP – nucleation-dependent polymerization

NMDA - N-methyl-D-aspartate

PBS – phosphate buffer solution

PKC – protein kinase C

PF – protofibrils

PP – perforant pathway

PPF – paired pulse facilitation

PrPc – cellular prion protein

PrPsc – scrapie isoform

$PrP_{106-126}$ – toxic prion peptide amino acid sequence 106-126

SAD – sporadic forms of Alzheimer's disease

SAPK - stress-activated protein kinase

Sb – subiculum

SEC – size exclusion chromatography

SEM – standard error of the mean

STP – short-term potentiation

VFT – venus flytrap

Chapter 1 Introduction

Alzheimer's disease (AD) is the fourth leading cause of death in adults, after heart disease, cancer and stroke (Arendt, 2001). AD has become a major public health concern due to the heavy costs it entails; a development that is compounded by a steady growth in the population most at risk – the elderly. After the age of 60, the risk of AD increases exponentially doubling every five years (Finch, 2003). Dr. Alois Alzheimer was one of the first to describe the disease in 1906. Since then, researchers have developed a deeper understanding of the changes in the brain and behavior that characterize this disease.

AD is a heterogeneous neurodegenerative disorder with insidious onset and irreversible progression that affects men and women equally. The prognosis of the disease is a steady decline of cognitive functions, and to date, there are no effective disease modifying therapies (Golde, 2003). Several drugs are currently approved to treat Alzheimer's disease. The current most common treatment for AD sufferers includes use of acetylcholinesterase inhibitors to improve cognitive function (Doody, 1999) and a N-methyl-D-aspartate antagonist (Mount and Downton, 2006). At best, these drugs lead to only modest improvements in the cognitive functions of patients; none can stop the destruction of brain cells that underlies the illness

Clinically, AD is characterized by loss of memory, followed by a progressive deterioration of all the mental functions and pathologically by the progressive neuronal degeneration of both cerebral and limbic cortices, reactive gliosis, activated phagocytic microglia, amyloid plaques and intraneuronal aggregates of disrupted microtubuli, known as neurofibrillary tangles. AD is diagnosed histopathologically by the presence, in sufficient numbers, of deposits of amyloid beta (Aβ) in plaques and tau in NFTs. The regions of the brain most affected by AD are the temporal lobe and in particular the hippocampus which is the seat of declarative memory functioning.

There is no single gene that accounts for AD heritability, despite some clues that have been provided by genetic analysis of the rare cases of early-onset familial Alzheimer's disease. The vast majority of late-onset AD cases are sporadic forms of the disease.

Mutations and polymorphisms in multiple genes are likely to contribute to sporadic AD pathogenesis together with non-genetic factors.

The Aβ protein is a key molecule in the pathogenesis of AD. The tendency of the Aβ peptide to aggregate, its reported neurotoxicity, and genetic linkage studies, has led to a hypothesis of AD pathogenesis that many AD researchers term the amyloid cascade hypothesis. In this hypothesis, an increased production of $A\beta_{1-42}$ results in neurodegeneration and ultimately dementia through a cascade of events. In the past 15 years, debate amongst AD researchers has continued unabated as to whether Aβ is a cause or an effect of the pathogenic process (Verdile et al.; 2004).

Since the reformulation of the amyloid cascade hypothesis (Hardy and Selkoe; 2002) to focus on oligomeric aggregates of amyloid beta as the prime toxic assemblies causing AD, many researchers refocused on detecting a specific molecular assembly of defined size that is the main trigger of AD. The result has been the identification of a host of molecular assemblies containing from two up to a hundred molecules of the Aβ peptide, which were all found to impair memory formation in mice. This clearly demonstrates that size is insufficient to define toxicity and peptide conformation has to be taken into account. The major focus of my research will be to investigate the interplay between oligomer size and peptide conformation as the key determinants of the neurotoxicity of the Aβ peptide. I will also investigate possible putative receptors involved in Aβ-mediated synaptic dysfunction.

1.1 Alzheimer's disease, the hippocampus and long-term potentiation

1.1.1 Vulnerable Networks—Entorhinal Cortex/Hippocampal Pathways

As AD progresses, extensive disruption of connectivity throughout the cortex and many subcortical areas occurs; two of the earliest areas affected are the hippocampus and entorhinal cortex which form a network that is essential for the normal function of episodic memory, thus providing an explanation for why memory problems which rely on this network are a very early and core symptom of AD (for a review see Ondrejcak et al. 2010). This mnemonic function is thought to require a continuous comparison of incoming integrated perceptual content via the entorhinal cortex with information and

related predictive schemata initially stored/generated in the hippocampus. The network's circuitry is mainly comprised of glutamatergic neurons and synapses, which are under tight control from intrinsic GABA-ergic inhibitory interneurons and external inputs including cholinergic neurons. Extensive deposition of Aβ is associated with the disruption of glutamatergic synapses in this network at an early stage of AD (Reitz et al.; 2009). Such marked and early deposition of Aβ may be at least partly the result of the relatively high excitatory drive through the network, since Aβ aggregation in the brain is driven by activity at these synapses (Deshpande et al.; 2009).

1.1.2 The hippocampal network

The hippocampal formation is a collection of laminated structures that lie below the parahippocampal gyrus on the ventral aspect of the temporal lobe, and includes the dentate gyrus (DG), the hippocampus proper, the subiculum (Sb) and the entorhinal cortex (EC). The size of the hippocampal formation is species dependent (Stephan et al. 1981). The hippocampus receives inputs from numerous limbic, cortical, and subcortical areas, and forms a largely unidirectional loop network. The primary pathway of neural activity entering the hippocampus is from the entorhinal cortex via the perforant path (PP; MPP-medial and LPP-lateral) to the dentate granule cells, with collaterals CA1 and CA3 pyramidal cells (see Figure 1.1 for graphic detail). The hippocampus has three *Cornu Ammonis* subfield areas, CA1, adjacent to the subiculum, CA2, and CA3. The DG and the hippocampus form interlocking C-shaped structures when viewed in transverse section. While the dentate fascia is trilaminated with a molecular layer, a granule cell layer, and a distinct polymorphic layer, the ammonic subfields are subdivided into stratum lacunosum molecular, stratum radiatum, stratum lucidum or mossy fiber layer (restricted to the CA3 region), pyramidal cell layer, and stratum oriens. In Ammon's horn, only subfields CA1 and CA3 are clearly discernible, whereas the CA2 region is indistinct.

The cerebral neocortex, the septal area, the contralateral hippocampus and the nuclei in the reticular formation of the brain stem are the four main sources of afferents to the hippocampal formation. The fornix is the largest efferent pathway of the hippocampal formation. Mossy fibers (MF) from the DG granule cells excite CA3 pyramidal cells and hilar interneurons. CA3 pyramidal cells excite CA1 pyramidal cells via the Schaffer

collateral (SC), as well as the CA1 cells in the contralateral hippocampus via the associational commissural pathway (AC). CA1 pyramidal cells send efferent fibers to subiculum, entorhinal cortex, and several subcortical areas. These neurons in turn send the main hippocampal output back to the EC, forming a loop.

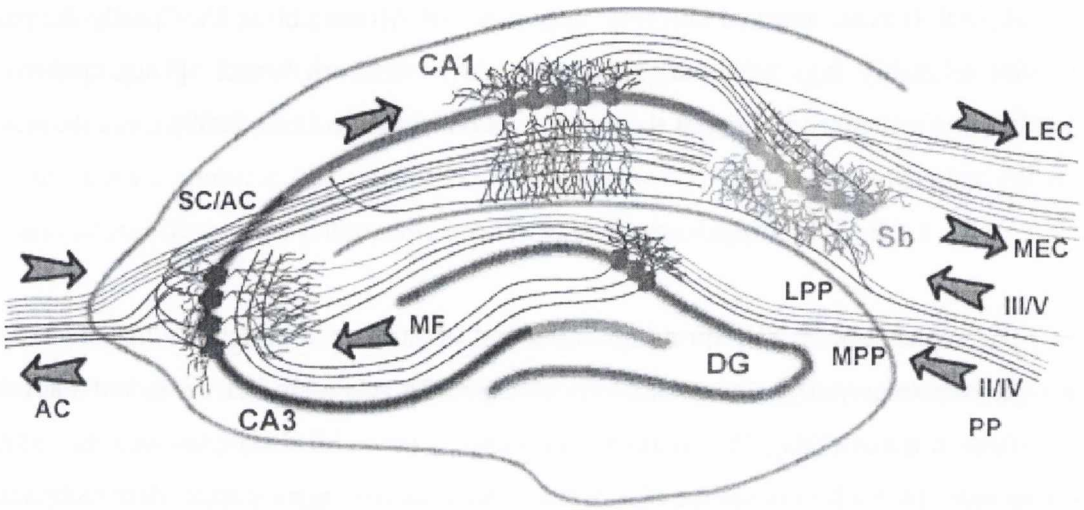


Figure 1.1 – The hippocampal formation (adapted from Wang and Storm 2005).

Analysis of clinical cases and the effects of similar lesions induced in animal models suggest that the severity of memory impairment correlates with the extent of damage to the hippocampal formation (Parkin, 1996, Zola-Morgan et al., 1986, Zola and Squire, 2001). However, more severe memory impairments were produced when the damage was increased to include the adjacent entorhinal and parahippocampal cortices (Jarrad, 1995 and Zola-Morgan et al., 1994). Interestingly lesions of the perirhinal and parahippocampal cortex, that spare the amygdala and hippocampal formation, also produce severe memory impairment (Zola-Morgan et al., 1989a; Zola-Morgan et al., 1989b). In conclusion, the communication via the bidirectional circuitry between the neocortex, the parahippocampal region and the hippocampus proper appears to play a critical role in memory formation (Eichenbaum et al., 1996 and Witter et al., 2000).

1.1.3 Properties and induction of long-term potentiation

The hippocampus plays a pivotal role in the storage of memory (Kandel, 2004). Activity -dependent persistent increases and decreases in the strength of communication at synapses (synaptic plasticity) provide an attractive biologically plausible means of

information storage in the brain. One such plasticity is called long-term potentiation (LTP) (Bliss and Gardner-Medwin 1973). Trains of high frequency stimulation (HFS) applied to the major synaptic pathways increase the amplitude of the excitatory postsynaptic potentials (EPSPs) in the target hippocampal neurons. This sustained increase in synaptic activity is termed LTP. LTP displays three fundamental properties, that of 'cooperativity' (Bliss and Collingridge, 1993), 'input specificity' (Lynch et al. 1977) and 'associativity' (Levy and Steward, 1979).

LTP shares many features with long-term memory that make it an attractive candidate for a cellular mechanism of learning. LTP, like memories can be generated rapidly and each depends upon the synthesis of new proteins. It shows a high degree of synapse specificity, which allows for a huge storage capacity given the number of synapses in the mammalian brain. In addition LTP appears associative in that activity at one point in the dendritic tree can influence the probability of a change in synaptic strength at another (Wigstrom et al., 1986; Malinow and Miller, 1986). Furthermore LTP is most reliably induced in brain areas thought to be involved in learning and memory while normal neuronal activity seen in learning and memory appears able to induce LTP (Power et al. 1997).

LTP is commonly divided into three phases that occur sequentially: short-term potentiation (STP), early LTP (E-LTP) and late LTP (L-LTP). Each phase of LTP is governed by a set of mediators- small molecules that dictate the events of that phase (Malenka and Bear, 2004). These molecules include protein receptors that respond to events outside of the cell, enzymes that carry out chemical reactions within the cell, and signaling molecules that allow for the progression from one phase to the next. In addition to these mediators, there are also modulator molecules that interact with mediators to finely vary the LTP that is generated. The E-LTP and late L-LTP phases of LTP are each characterized by a series of three events: induction, maintenance, and expression. Induction is the process by which a short-lived signal triggers that phase of LTP to begin. Maintenance corresponds to the persistent biochemical changes that occur in response to the induction of that phase. Expression entails the long-lasting cellular changes that result from activation of the maintenance signal (Sweatt, 1999). Thus the mechanisms of LTP can be discussed in terms of the mediators that underlie the induction, maintenance, and expression of E-LTP and L-LTP.

The induction of E-LTP is governed by glutamate receptors with specific reference to N-methyl-D-aspartate (NMDAR) / amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPAR) and kainate receptors. Cumulative evidence has shown that generation of LTP in most afferent pathways of the hippocampus to be NMDAR dependent (Collingridge et al., 1983) but receptor activation alone does not guarantee the induction of LTP. Ca^{2+} influx is also necessary for LTP induction (Bortolotto Collinridge, 1993), as is a transient activation of calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) and to a lesser extent other protein kinases (Lynch 2004). Maintenance of E-LTP is characterized by the persistent activation of CaMKII and PKC. During this stage, CaMKII and PKC lose their dependence on calcium and become autonomously active. Consequently they are able to carry out the phosphorylation events that underlie E-LTP expression. Autonomously active CaMKII and PKC use phosphorylation to carry out the two major mechanisms underlying the expression of E-LTP. First they phosphorylate existing AMPARs to increase their activity (Malenka and Bear, 2004). Second, they mediate or modulate the insertion of additional AMPARs into the postsynaptic membrane. Importantly, the delivery of AMPA receptors to the synapse during E-LTP is independent of protein synthesis.

Late LTP is induced by changes in gene expression and protein synthesis brought about by the persistent activation of protein kinases activated during E-LTP, such as mitogen-activated protein kinase (MAPK). In fact, MAPK—specifically the extracellular signal-regulated kinase (ERK) subfamily of MAPKs—may be the molecular link between E-LTP and L-LTP, since many signaling cascades involved in E-LTP, including CaMKII and PKC, can converge on ERK (Kelleher et al., 2004). Upon activation, ERK may phosphorylate a number of cytoplasmic and nuclear molecules that ultimately result in the protein synthesis and morphological changes observed in L-LTP. ERK-mediated changes in transcription factor activity may trigger the synthesis of proteins that underlie the maintenance of L-LTP. The identities of only a few proteins synthesized during L-LTP are known. Regardless of their identities, it is thought that they contribute to the increase in dendritic spine number, surface area, and postsynaptic sensitivity to neurotransmitter associated with L-LTP expression (Lynch, 2004).

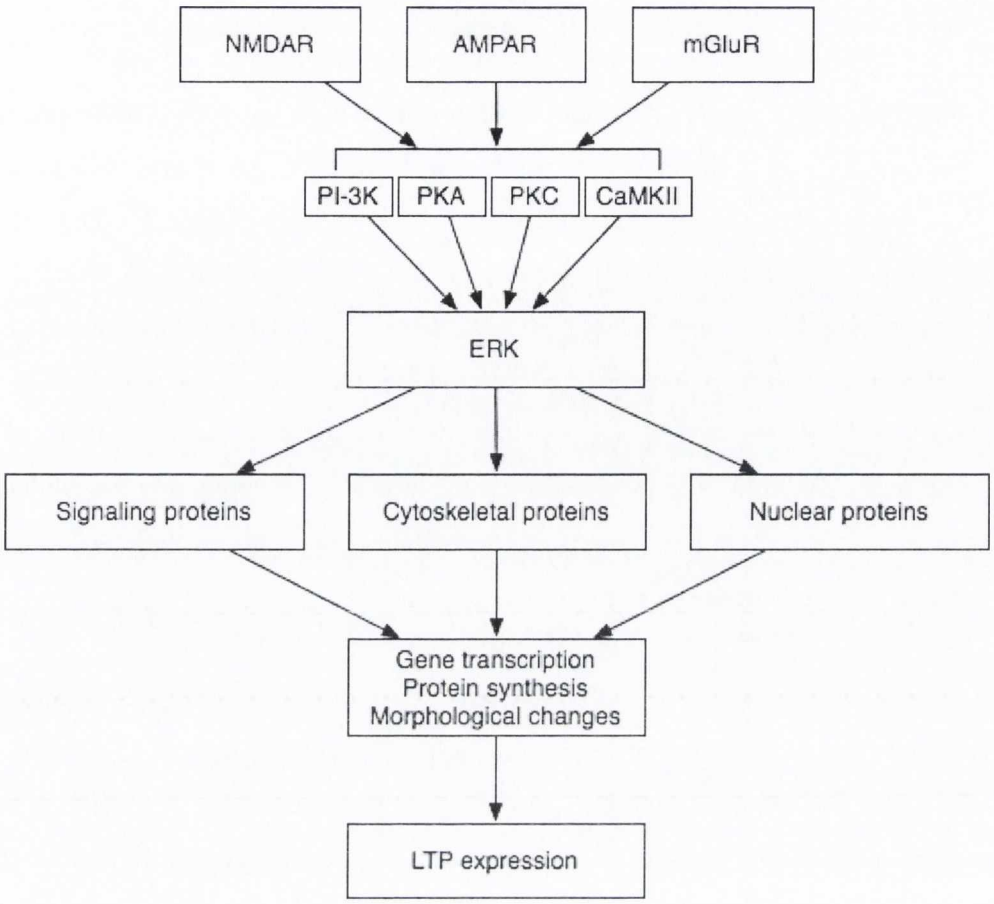


Figure 1.2 – LTP expression- the early and late phases of LTP are thought to communicate via extracellular signal-regulated kinases (adapted from Lynch 2004).

Metaplasticity is a term originally coined by W.C. Abraham and M.F. Bear (1996) to refer to the plasticity of synaptic plasticity. Until that time synaptic plasticity had referred to the plastic nature of individual synapses. However this new form referred to the plasticity of the plasticity itself, thus the term meta-plasticity. Conceptually, the synapse's previous history of activity determines its current plasticity. This may play a role in some of the underlying mechanisms thought to be important in memory and learning such as LTP, long-term depression (LTD) and so forth. These mechanisms depend on current synaptic "state", as set by ongoing extrinsic influences such as the level of synaptic inhibition, the activity of modulatory afferents such as catecholamines, and the pool of hormones affecting the synapses under study. There is much research currently underway on the subject, despite its difficulty of study, because of its theoretical importance in brain and cognitive science (Montgomery and Madison, 2004; Zelcer et al., 2006; MacDonald et al., 2007).

1.1.4 Glutamate receptors in the CNS

Glutamate is the most prominent neurotransmitter in the body, being present in over 50% of nervous tissue. The two primary glutamate receptors are named after agonists that bind to them with high specificity: AMPAR and NMDAR. One of the major functions of glutamate receptors appears to be the modulation of synaptic plasticity. Both metabotropic and ionotropic glutamate receptors have been shown to have an effect on synaptic plasticity. An increase or decrease in the number of ionotropic glutamate receptors on a post-synaptic cell may lead to LTP or LTD of that cell, respectively. Additionally, metabotropic glutamate receptors may modulate synaptic plasticity by regulating post-synaptic protein synthesis through second messenger systems (Adamson et al., 1990).

Glutamate receptors can be divided into two groups according to the mechanism by which their activation gives rise to a postsynaptic current. Ionotropic glutamate receptors (iGluRs) form the ion channel pore that activates when glutamate binds to the receptor. Metabotropic glutamate receptors (mGluR) indirectly activate ion-channels on the plasma membrane through a signaling cascade that involves G proteins. Ionotropic receptors tend to be quicker in relaying information but metabotropic are associated with a more prolonged stimulus. This is due to the usage of many different messengers to carry out the signal but since there is a cascade just one activation of a G-protein can lead to multiple activations. The glutamate receptor is usually not exclusively geared towards glutamate as the ligand and sometimes even requires another agonist.

There are many specific subtypes of glutamate receptors, and it is customary to refer to primary subtypes by a chemical that binds to it more selectively than glutamate. The research, though, is ongoing as subtypes are identified and chemical affinities measured. There are several compounds that are routinely used in glutamate receptor research and associated with receptor subtypes:

Type	Name	Agonist(s)
Ionotropic	NMDA receptor	NMDA
	Kainate receptor	Kainate
	AMPA receptor	AMPA
Metabotropic	mGluR	L-AP4, ACPD, L-QA

Figure 1.3 - Summary of glutamate receptors types and agonists.

Glutamate receptors exist primarily in the central nervous system and can be found on the dendrites of post-synaptic cells where they bind to glutamate released into the synaptic cleft by pre-synaptic cells. They are also present on both astrocytes and oligodendrocytes. The glutamate binds to the extracellular portion of the receptor and provokes a response, with the various types of receptors producing different responses. Ionotropic and metabotropic glutamate receptors, with the exception of NMDA, are found on cultured glial cells that can open in response to glutamate and cause cells to activate second messengers to regulate gene expression and release neuroactive compounds. Furthermore, brain slices show glutamate receptors are ubiquitously expressed in both developing astrocytes and oligodendrocytes *in vivo*. Because of this, glial glutamate receptors are thought to be vital for glial cell development.

All ionotropic glutamate receptors are ligand-gated nonselective cation channels that allow the flow of K+, Na+ and sometimes Ca^{2+} in response to glutamate binding. Upon binding, the agonist will stimulate direct action of the central pore of the receptor, an ion channel, allowing ion flow and causing EPSC. This current is depolarizing and, if enough glutamate receptors are activated, may trigger an action potential in the post-synaptic neuron. All produce excitatory post-synaptic currents, but the speed and duration of the current is different for each type. NMDA receptors have an internal binding site for Mg^{2+} ions creating a voltage dependant block that is removed by outward flow of positive current. Since the block must be removed by outward current flow, NMDA receptors rely on the EPSC produced by AMPA receptors to open. NMDA receptors are permeable to Ca^{2+} that is an important cation in the nervous system and has been linked to gene regulation. It is thought that the flow of Ca^{2+} through NMDA receptors can cause both LTP and LTD by transducing signaling cascades and regulating gene expression.

Metabotropic glutamate receptors, which belong to subfamily C of G protein-coupled receptors, are divided into three groups, with a total of eight sub-types. The mGluRs are composed of three distinct regions: the extracellular region, the transmembrane region, and the intracellular region. The extracellular region is composed of a Venus Flytrap (VFT) module that binds glutamate, and a cysteine-rich domain that is thought to play a role in transmitting the conformational change induced by ligand binding from in the VFT module to the transmembrane region. The transmembrane region consists of seven transmembrane domains and connects the extracellular region to the intracellular region where G protein coupling occurs. Glutamate binding to the extracellular region of an mGluR causes G proteins bound to the intracellular region to be phosphorylated, affecting multiple biochemical pathways and ion channels in the cell. Because of this, mGluRs can both increase or decrease the excitability of the post-synaptic cell, thereby causing a wide range of physiological effects.

1.1.5 Conclusions

Although the specific molecular initiators of the AD process remain unknown in most patients, biochemical studies indicate that the severity of cognitive impairment in AD correlates more strongly with the cortical levels of soluble Aβ protein assemblies than with the burden of insoluble amyloid plaques (Lue et al., 1999; McLean et al., 1999; Shankar et al., 2008). Studies from numerous laboratories have now established that soluble Aβ oligomers can inhibit LTP (Walsh et al. 2002), and that LTD induction is facilitated by pathophysiologically relevant low concentrations of soluble Aβ oligomers through activation of either NMDA receptors or mGluRs, depending on the electrical stimulation protocol used to induce the LTD (Li et al. 2009). Research on hippocampal plasticity has also indicated that the induction of LTD results in decreased dendritic spine volume or outright elimination of spines (Matsuzaki et al. 2004; Nägerl et al. 2004; Zhou et al. 2004), changes that may parallel molecular and structural aspects of synaptic failure in AD. Therefore, understanding how Aβ impairs hippocampal synaptic function at the molecular level could enable the development of specific neuroprotective therapies for AD.

1.2 The role of amyloid beta in Alzheimer's disease

1.2.1 APP, amyloid beta and Alzheimer's disease

The amyloid precursor protein (APP) is a type I membrane phosphoprotein, and is encoded by a single gene on chromosome 21 (individuals with an extra copy of this chromosome, as in trisomy 21 (Down's syndrome) develop premature AD pathology). Cleavage of APP by either α- or β-secretases produces large soluble N-terminal fragments sAPPα and sAPPβ, and membrane-bound C-terminal fragments (C83 and C99). The release of sAPP (α and β) occurs constitutively, but it is also regulated by neural activity and can be influenced by agonists of various neurotransmitter receptors. The application of agonists of the type 1 metabotropic glutamate receptor to cultured cells expressing the receptors increases sAPP release (Nitsch et al., 1992). Similarly, activation of M1 and M3 muscarinic acetylcholine (mACh) receptor subtypes increases the release of sAPP from cultured cells (Nitsche et al., 1997).

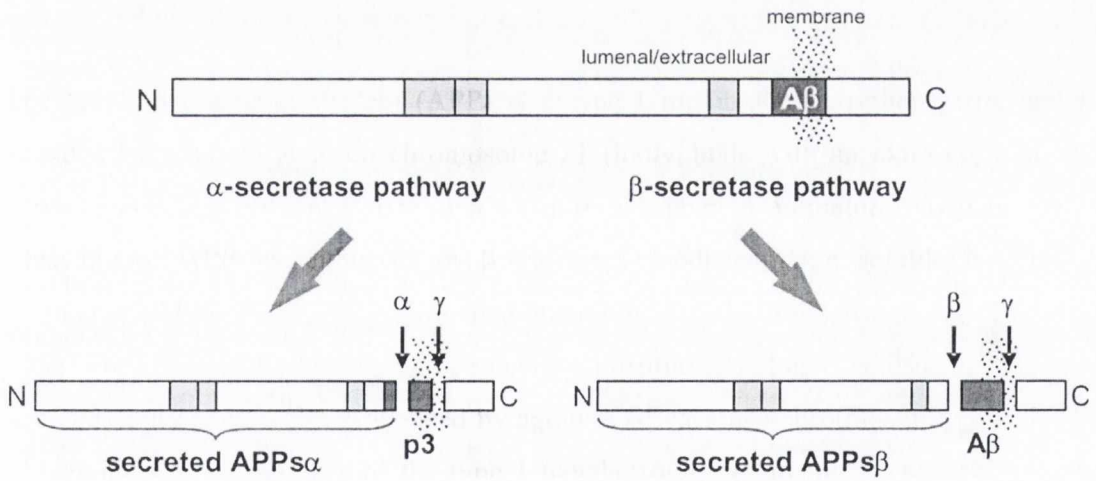


Figure 1.4 – The putative intracellular pathways of APP processing. Arrows indicate the two pathways- α-secretase and β-secretase mediated. Aβ domain (dark grey) and alternatively spliced regions (pale grey) represented.

It is understood that proteolytic cleavage of APP is mediated by (at a minimum) two competing pathways. In the non-amyloidogenic pathway (Fig. 1.4) α-secretase (now thought to be the metalloprotease TACE [ADAM 17] or ADAM 10) cleaves within the Aβ domain (dark greay) of APP to release αAPPs, thus precluding the formation of Aβ

(Maccioni et al., 2001). The C-terminal fragment products are further cleaved by γ-secretase to yield non-amyloidogenic fragments (p3). This pathway can be regulated by phosphorylation processes, mediated by protein kinase C.

In the amyloidogenic pathway (Fig. 1.4), β-secretases (BACE) cleaves near the N-terminus of the Aβ domain to generated βAPPs. The C-terminal fragment containing the whole Aβ domain is cleaved by γ-secretase to liberate the Aβ peptide, typically ending at residue 40 or 42. It is thought that MAPK or stress-activated protein kinase (SAPK) may play a role in modulating this pathway (Maccioni et al., 2001).

The most common isoforms of Aβ are $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network. The $A\beta_{1\text{-}40}$ form is the more common of the two, but $A\beta_{1\text{-}42}$ is the more fibrillogenic and is thus associated with disease states (Crouch et al., 2008). Mutations in APP associated with early-onset Alzheimer's have been noted to increase the relative production of $A\beta_{1\text{-}42}$, and thus one suggested avenue of Alzheimer's therapy involves modulating the activity of β- and γ-secretases to produce mainly $A\beta_{1\text{-}40}$ (Golde, 2003).

1.2.2 The amyloid cascade hypothesis

The amyloid cascade hypothesis of AD pathology has largely guided the direction of research (and funding) into this neurodegenerative disorder for over a decade now. By this model, AD is a clinico-pathological syndrome in which different gene defects can lead (directly or indirectly) to altered APP expression and proteolytic processing and/or to changes in Aβ peptide stability and aggregation. These changes result in a chronic imbalance between Aβ production and clearance, creating a neurotoxic environment that leads to AD dementia (Hardy and Selkoe, 2002). In the amyloid cascade model of AD, the gradual accumulation of aggregated Aβ initiates a very complex, multi-step cascade that includes gliosis, inflammatory changes, oxidative responses, neuritic/synaptic change, transmitter loss, and the production of plaques and tangles (Selkoe and Schenk, 2003). The oligomerization and subsequent fibrillogenesis of Aβ is considered to be the primary event that leads to all subsequent events in AD pathology.

1.2.3 Glutamatergic Mechanisms—Effects of Aβ

Given the initial emphasis of the amyloid cascade hypothesis on neurodegeneration, much early research focused on the ability of Aβ to increase excitotoxicity mediated through glutamate receptors, especially NMDAR (Greenamyre and Young, 1989; Koh et al., 1990; Lawlor and Davis, 1992; Mattson et al., 1992; Hynd et al., 2004). Consistent with these reports, relatively low doses of Aβ were found to exacerbate delayed cognitive impairment caused by activation of NMDAR (Dornan et al., 1993; Nakamura et al., 2006). Possible mechanisms for the Aβ-mediated enhanced excitotoxicity include the ability of Aβ to reduce glutamate uptake (Harris et al., 1995; Keller et al., 1997; Harkany et al., 2000; Fernandez-Tome et al., 2004; Matos et al., 2008), or to increase glutamate release (Arias et al., 1995; Noda et al., 1999; Bobich et al., 2004; Chin et al., 2007; Kabogo et al., 2008; Puzzo et al., 2008).

The important role of glutamatergic mechanisms, and in particular NMDAR in causing clinical dementia in AD, was validated when the low affinity open channel NMDA receptor antagonist memantine (Lipton, 2007; Parsons et al., 2007) was licensed for the treatment of patients. Combined with the recognition that mechanisms other than neurodegeneration contribute significantly to the cognitive symptoms of AD, glutamatergic transmission and plasticity of that transmission, rather than solely excitotoxicity, have become a major focus of interest.

1.2.4 Amyloid beta oligomers/proto-fibrils disrupt LTP and synaptic function

Alzheimer's disease is a slowly progressive disorder at both the histopathological and clinical levels. Early symptoms of mild memory loss and minimal cognitive impairment lead gradually over 5-15 years to profound dementia and death. Biochemical and morphological studies suggest that clinical impairment in AD involves early synaptic dysfunction (Anderton et al., 1998; Cummings et al., 1998), followed by more severe neuronal changes that include increased synaptic loss, widespread neuritic dystrophy, neurofibrillary tangles, and frank neuronal death (Terry et al., 1991; Gómez-Isla et al., 1996; Sze et al., 1997; Anderton et al., 1998).

There is now ample evidence that Aβ oligomers interfere specifically with synaptic function (Klein et al., 2001; Walsh and Selkoe, 2004; Selkoe, 2008). Over the past two decades researchers have built up a detailed picture of the natural economy of brain Aβ. The steady state level of Aβ is controlled by its production, degradation and clearance (Selkoe, 2001) and it is proposed that in disease a defect leading to over-production or decreased clearance causes an accumulation of Aβ. This in turn triggers a pathogenic cascade culminating in the cognitive deficits that characterize AD.

Like several other disease-associated proteins, Aβ has the ability to self-associate, and can form an array of different assembly forms ranging from dimers to aggregates of fibrils. Initially, it was assumed that toxicity was mediated by fibrillar Aβ similar to that present in amyloid plaques. However, the quantity and temporal progression of amyloid plaques do not correlate well with the clinical progression of the disease (Terry et al., 1991; Yen et al., 1995), thus raising the simple question: if Aβ causes AD, then why doesn't the amount of Aβ in the form of amyloid plaques relate to the severity of dementia?

Over a decade ago research began to look at soluble forms of Aβ. In Michael Rowan's lab *in vivo* experiments (Cullen et al., 1996) using $A\beta_{1\text{-}40}$ showed delayed onset reductions in baseline synaptic transmission in the CA1 area of the rat hippocampus. This effect was prevented by treatment with an NMDAR antagonist CPP. Cullen et al. (1997) also showed that acute intracerebroventricular (i.c.v) injection of either $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ rapidly inhibited LTP in the intact hippocampus. Other laboratories showed that continuous i.c.v infusion of $A\beta_{1\text{-}40}$ for 10-11 days inhibited LTP in CA1 (Itoh et al. 1999), and that the administration of synthetic Aβ oligomers blocked rat hippocampal LTP (Lambert et al., 1998). Walsh et al., (2002) have provided evidence that a natural human Aβ, secreted into the conditioned culture medium of Chinese hamster ovary (CHO) cells that stably express a mutated form of APP (7PA2 cells), can perturb LTP. The conditioned medium from these 7PA2 cells contains monomeric Aβ and SDS-stable oligomeric Aβ without larger insoluble aggregates. The concentration of Aβ in 7PA2-conditioned medium is physiologically relevant (approximately 1–2 nM), that is, it is approximately the concentration found in normal human CSF (Walsh et al., 2000). Of interest, a ~100-fold lower potency of standard preparations of soluble synthetic Aβ to inhibit plasticity may be due to the lower relative abundance of stable Aβ oligomers

to less active Aβ assemblies, in particular Aβ monomers, which have been reported not to inhibit LTP (Walsh et al., 2002; Wang et al., 2004; Klyubin et al., 2005).

Soluble Aβ assemblies (monomers, dimers, trimers, etc.) became more intensely studied in AD once it was shown that Aβ in the soluble fraction of AD brain samples correlated best with AD severity (Lue et al., 1999; McLean et al., 1999). It is now generally accepted that the major toxicity of Aβ comes from neither the monomeric nor the insoluble fibrils, but rather the oligomeric assemblies. These soluble aggregated structures range from small oligomers (Podlisny et al., 1998; Walsh et al., 2005; Deshpande et al., 2006) to larger assemblies variously called Aβ-derived diffusible ligands (Lambert et al., 1998) and presumed dodecameric oligomers Aβ*56 (Lesné et al., 2006).

Thus, multi-disciplinary research provides evidence that Aβ plays an important role in AD. Taken together with the findings that monomer is innocuous and that amyloid plaques alone cannot account for disease, many have concluded that some other intermediate form of Aβ must be important (Hardy and Selkoe, 2002). Similarly, it seems reasonable that Aβ assemblies that can readily diffuse and access the space in and surrounding the synaptic cleft mediate the synaptic and neuronal compromise seen at sites distant from plaques. Given the available evidence, Selkoe and colleagues (2002, 2004) argued that AD is a form of synaptic failure. Long before the onset of clinical AD soluble (non-fibrillar) assembly states of Aβ peptides are believed to cause cognitive problems by disrupting synaptic function in the absence of significant neurodegeneration. AD therefore involves compromise of synaptic function and memory, where certain forms of the AD-associated Aβ can compromise LTP.

Studies from the last decade suggest that soluble non-fibrillar Aβ assemblies going by various names (e.g. Aβ derived diffusible ligands, oligomers, paranuclei, and protofibrils) may provide the missing link, but as yet the specific form(s) of Aβ which causes injury to neurons *in vivo* has not been identified (Harper et al., 1997; Lambert et al., 1998; Walsh et al., 1999; Hoshi et al., 2003; Gong et al., 2003; Bitan et al., 2003). Alzheimer researchers have gradually built the argument that small assemblies of the Aβ peptide might be harming neurons in ways quite separate from the damage done by fibrillized forms. It has been shown that soluble oligomers have a unique distribution in

human AD brain that is distinct from fibrillar amyloid (Kayed et al., 2003). Recently, Townsend et al. (2006) have suggested that there exists a varying degree of toxicity within naturally occurring oligomeric assemblies. Their *in vitro* studies showed that trimeric Aβ assemblies inhibited LTP with the highest potency compared with dimers and tetramers. Human CSF that contains small oligomers such as dimers can also inhibit LTP at extremely low Aβ concentrations (Klyubin et al., 2008).

Although considerable evidence supports a causal role for Aβ oligomers in AD, a direct link between a specific form of Aβ and synapse loss had not, until recently, been established. Recent research using confocal microscopy has aimed to visualize what oligomers do to dendritic spines of fluorescently labeled neurons. Shankar et al., (2007) reported that naturally secreted Aβ oligomer-containing fractions at picomolar concentrations separated by size exclusion chromatography (SEC) caused a reduction in the density of spines on dendrites of cultured rat hippocampal slices. The dendrites stayed in place throughout the experiment but were no longer studded with spines a few days after oligomer application. Spine numbers began to drop the day after oligomer exposure and recovered once the oligomers were washed out. Anti-Aβ antibodies restored spine number, much like they appear to rescue previously observed oligomer effects on LTP in rats (Klyubin et al., 2005).

Roselli et al., (2005) demonstrated that soluble Aβ interfered with postsynaptic function, providing further support to the view that soluble Aβ may be responsible for the cognitive impairments observed in early-phase AD patients (Walsh and Selkoe, 2004). They examined the possibility that soluble $A\beta_{1\text{-}40}$ exerts its effects on synaptic plasticity by regulating the molecular composition and stability of excitatory synapses. They focused their studies on postsynaptic density protein-95 (PSD-95), member of the membrane-associated guanylate kinase family of PDZ domain-containing proteins, and a key player in the organization, function, and plasticity of excitatory synapses (Ehrlich and Malinow, 2004; Kim and Sheng, 2004). The importance of PSD-95 in cognitive processes is demonstrated by the observation that PSD-95 knockout mice show impaired learning abilities (Migaud et al., 1998). The dynamic manner in which PSD-95 levels are regulated contributes to the key role of the protein in synaptic plasticity: brief NMDAR activation induces rapid PSD-95 proteasomal degradation, an event accompanied by AMPAR internalization (Colledge et al., 2003). Experiments from this

study indicated that soluble Aβ induces a decrease in PSD-95 levels in a time- and dose-dependent manner, without altering the expression of the presynaptic protein synapsin I, the AMPAR subunit GluR2, or the kinases CaMKII and cdk5. These findings indicate that PSD-95 is a specific target of Aβ. Interestingly, the dose of Aβ that affected PSD-95 was within the range that blocks LTP (Kamenetz et al., 2003; Wang et al., 2004), suggesting a correlation between the reduction of PSD-95 levels and the inhibition of LTP. Experimental results were consistent with the view that exposure of neurons to Aβ peptides alters the availability of PSD-95 and, most likely, dendritic spine morphology.

LTP has long been considered a physiological surrogate for learning and memory. Recently, Fedulov and colleagues (2007) showed that LTP indeed occurs within individual synapses during learning. LTP was also recently induced in humans, indicating that this type of plasticity does occur during learning in man (Cooke and Bliss, 2006). Other recent findings have demonstrated that oligomers can cause cognitive impairment in the absence of neurodegeneration in animals (Cleary et al., 2005; Lesne et al., 2006). Soluble oligomeric Aβ had been hypothesized, but never directly proven, to mediate aspects of memory loss in APP transgenic mice (Westerman et al., 2002, Hsia et al., 1999). The research team led by Jim Cleary addressed the unanswered question of whether cognitive deficits associated with Aβ are directly caused by soluble Aβ oligomers. Building upon the work of Walsh et al. (2002), the Cleary team showed that physiological (1-2 nM range) amounts of naturally secreted human Aβ rapidly, potently and transiently disrupted cognitive function of a learned behavior, by microinjecting 7PA2 conditioned medium i.c.v into rats. A highly sensitive operant task, that assesses very subtle cognitive effects of very small doses of psychoactive drugs, was used- the alternating lever cyclic ratio test (Weldon et al. 1996).

Although the detrimental effect of different oligomeric assemblies on synaptic plasticity is well described, less is known about the molecular mechanism that leads to malfunctioning of synapses. Shankar et al. (2007) suggested that oligomeric Aβ might disturb synaptic function via NMDA-related cascades. A recent publication (Nimmrich et al. 2008) has examined the underlying cellular mechanism of Aβ oligomer-induced synaptic modifications by using a stable oligomeric Aβ preparation called "$A\beta_{1-42}$ globulomer." Synthetically prepared $A\beta_{1-42}$ globulomer has been shown to localize to

neurons and impairs LTP (Barghorn et al., 2005). Results demonstrated that $A\beta_{1-42}$ globulomer does not affect intrinsic neuronal properties, as assessed by measuring input resistance and discharge characteristics, excluding an unspecific alteration of membrane properties. $A\beta_{1-42}$ globulomer, at concentrations as low as 8 nM, was shown to specifically suppress spontaneous synaptic activity resulting from a reduction of vesicular release at terminals of both GABAergic and glutamatergic synapses. Miniature EPSCs and IPSCs were primarily unaffected. A detailed search for the precise molecular target of $A\beta_{1-42}$ globulomer revealed a specific inhibition of presynaptic P/Q calcium currents, whereas other voltage-activated calcium currents remained unaltered. Intact P/Q calcium currents are needed for synaptic plasticity. Nimmrich and colleagues postulated that the disruption of such currents by $A\beta_{1-42}$ globulomer might cause deficits in cellular mechanisms of information storage in brains of AD patients. The inhibitory effect of Aβ1–42 globulomer on synaptic vesicle release could be reversed by roscovitine, a specific enhancer of P/Q currents. Selective enhancement of the P/Q calcium current may provide a promising strategy in the treatment of AD.

It can be concluded that the disruption of LTP or LTP-like processes by Aβ oligomers may, at least in part, be causal to memory deficits in AD. This finding is important for the development of new AD-effective drugs, as antibodies or molecules reversing such deficits at the neuronal level may be useful for the treatment of cognitive deficits in AD. An understanding of the cellular mechanisms of Aβ- oligomer-induced LTP impairment could lead to a primary molecular target, leading to effective treatment strategies.

1.2.5 Defining oligomers/proto-fibrils

Data from many laboratories now supports the once controversial hypothesis that the accumulation and aggregation of Aβ initiates a complex cascade of molecular and cellular changes that gradually leads to the clinical features of Mild Cognitive Impairment-amnestic type and then frank AD. As a result, understanding precisely how Aβ accumulation and assembly compromise synaptic structure and function has become the centerpiece of therapeutically oriented research on the disease.

Aβ is thought to start accumulating *in vivo* as low molecular weight assemblies (LMW) consisting principally of monomers that are constitutively secreted from brain cells (Figure 1.5). These may, under certain circumstances, progress to oligomers and ultimately to mature 7- to 10-nm-wide amyloid fibrils. Aβ oligomers (dimers, trimers, tetramers, and possibly larger assemblies) have been identified in the conditioned media of certain cell lines that constitutively secrete Aβ (Podlisny et al., 1995, 1998; Xia et al., 1997), and in CSF (Pitschke et al., 1998). Fibril formation by synthetic Aβ peptides is believed to proceed *in vitro* via a transition of LMW Aβ to intermediate assemblies that go on to form fibrils (Harper and Lansbury, 1997; Teplow, 1998). Two independent laboratories identified such intermediates in the formation of synthetic Aβ fibrils that are referred to as protofibrils (PF) (Harper et al., 1997; Walsh et al., 1997). From an operational perspective, soluble oligomers/proto-fibrils are defined as Aβ assemblies that are not pelleted from physiological fluids by high-speed (100,000 x *g*) centrifugation.

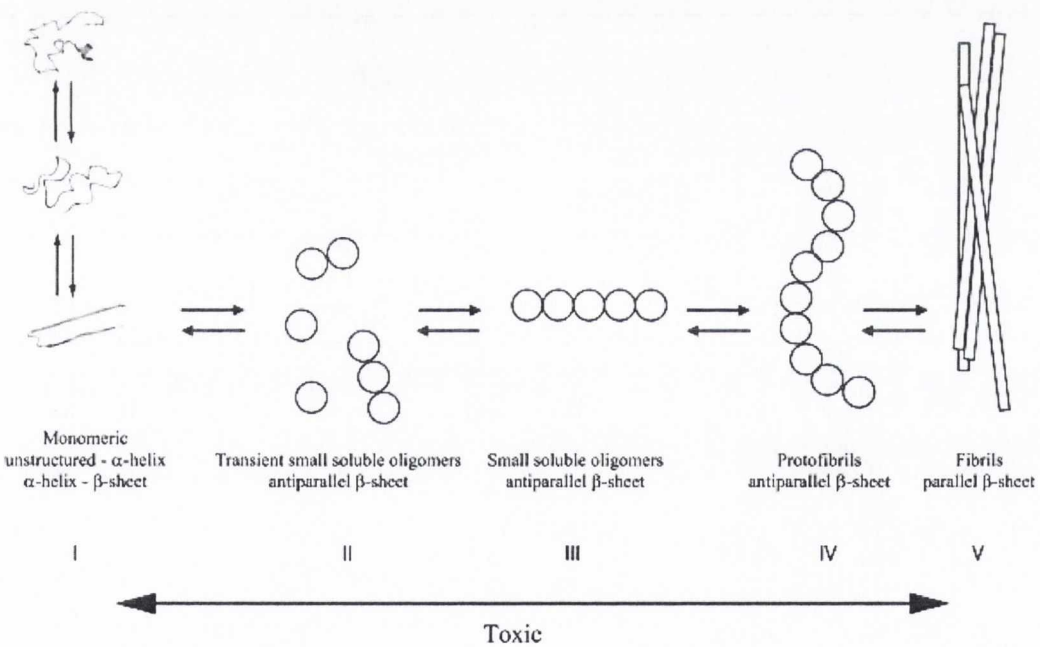


Figure 1.5 – Aggregation scheme of amyloid beta peptide involving the transformation of monomeric Aβ peptide through a variety of toxic oligomeric stages to a mature fibril (adapted from Broersen at al., 2009).

Size exclusion chromatography and photo-induced cross-linking have identified a LMW Aβ components containing monomers, dimers, trimers and tetramers in rapid equilibrium, as well as high molecular weight (HMW) globular oligomers in solutions

of freshly prepared peptide (Walsh et al., 1997; Lambert at al., 1998). They are flexible structures that can continue to polymerize *in vitro* to form amyloid fibrils or can de-polymerize to lower-order species. The PF/HMW oligomer peak (as shown by size exclusion chromatography) decreases concomitantly with the appearance of more rigid and longer mature fibrils (Walsh et al., 1999; Goldsbury et al., 2000). PFs are narrower than bona-fide amyloid fibrils (~4–5 nm versus 7–10 nm). Ultrastructural analyses of synthetic protofibril preparations by electron microscopy and atomic force microscopy have revealed both straight and curved assemblies up to 150-200 nm in length. Synthetic Aβ PFs have been shown to contain substantial β-sheet structure, as they are able to bind Congo red or Thioflavin T in ordered fashion. HMW oligomers are heterogeneous, containing from 50 to 200 Aβ peptides as measured by quantitative scanning transmission electron microscopy (Goldsbury et al. 2000). As assembly proceeds, short flexible PF structures <200 nm in length also appear (Walsh et al. 1999; Harper et al. 1999).

Many different types of assembly forms of synthetic Aβ, including amyloid fibrils, protofibrils, annular structures, paranuclei, Aβ-derived diffusible ligands and globulomers, have been described over the last two decades (for review see Caughey and Lansbury 2003). Current research investigating the relative importance of the various soluble Aβ assembly-states in causing synaptic and cognitive deficits has emphasized the importance of both low-n oligomers (such as dimers and trimers) and larger oligomers (including some that may form globular structures independent of Aβ aggregation into fibrils). A major difficulty has been the challenge of obtaining sufficiently pure forms of Aβ for quantitative study.

1.2.6 Fibrils of Aβ

The mechanism underlying the initiation of this progressive pathophysiology is believed to involve the age-related accumulation of Aβ, which can form the abundant amyloid fibrils observed in neuritic plaques at autopsy (Esiri et al., 1997). The observation of these end-stage lesions in postmortem brain tissue has led to an assumption that accumulation of fibrils per se underlies the progression of AD. This impression has been supported in part by studies of neuronal cultures, in which progressive neurodegeneration can be induced by highly aggregated, fibrillar Aβ but not by

equivalent concentrations of Aβ monomers (Mattson et al., 1993; Pike et al., 1993; Lorenzo and Yankner, 1994).

AD is neuropathologically defined, in part, as extracellular amyloid deposits in the brain containing polymerized Aβ fibrils. Amyloid fibril formation is an extremely complex reaction (for a review see Kumar and Udgaonkar 2009). A protein can assemble into multiple structurally distinct fibrils and structural heterogeneity also appears to be prevalent in the assembly intermediates formed at initial times of the reaction (Kodali and Wetzel, 2007). Amyloid fibrils are ~10 nm in diameter, and are composed typically of 2–6 protofilaments. Amyloid fibrils of all proteins possess the same structural motif, the cross-β motif, wherein the β-strands are oriented perpendicular to, and the β-sheets parallel to the fibril axis (Sunde and Blake, 1998). In cross-β motifs, the separation between hydrogen-bonded β-strands is ~0.48 nm, and that between β-sheet layers is ~ 1.0–1.3 nm (Serpell et al., 2000). Amyloid fibril formation involves a structural rearrangement of the native state into a β-sheet rich fibrillar conformation (Frare et al., 2006). β-sheets seem to provide a scaffold that is favorable for protein assembly: the edge strands of β-sheet structures are unstable, and the sheet can grow by interacting with any other β-strands it encounters. Natural β-sheet proteins are seen to utilize a number of mechanisms to avoid the edge-to-edge aggregation of their β-sheets (Richardson and Richardson, 2002).

The kinetics of mature fibril formation by the Aβ protein displays an initial lag phase, which is shortened upon seeding (for a review see Goldsbury et al., 2005). It appears therefore that the formation of mature fibrils by the Aβ protein involves nucleation-dependent polymerization (NDP). NDP is expected to be cooperative and to proceed without any accumulation of stable pre-fibrillar oligomers. The accumulation of spherical oligomers and protofibrils precedes the formation of mature fibrils by amyloid-β. It appears that the fibril formation mechanism by Aβ is much more complex than that expected from either the isodesmic (linear Figure 1.5) or the NDP mechanism.

In AD, Aβ peptides accumulate extracellularly and polymerize into fibrils, resulting in amyloid deposits in the vasculature and parenchyma of the hippocampal and cortical regions of the brain. It has been speculated that the mature fibrils themselves, as well as oligomers and/or protofibrils transiently appearing during fibril formation, could

contribute to neurotoxicity, manifesting in the cognitive decline evident in AD (Walsh and Selkoe, 2004). In cell culture models fibrils have been shown to be neurotoxic (Busciglio at al., 1992; Pike et al., 1993; Ward et al., 2000; Petkova et al., 2005). Other experiments characterized the dystrophic effect of fibrillar Aβ on rat cortical and hippocampal neurons (Grace et al., 2002; Grace and Busciglio, 2003). A similar dystrophic neurite-inducing effect of fibrillar Aβ was observed on human cortical neurons (Deshpande et al., 2006).

Some researchers have questioned the importance of fibrils in AD- hypothesizing that not all fibril morphologies are equally toxic, leading to variable results with regard to cytotoxicity. Puzzo and Arancio (2006) showed that synthetically derived fibrillar Aβ could impair the late phase of LTP, further emphasizing fibrils in the disease state. Critics comment that it is very difficult to ensure the purity of a fibrillar Aβ solution, without contamination of either preseeds or pre-fibrillar material. Therefore it cannot be excluded that the toxicity observed for fibrillar Aβ is actually the result of contamination. *In vivo* experiments in transgenic models show the presence of widespread dystrophy in neuronal processes in contact with fibrillar Aβ deposits (Tsai et al., 2004; Spires et al., 2005). Schmid et al., (2007) further tested fibrillar Aβ in an *in vivo* rat model. The fibrillar $A\beta_{1-40}$ peptides were applied via i.c.v injection and the effects on synaptic transmission and LTP were observed. Results demonstrated that fibrillar Aβ can attenuate LTP and may also influence transmitter release.

In 2008, a team of Belgian researchers (Martins et al., 2008) suggested that mature fibrils might not be the inert end products of a pathway involving the formation of a heterogeneous population of transient toxic Aβ oligomeric assemblies. They found that co-incubation of mature Aβ fibrils with biomimetic membrane particles results in the release of toxic Aβ oligomers, suggesting a fibril-to-oligomer pathway. Incubation of released Aβ oligomers from fibrils on hippocampal primary neuronal cell cultures resulted in profound cytotoxicity, and animals injected with these oligomers showed significant cognitive impairment compared with control animals. The main question arising from these observations is whether this fibril to oligomer pathway might occur *in vivo*.

The underlying cause of AD is thought to be the accumulation and aggregation of Aβ. A promising strategy against AD is the application of protective, peptide-based neuroprotective agents that selectively bind to Aβ, arresting its entry into a toxic aggregation pathway. Aβ polymerization *in vivo* is far from understood. However, numerous *in vitro* studies have been conducted with the aim of eventually establishing *in vivo* inhibition strategies (for a review see Bitan et al., 2003).

1.2.7 Conclusions

The studies reviewed above highlight the challenging nature of identifying the critical component of the complex and constantly evolving reaction mixture of different Aβ assemblies. However, the potential result may be highly rewarding as it should allow us to understand how known risk factors for AD map onto the formation of toxic intermediates of amyloid formation: do well established risk factors such as carboxy-terminal heterogeneity of Aβ, the apolipoprotein E allele and age act by stabilizing the toxic substructures, and if so, which therapeutic interventions are best suited for counter acting these effects? Moreover, as long as we cannot accurately model the accumulation of the neurotoxic assemblies, we cannot be certain that therapeutic intervention will produce the desired outcome. For example, an overall decrease in the concentration of Aβ may stabilize low-n oligomers, thereby potentially increasing rather than decreasing toxicity.

1.3 A role for anti- Aβ antibodies in Alzheimer's disease?

1.3.1 Therapeutic strategies for AD

Modern therapeutic strategies in AD are addressed to interfering with the main pathogenic mechanisms potentially involved in AD. Major pathogenic events and their respective therapeutic targets include the following: genetic defects, β-amyloid deposition, tau-related pathology, apoptosis, neurotransmitter deficits, neurotrophic deficits, neuronal loss, neuroinflammation, oxidative stress, calcium dysmetabolism, neuronal hypometabolism, lipid metabolism dysfunction, cerebrovascular dysfunction, neuronal dysfunction associated with nutritional and/or metabolic deficits, and a miscellany of pathogenic mechanisms potentially manageable with diverse classes of

chemicals or biopharmaceuticals (for a review see Cacabelos, 2009). Since the early 1980s, the neuropharmacology of AD was dominated by the acetylcholinesterase inhibitors- represented by tacrine, donepezil, rivastigmine and galantamine (Loveman, 2006). Memantine, a weak NMDA antagonist, was introduced in the 2000s for the treatment of severe dementia (Reisberg et al. 2001); and the first clinical trials with immunotherapy, to reduce Aβ burden in senile plaques, were withdrawn due to severe adverse drug reactions (Shenk et al. 2004).

Researchers of AD have identified five strategies as possible interventions against Aβ (for a review see Citron, 2004). β-Secretase inhibitors are one strategy that would work to block the first cleavage of APP. γ-Secretase inhibitors (e. g. semagacestat) work to block the intra-membrane cleavage of APP and stop the subsequent formation of Aβ. A third therapeutic intervention is delivering selective $A\beta_{1-42}$ lowering agents (e.g. tarenflurbil) that modulate γ-secretase to reduce $A\beta_{1-42}$ production in favor of other (shorter) Aβ versions. Strategies that deliver anti-aggregation agents may prevent Aβ from aggregating or clear aggregates once they are formed. Finally, immunotherapeutic strategies that passively stimulate the host immune system to recognize and attack Aβ or actively administering antibodies that either prevent plaque deposition or enhance clearance of plaques, is another strategy. During the past few years no relevant drug candidates have been approved for the treatment of AD, despite the initial promises of β- and γ-secretase inhibitors.

1.3.2 Targeting Aβ pathology in Alzheimer's disease with Aβ immunotherapy

Immunization against the Aβ protein as a disease-modifying treatment for AD is gaining experimental support from numerous laboratories. Walsh et al. (2002) observed that an *ex vivo* removal of Aβ prevented the inhibition of hippocampal LTP in anaesthetized rats. Recent studies have demonstrated the potential utility of antibodies for the treatment of AD. In transgenic mouse models of AD, peripheral and intracerebral administration of Aβ -specific antibodies reduce amyloid burden to varied extents (Tucker et al. 2008). Tucker and colleagues reported that intracerebral injection of Aβ antibodies produces modest reductions in amyloid deposition in two transgenic models and that the mechanism may involve prevention of amyloid formation rather

than clearance of pre-existing plaques. Klyubin et al. (2005) reported that injection of a monoclonal antibody (mAb) to Aβ completely prevented the inhibition of LTP, in rat hippocampus, when injected after Aβ. This laboratory also showed that active immunization against Aβ was partially effective in a rat model. Furthermore, they reported that the partial LTP rescuing effects correlated positively with levels of antibodies to Aβ oligomers. The ability of both forms of *in vivo* immunization to neutralize plasticity-disruptive solube Aβ oligomers suggests that treatment with such antibodies could target potentially reversible cognitive deficits in early AD.

The clinical efficacy of active immunization with Aβ comes with a note of caution. In 2002 a Phase II safety, tolerability, and pilot efficacy study was abandoned after four early reports of meningoencephalitis, but follow-up continued (Schenk, 2002). Symptoms and laboratory findings consistent with meningoencephalitis occurred in 18 of 298 (6%) patients treated with AN1792 (an experimental preparation of pre-aggregated Aβ) compared with 0 of 74 on placebo).

Aware of such problems, Chauhan and Siegel (2002) found success with a modified passive immunization method in transgenic mice models of the disease (Tg2576 and TgCRND8). In a single small-dose injection, they delivered the antibody (Ab) directly into the third ventricle and examined brain tissue at one, four and eight weeks. At one and four weeks, the density of amyloid protein was 67% less than in control animals. But by eight weeks, with no further Ab injections, the protein had again accumulated. No side effects, such as hemorrhaging or inflammation, were evident. The results suggest that periodic administration of Abs directly into the brain might offer a safer method for treating Alzheimer's. The vaccine reduces the accumulation of amyloid proteins for at least four weeks, providing a window during which other treatments could be used to prevent the formation of new plaques.

As of 2008, there were more than 10 clinical trials of Aβ immunotherapy underway in patients with AD (Nitsch and Hock, 2008). The aim is to identify safe approaches for the efficacious antibody-mediated removal of aggregated brain Aβ or its neurotoxic oligomeric precursors. Initial experimental and neuropathological evidence for clearance of brain β-amyloid in response to Aβ immunotherapy is associated with structural and functional rescue of neurons, as well as initial signs of clinical

stabilization and reduced rates of dementia progression. For the next steps in the future improvement of Aβ immunotherapy, major challenges in pharmacokinetics, safety, and tolerability need to be addressed. These include the low penetrations rates of IgG molecules through the blood-brain barrier, possible reductions in brain volume, the possibility of autoimmune disease related to unwanted cross-reactivity with endogenous antigens on physiological structures, micro-hemorrhages related to cross-reaction with pre-existing vascular amyloid pathology, possible relocalization of Aβ from Aβ-plaques to brain blood vessels resulting in increased amyloid angiopathy, and the lacking activity of Aβ antibodies on pre-existing neurofibrillary tangle pathology, as well as the lacking molecular identification of the forms of Aβ to be therapeutically targeted (for a review see Bates et al., 2009).

The solutions to these problems will be guided by the fine lines between tolerance and immunity against physiological and pathological structures, respectively, as well as by the understanding of the pathogenic transition of soluble Aβ into toxic oligomeric aggregation intermediates in the dynamic equilibrium of Aβ fibril assembly. Provided that the ongoing and planned clinical trials address these issues in a timely manner, there is a good chance for Aβ immunotherapy to be one of the first disease-modifying therapies of Alzheimer's disease to be introduced into clinical practice.

1.3.3 Aβ related neurotoxicity in AD: oligomer size or conformation?

As previously mentioned, the oligomeric form of Aβ is highly toxic to the brain and is the trigger for loss of synapses and neuronal damage. Because of this, many laboratories have been hunting for a specific molecular assembly of defined size that is the main trigger of AD. The result has been the identification of a host of molecular assemblies of Aβ, ranging from dimers (Klyubin et al., 2008; Shankar et al., 2008), trimers (Townsend et al., 2006) and Aβ assemblies with a molecular weight of 56 kDa (Lesne et al., 2006) to Aβ-derived diffusible ligands (Gong et al., 2003) and protofibrils (Kayed et al., 2009) in potent neurotoxic fractions. All are capable of impairing memory formation in mice and their formation and significant accumulation in the brain should thus be considered a potential cause of AD.

An operational definition of amyloid-fibrils is Aβ assemblies that are pelleted from physiological fluids by high-speed (100,000 x *g*) centrifugation. Oligomers-ptotofibrils were considered to be all the assembly species other than this, except for monomers. Charles Glabe has taken this assembly classification a step further by introducing an Ab-specificity element, whereby amyloids are classified on their ability to react with conformation-dependent antibodies (Glabe, 2008). Aβ assemblies are considered to acquire surface tertiary structures that are not present in physiologic Aβ monomers and that induce synaptic impairment and neuronal loss through interactions with neuronal cells. Therefore, as recently suggested by Charles Glabe, it is reasonable to classify seemingly different Aβ assemblies in terms of their immunoreactivity to antibodies that recognize particular surface tertiary structure.

Because the surface tertiary structure mediates the binding of Aβ assemblies to their target(s) and is therefore responsible for exerting the toxic effects, Aβ assemblies having distinct surface tertiary structures are likely to have distinct mechanisms of neurotoxicity and may contribute differently to the disease development. Charles Glabe and colleagues have suggested the existence of two distinct types of soluble oligomer conformations, designated as prefibrillar oligomers and fibrillar oligomers, which overlap broadly in size but are immunologically different (Kayed et al. 2007).

The assembly of Aβ amyloid is generally described as a nucleation-dependent polymerization reaction. Like any chemical process, the characterization of this assembly process requires the description of the order in which the relevant molecular species occur along the reaction pathway. However, as aggregation is a stochastic process, molecules will not synchronize during the reaction and, as a result, the reaction mixture will be highly complex and composed of several assemblies (polymorphic) at any given time. Homogeneity of the sample is not thought to occur until after the polymerization reaction is complete and even then it is possible that the formed mature fibrils or plaques are not eternally stable. To make matters worse, the composition of the reaction will be significantly modulated by peptide concentration and physicochemical parameters, such as temperature, ionic strength and pH. It is thus not surprising that independent studies of this highly dynamic reaction mixture have yielded a multitude of transient molecular assemblies that have been claimed to occupy an essential position along this pathway. The transient nature of the intermediate oligomers

is equally challenging for the characterization of the toxic potential of these assemblies (Bitan et al. 2003), and thus a number of differently sized oligomers have been suggested as the cause of AD.

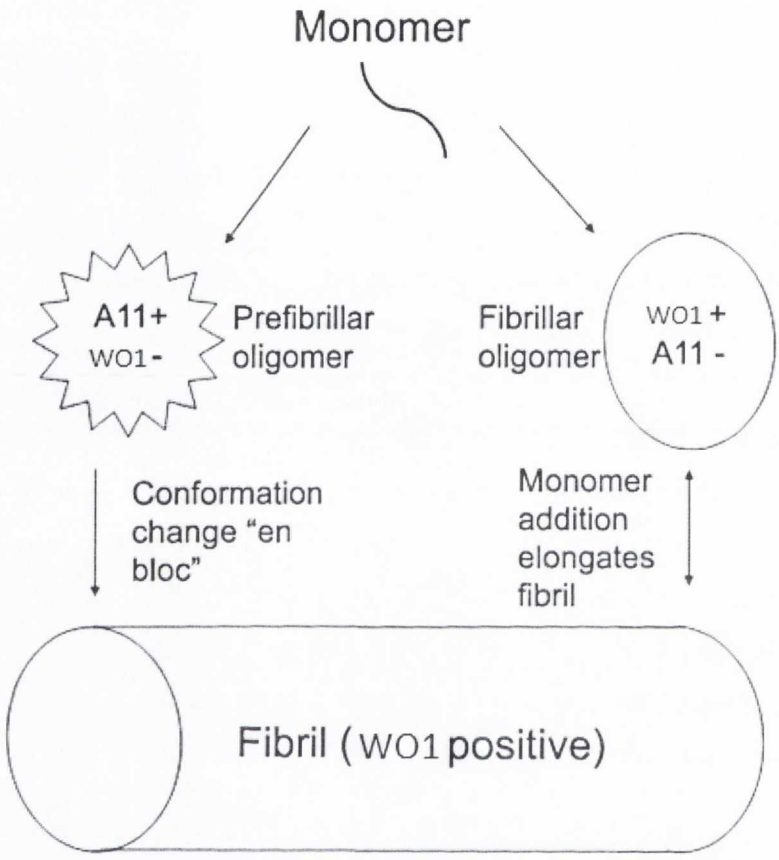


Figure 1.6 - Schematic representation of the distinct types of amyloid oligomers and fibrils (adapted from Glabe, 2008). See text for description.

The aggregation pathway (Figure 1.6) begins with a misfolded amyloidogenic monomer (top) and can diverge into two paths depending on which conformation it adopts (Glabe, 2008). Monomers can aggregate to form prefibrillar oligomers that are A11-positive and WO1-negative (left pathway). These prefibrillar oligomers may then align to form protofibrils (Figure 1.6) and undergo a concerted conformation change "en bloc" to form fibrils. They are termed prefibrillar oligomers because they are transient intermediates that ultimately become fibrils. In the other pathway, amyloidogenic monomers aggregate to form a fibrillar conformation or lattice that is WO1-positive and A11-negative (right pathway). These fibrillar oligomers may represent fibril nuclei or seeds that are aggregates capable of elongating by recruiting additional monomers at

their ends. Addition of monomers ultimately elongates the fibrils to a size that satisfies an arbitrary operational definition of insolubility and would be recognized as fibrillar under the electron or atomic force microscope, although no conformational difference is apparent by Ab reactivity. Fibrils may be distinct from fibrillar oligomers on the basis of their content of multiple protofilaments (Figure 1.5), but this does not imply that a fundamental conformation change in their integral peptide-building blocks is necessary for fibrillar oligomers to convert to fibrils. They may simply coalesce or grow by monomer addition to form fibrils.

1.3.4 The anti-oligomer antibody A11

Aβ oligomers have been known to be present in Aβ preparations for many years (Masters et al., 1985). Their significance and their relationship with fibrils has been a longstanding question that is only now becoming clearer as techniques improve. Antibody M16- specific for aggregated forms of Aβ- was one of the first conformation-dependent antibodies created in a lab (Yang et al., 1995). On Western blots, this Ab recognized SDS-resistant aggregates of dimers or tetramers or higher-order Aβ oligomers, but not monomer or APP, and these antibodies stain plaques intensely, suggesting that they are conformationally related to fibrils. This Ab was useful for distinguishing pathological Aβ deposits from native APP, which is important for identifying intracellular Aβ deposits.

Charles Glabe's research group suggested that not all oligomers are obligate precursors to fibrils, but instead follow their own, separate pathway (Yang et al., 1995). Recently, this idea has received some support from others. For example, research on other neurodegenerative diseases involving deposition of proteins such as α-synuclein and huntingtin suggests that chaperones importantly influence which pathway the monomeric protein will enter (Muchowski and Wacker, 2005).

The antibody, A11 (pan-anti-oligo), was developed and used to show that there are also oligomers that have a conformation that is distinct from fibrils. Antibody A11 is selective for prefibrillar Aβ oligomers (Kayed et al., 2003), recognizing a generic epitope common to prefibrillar oligomers and not fibrils, monomers or natively folded precursor proteins. These oligomers are widely believed to represent a primary toxic or

pathological species and are called "prefibrillar" because they kinetically precede fibril formation and disappear after fibrils have formed (Baglioni et al., 2006). This Ab was produced in rabbits by immunization with a molecular mimic of oligomeric Aβ. While A11 stains small focal or punctuate deposits in AD tissue, it does not stain diffuse plaques or other plaque types, suggesting that diffuse deposits are not accumulations of prefibrillar oligomers (Kayed et al., 2003). Glabe and his colleagues (Kayed et al., 2007) compared fibril-specific and oligomer-specific antibodies and showed that they recognize separate and distinct assemblies, and yet on Western blots some of these soluble oligomers run at the same size (~50 KD–200 KD). This indicates that there are two distinct types of soluble oligomers and that size is a poor indicator of whether something is fibrillar or prefibrillar (conformation).

Remarkably, the antibody binds (and blocks the toxicity of) not only oligomers of Aβ, but also amyloidogenic proteins with diverse amino acid sequences, such as oligomeric aggregates formed by α-synuclein, islet amyloid polypeptide, polyglutamine, lysozyme, insulin, and prion protein. It is interesting that the same concentrations of these different proteins like insulin, prion protein, α-synuclein all led to an oligomeric state that was recognized by the same antibody at the same concentrations.

A11 did not detect the monomeric or fibrillar versions of these proteins, findings similar to those of Aβ experiments. It would appear that A11 recognizes a common structural peptide backbone epitope independent of amino acid side chains- effectively targeting structure and not sequence. A potential explanation for this unusual specificity is the possibility that the anti-oligomer antibody recognizes a unique ''alpha extended chain'' pleated sheet structure (Kayed and Glabe, 2006). Kayed and colleagues have recently (2009) used the A11 antibody to further characterize sub-species of oligomers. Using A11 and other conformation-specific antibodies (αAPF and OC) they were able to distinguish annular protofibrils from pre-fibrillar oligomers. In another recent paper, researchers used A11 to provide novel structural insight into the relationship between fibrils and fibrillar oligomers- suggesting that the increased toxicity of fibrillar oligomers may be due to their ability to replicate and the exposure of hydrophobic sheet surfaces that are otherwise obscured by sheet-sheet interactions between protofilaments in a fibril (Wu et al., 2009).

1.3.5 The (proto)-fibrillar antibodies WO1 and WO2

Disease-related amyloid fibrils appear to share a common, but poorly understood, structure. O'Nullain and Wetzel (2002) reported the generation and preliminary characterization of two conformation-specific mAbs, WO1 and WO2, that bind to the amyloid fibril state of the Alzheimer's peptide Aβ1–40 but not to its soluble, monomeric state. Interestingly (like A11), these mAbs also bind to other disease-related amyloid fibrils and amyloid-like aggregates derived from other proteins of unrelated sequence, such as transthyretin, islet amyloid polypeptide, β2-microglobulin, and polyglutamine. At the same time, WO1 and WO2 do not bind to the native protein precursors of these amyloids, nor do they bind to other kinds of protein aggregates. This new class of mAbs, associated with a fundamental amyloid-folding motif, appears to recognize a common conformational epitope with little apparent dependence on amino acid side chain information.

Follow-up studies indicated that the anti-fibril Ab WO1 also recognizes protofibrils, indicating that they may share a common structure to fibrils (Williams et al., 2005). This group used a molecule that stimulated Aβ aggregation, calmidazolium chloride (CLC), which rapidly and efficiently converted $A\beta_{1\text{-}40}$ monomers into clusters of protofibrils. As monitored by electron microscopy, these protofibrils persisted for days when incubated in phosphate buffer solution (PBS) at 37°C, with a slow transition to fibrillar structures apparent only after several weeks. Like normal protofibrils, the CLC-Aβ aggregates exhibit a low thioflavin T response.

Metastable oligomeric and protofibrillar forms of amyloidogenic proteins have been implicated as on-pathway assembly intermediates in amyloid formation and as the major toxic assemblies in a number of amyloid diseases including Alzheimer's disease. These conformation-specific mAbs, WO1 and WO2, should contribute to the understanding of amyloid structure, assembly, and toxicity.

1.3.6 Conclusions

In conclusion, a wide range of AD-related synaptotoxic Aβ oligomeric sizes have been identified. How oligomer size precisely relates to the disease process has not been

clarified and recent work shows that the wide range of Aβ oligomers may have a specific conformation in common. Recent findings suggest that Aβ oligomer size may not be the only AD-inducing factor and paradigms in which both oligomer size and structural arrangement act as toxic parameters in AD development should be adopted. Studies targeting the toxic contributor to AD in the past have usually highlighted only one of these aspects, but future studies should consider a multi disciplinary approach in which oligomer size, structural characteristics and synaptic activity results are recorded simultaneously.

1.4 A role for the cellular prion protein receptor in Alzheimer's disease?

1.4.1 Putative physiological role of Aβ in synaptic plasticity

The extremely high potency of Aβ oligomers in disrupting synaptic plasticity has led to extensive studies into the cellular mechanisms (Pena et al., 2006; Rowan et al., 2007), including putative receptor sites (Verdier and Penke, 2004ab; Wang et al., 2007; Origlia et al., 2008; Yang et al., 2008; Lauren et al., 2009; Yan et al., 2009). Despite well-established deleterious effects of certain Aβ assemblies, there is growing evidence that APP and its fragments, including Aβ itself, may play a role in normal neuronal functioning (Bishop and Robinson, 2004; Senechal et al., 2006; Wasling et al., 2009). Indeed, APP-deficient mice show compromised neuronal function, reduction in synaptic markers and deficits in learning and memory as well as synaptic plasticity, although there is some lack of agreement as to the relative importance of different APP products and the role of compensatory changes (Muller et al., 1994; Zheng et al., 1995; Dawson et al., 1999; Phinney et al., 1999; Seabrook et al., 1999; Ring et al., 2007; Senechal et al., 2008). Somewhat similar considerations arise in the case of BACE knockout mice (Ma et al., 2007; Wen et al., 2008). Remarkably, endogenous Aβ has been implicated even in neuronal survival in cultured neurons (Plant et al., 2003). Of particular relevance to synaptic mechanisms, Kamenetz et al., (2003) suggested that Aβ might serve as a normal negative feedback mechanism in the regulation of synaptic activity. Since several potential therapeutic approaches of AD treatment are designed to target APP, it is important to understand the physiological functions of the different APP breakdown products in synaptic plasticity.

1.4.2 Amyloid-receptor interactions

Interactions between Aβ and various receptors have been shown through biochemical and pharmacologic techniques. Given the profound loss of cholinergic transmission in AD nicotinic and muscarinic acetylcholine receptors have drawn considerable attention. Synthetic Aβ has been shown to bind the calcium permeable α7 nicotinic acetylcholine receptors with high affinity (Wang et al., 2000). Functionally, this interaction has been proposed to account for the internalization of NMDA receptors through a calcineurin dependent pathway (Snyder et al., 2005; Dewachter et al., 2009).

Recently, Wang et al., (2008) investigated the role of integrin receptors in Aβ-mediated block of LTP in the dentate gyrus *in vitro* and in the CA1 *in vivo*. Selective antibodies to the αv-integrin subunit were found to prevent the Aβ inhibition of LTP, both in the dentate gyrus *in vitro* and in the CA1 *in vivo*. In contrast, two control antibodies did not prevent such action of Aβ. In addition, a small molecule nonpeptide antagonist of αv-containing integrins and two other antagonistic ligands of integrins, superfibronectin and the disintegrin echistatin, also prevented the Aβ inhibition of LTP. These studies indicated that αv-integrins might be important mediators of synaptic dysfunction prior to neurodegeneration in AD.

In 2009, Nikolaev and colleagues described a novel role for APP in neurodegeneration, a role that appears completely independent of Aβ toxicity and that might finally explain why only certain neurons bear the brunt of Alzheimer disease pathology (Nikolaev et al., 2009). This laboratory reported that the extracellular domain of APP serves as a ligand for death receptor 6 (DR6), an orphan member of the tumor necrosis factor receptor superfamily. They showed that when the N-terminus of sAPPα (N-sAPPα) binds DR6, it sets off an apoptotic cascade in embryonic spinal neurons that targets both axons and cell bodies. The work indicates that N-sAPPα has a role in axonal pruning and neural cell death in development, but it also raises the possibility that similar events occur in mature neurons in the brain.

Progressive decrease in neuronal function is an established feature of AD. Previous studies have shown that Aβ peptide induces acute increase in spontaneous synaptic

activity accompanied by neurotoxicity, and Aβ induces excitotoxic neuronal death by increasing calcium influx mediated by hyperactive AMPA receptors. An *in vivo* study has revealed subpopulations of hyperactive neurons near Aβ plaques in mutant APP-transgenic animal model of AD that can be normalized by an AMPA receptor antagonist. Wang et al. (2010) determined that soluble Aβ acutely induces hyperactivity of AMPA receptors by a mechanism involving β2-adrenergic receptor (β2AR), and that extra cellular N terminus of β2AR is critical for the binding. The binding is required to induce G-protein/cAMP/PKA signaling, which controls PKA-dependent phosphorylation of GluR1 and β2AR, and AMPA receptor-mediated EPSCs. β 2AR and GluR1 also form a complex comprising postsynaptic density protein 95, PKA and its anchor AKAP150, and protein phosphotase 2A. Both the third intracellular loop and C terminus of β2AR are required for the β2AR/AMPA receptor complex. Aβ acutely induces PKA phosphorylation of GluR1 in the complex without affecting the association between two receptors.

For the cellular basis of the Aβ-induced synaptic changes, previous studies have suggested the involvement of postsynaptic signaling mechanisms. For example, the binding of amyloid-beta derived diffusible ligands (ADDLs) and Aβ oligomerss has been reported to co-localize with PSD-95 (Lacor et al,. 2004; Deshpande et al., 2006; Lacor et al., 2007). As expected from the postsynaptic locale of their binding, ADDLs bind close to or at NMDA-R, and NMDA-R antagonists inhibit ADDL-induced dendritic changes (Lacor et al. 2007), reactive oxygen species formation (De Felice et al. 2007), and insulin receptor impairment (Zhao et al. 2008). NMDA-R antagonists have also been reported to inhibit Aβ dimer-induced synaptic loss (Shankar et al., 2007; Shankar et al., 2008). Interestingly, cellular prion protein (PrPc), which interacts with NMDAR (Khosravani et al. 2008), has recently been reported to serve as a high affinity postsynaptic receptor mediating ADDL-induced synaptic dysfunction (Lauren et al. 2009). Taken together, these studies are consistent with the idea that Aβ dimers, ADDLs, and AβOs perturb postsynaptic transmission.

1.4.3 The putative cellular prion protein Aβ receptor

The APP-processing pathway is a pathological component of AD, but there is no consensus regarding the physiological functions of APP and its products. Two studies (Nikolaev et al., 2009; Lauren et al., 2009) link the physiological and pathological aspects of APP processing. They show that the APP products, N-sAPPα and $A\beta_{1-42}$, are ligands for death receptor 6 and PrPc, respectively, which are important in nervous system development and synaptic suppression.

Since most PrPc is localized to the cell surface, it is reasonable to hypothesize that the protein could participate in transmembrane signaling processes. Like other GPI-anchored proteins, PrPc resides in lipid raft domains on the plasma membrane, which are known to serve as molecular scaffolds for signal transduction (for a review see Westergard et al., 2007). Since its polypeptide chain is entirely extracellular, PrPc would presumably need to interact with transmembrane adaptor proteins in order to transmit signals into the cytoplasm. Clearly, a crucial challenge in the field is to identify the molecular components of putative PrPc-mediated signal transduction pathways.

There are now a number of studies suggesting that PrPc can activate transmembrane signaling pathways involved in several different phenomena, including neuronal survival, neurite outgrowth, and neurotoxicity (Schneider et al., 2003; Chen et al., 2003; Forloni et al., 1993). In some of these cases, signal transduction is initiated by interaction of PrPc with specific protein or peptide ligands. In other cases, PrPc appears to act constitutively.

In 2009, Lauren et al. startled the research world, when they showed that oligomers of Aβ bind cellular prion protein. Furthermore, in the absence of PrPc, Aβ oligomers no longer suppressed LTP—one of the best-characterized Aβ toxicities. The discovery that PrPc may be a mediator in the development of Alzheimer's disease is fascinating, not least from a therapeutic perspective.

The Strittmatter lab had previously shown that the Nogo receptor, known for limiting axon regeneration and repair, binds to APP and to Aβ, but not necessarily oligomers (Park et al., 2006a and Park et al., 2006b). To find proteins that specifically bind to

oligomeric assemblies of the peptide, which are widely believed to be the most toxic Aβ entities, Lauren and colleagues used biotin-labeled oligomers (prepared as per the ADDL method of Bill Klein's group [Chromy et al., 2003]) to screen COS-7 cells expressing a mouse adult brain cDNA library. Screening 200,000 clones generated two strong hits—both expressing the prion protein.

To test whether this oligomer-prion interaction was real, Lauren and colleagues took advantage of PrPc knockout mice (Prnp-/-), which seemed to have normal synaptic plasticity as judged by LTP measurements (Lauren et al. 2009). They observed that after 15-20 days *in vitro*, cultured hippocampal neurons from wild-type mice bound Aβ oligomers. This timing corresponds to the emergence of PrPc expression in Map2-positive dendrites on the neurons, suggesting PrPc is a major Aβ binding site in these cells. The Aβ oligomers and the PrPc colocalized, and while the researchers did detect Aβ oligomers on PrPc-negative neurons, binding was reduced by about 50 percent, again indicating PrPc as a major site for oligomer binding.

To test the functional significance of this prion-Aβ interaction, Lauren and colleagues measured LTP in the Schaeffer collateral pathway of the hippocampus. In hippocampal slices from normal mice, $A\beta_{1-42}$ oligomers reduced LTP significantly, but in slices from PrPc-null animals the oligomers did not. In addition, wild-type slices were protected from the toxic effects of Aβ oligomers if they were first treated with the PrPc antibody 6D11. The authors concluded that PrPc exerts a receptor action acutely to mediate $A\beta_{1-42}$-oligomer inhibition of synaptic plasticity in the hippocampal slice. Very recently, Chen et al. (2010) reported that $A\beta_{1-42}$ oligomers, and not monomers or fibrils, showed a remarkably high binding affinity to PrPc.

This year, the Strittmatter laboratory (Gimbel et al. 2010) published another paper supporting their hypothesis that PrPc is essential for the ability of brain-derived Aβ to suppress cognitive function. They crossed familial AD transgenes encoding APPswe and PSen1δE9 into Prnp–/– mice to examine the necessity of PrPc for AD-related phenotypes. Neither APP expression nor Aβ levels were altered by PrPc absence in this transgenic AD model. However, deletion of PrPc expression rescued 5-HT axonal degeneration, loss of synaptic markers, and early death in APPswe/PSen1δE9 transgenic mice. The AD transgenic mice with intact PrPc expression exhibited deficits

in spatial learning and memory. Mice lacking PrPc, but containing Aβ plaque derived from APPswe/PSen1δE9 transgenes, showed no detectable impairment of spatial learning and memory. Thus, deletion of PrPc expression dissociated Aβ accumulation from behavioral impairment in these AD mice, with the cognitive deficits selectively requiring PrPc.

This hypothesis has become a hotly debated issue. In a challenge of this work, Balducci et al. (2009) confirmed that $A\beta_{1-42}$ oligomers interact with PrPc, with nanomolar affinity, but that PrP-expressing and PrP knockout mice were equally susceptible to this Aβ-mediated impairment of recognition memory. Thus their data suggest that $A\beta_{1-42}$ oligomers are responsible for cognitive impairment in AD and that PrPc is not required. A second lab has also refuted the importance of PrPc in Aβ –mediated synaptic perturbations. Kessels et al. (2010) showed that Aβ-induced synaptic depression, loss of dendritic spines and blockade of LTP are present in hippocampal slices prepared from PrPc-deficient animals. As well, Aβ oligomers were shown to impair long-term memory equally in PrPc-lacking and PrPc-expressing mice. They concluded that although oligomeric Aβ can bind PrPc, it does not seem to be the receptor responsible for synaptic perturbations caused by oligomeric Aβ.

What is clear is that PrPc has a remarkably high affinity to Aβ- what is less clear is how this relates to Aβ-mediated synaptic impairment. Elucidation of the molecular mechanisms by which Aβ produces synaptic perturbations remains as a major goal in finding therapeutic treatments of AD. Whether inhibition of Aβ-mediated synaptic dysfunction via PrP down-regulation could represent a potential therapeutic approach against AD remains to be explored.

1.4.4 The prion protein peptide $PrP_{106-126}$

Prions are the transmissible pathogenic agents responsible for diseases such as scrapie and bovine spongiform encephalopathy. The fragment spanning the Human PrP region 106-126 ($PrP_{106-126}$) exhibits similar physical properties and neurotoxicity to the full-length PrPsc. For instance $PrP_{106-126}$ possesses a β-structure (Salmona et al., 1999), is resistant to enzymatic degradation (Selvaggini et al., 1993), forms amyloid fibrils and causes apoptosis of cultured neurons (Ettaiche et al., 2000; Forloni et al., 1993;

Thellung et al., 2000). For these reasons, it has been suggested that the fragment $PrP_{106-126}$ models the physiochemical and pathogenic properties of PrPsc (Florio et al., 1998). $PrP_{106-126}$ has been extensively studied as a potential mediator of PrPc conversion into scrapie isoform (PrPsc). Seemingly the cell membrane may have a role in both $PrP_{106-126}$ neurotoxicity and PrP conversion. Nevertheless, consensus on the mechanism by which $PrP_{106-126}$ exerts its effect has not been reached (Henriques and Castanho, 2010).

Several studies reported that the toxicity of $PrP_{106-126}$ is dependant on the expression of the host PrPc at the cell surface (Brown et al., 1994; Pietri et al., 2006) and a direct interaction of $PrP_{106-126}$ with PrPc, to mediate the conversion of PrPc into PrPsc, was suggested. On the contrary, Fioriti et al. reported that $PrP_{106-126}$ does not induce formation of abnormal species, but suggested that $PrP_{106-126}$ toxicity is the result of a modification in the physiological functions of PrPc (Fioriti et al., 2005).

Interestingly, Henriques and colleagues recently reported that $PrP_{106-126}$ has a low affinity for lipid membranes under 'physiological' conditions without evidence of membrane disturbances (Henriques et al. 2008). A reported lack of membrane insertion and leakage has led them to hypothesize that the physiological prion protein PrPc mediates $PrP_{106-126}$ toxic effects in neuronal cells. Modulation or inhibition of PrPc physiological function by $PrP_{106-126}$ suggests that $PrP_{106-126}$ toxicity is related to a loss of PrPc function instead of a gain of toxic properties.

1.4.5 Anti-PrPc antibody fragments

In the favored model of prion replication, direct interaction between the pathogenic isoform (PrPsc) template and endogenous PrPc is proposed to drive the formation of nascent infectious prions. Specifically, the fundamental event in prion disease is thought to be the posttranslational conversion of PrPc into PrPsc. The occurrence of PrPc on the cell surface and PrPsc in amyloid plaques in situ or in aggregates following purification complicates the study of the molecular events that underlie the disease process. Since mAbs are highly sensitive probes of protein conformation they have been used under these conditions.

Monoclonal antibody binding studies have demonstrated the innate structural flexibility of PrPc (Peretz et al., 1997; Williamson et al., 1998). Antibody epitopes, lying between amino acids 90–112, were found to be exposed in PrPc but altered (or buried) in infectious forms of PrP. In contrast, an epitope lying toward the C-terminus of PrP is present in both cellular and infectious forms of the protein, indicating that C-terminal regions of PrP may possess more conformational rigidity than N-terminal regions (Peretz et al., 1997; Williamson et al., 1998).

Peretz et al. (2001) showed that monoclonal antibodies binding cell-surface PrPc inhibit PrPsc formation in a dose-dependent manner. In cells treated with the most potent Abs, Fabs D18 and D13, prion replication was abolished and pre-existing PrPSc was rapidly cleared, suggesting that these antibodies may cure established infection. The potent activity of Fabs D18 and D13 was associated with their ability to better recognize the total population of PrPc molecules on the cell surface, and with the location of their epitopes on PrPc (residues 133-157 and 96-104 respectively). These observations supported the use of Abs in the prevention and treatment of prion diseases and identified a region of PrPc for drug targeting.

1.4.6 Conclusions

The relationship between PrPc and Aβ is controversial and ongoing. Lauren et al. reported the surprising identification by expression cloning that PrPc is a receptor for Aβ oligomers and the synaptotoxic consequences of this interaction. There is some evidence in support of these findings, and if confirmed by other labs, PrP could be an important new target for AD therapeutics.

1.5 Study design and objectives

The purpose of the present series of experiments was to further investigate the mechanisms underlying the acute Aβ-mediated disruption of synaptic plasticity *in vivo*. The main objectives of this thesis were as follows:

1.5.1 Purification of amyloid beta assemblies

The first was to characterize the effects of highly purified natural Aβ oligomers derived from cultured medium APP-over expressing (7PA2) cells- these natural oligomers are a more purified version of the ones published by Walsh et al. (2002). Here we wanted to test the LTP-inhibiting abilities of oligomers purified under three different methods: a one-step size exclusion chromatography method, a two-step purification method sequentially performing size exclusion chromatography and ion exchange and a 5-step method.

1.5.2 Amyloid beta and the pan-oligomeric antibody A11

The primary objectives of the second strand of research were to investigate the ability of a pan-oligomer antibody (A11) to abrogate the LTP-inhibiting effects of these soluble natural oligomers, soluble synthetic Aβ, aggregated Aβ and the prion protein peptide $PrP_{106-126}$.

1.5.3 Amyloid beta and the (proto)-fibrillar antibodies WO1/WO2

The third strand of research was to elucidate the activity of anti-fibrillar antibodies (WO1 and WO2) against the inhibition of LTP by soluble natural oligomers, soluble synthetic Aβ and aggregated Aβ.

1.5.4 Amyloid beta and the anti-PrPc antibody fragment D13

The final area of research was to determine if the *in vivo* pre-application of the anti-PrPc antibody fragment D13 could protect against the LTP-inhibitory effects of soluble synthetic Aβ, the prion protein peptide $PrP_{106-126}$ and Aβ dimer-rich human brain extract.

Chapter 2 Methods and Materials

2.1 Animals

The animals used in these studies were male Wistar rats (inbred strain, received from the Bioresources Unit of Trinity College Dublin). Rats weighed between 250 and 320 grams. These weights correspond to animals aged between two and four months at the time of surgery. Animals were stored on site in cages containing a maximum of 3 rats. There was free access to food (iradiated pellets) and water at all times. The on site holding facility was on a twelve h light / dark cycle. A constant temperature of 19-23°C was maintained to minimize stress in the rats. Animal experiments were licensed by the Department of Health and Children (Ireland) and approved by Trinity College Animal Ethics Committee.

2.2 Surgery

2.2.1 Surgical preparation

All rats were weighed before use to determine the amount of anesthetic required to induce anaesthesia. Rats were transferred from the holding cages to the laboratory via carry boxes that simulate these cages. Rats were anaesthetized with urethane (1.5 g/kg, i.p.). Anesthesia state would normally occur within 2-5 min, and the animal remained in an immobilized state for 4-7 h.

A subcutaneous injection of local anesthetic (Norocaine- Lidocaine 20% and adrenaline <1%; Norbrook Laboratories) was delivered to the top of the skull and allowed to absorb into the surrounding tissue for 2-5 min. A midline incision was made from a point between the eyes to the back of the skull, and excess skin was removed from either side of this incision. The periosteum was removed by scraping with a scalpel blade revealing the skull plates, cranial sutures and associated blood vessels. The cranial sutures were used to co-ordinate placement of electrodes (lambda, bregma and the midline). The area was sterilized with ethanol and dried with tissue paper before co-ordinates were marked on the skull.

2.2.2 Electrode implantation

Electrodes were constructed prior to each experiment. For bipolar stimulating electrodes two pieces of Teflon coated tungsten wire (75 μm total external diameter, Advent Research Materials) were soldered to a dual pin socket connector. These were twisted together and glued together with cyanoacrylate. The wires were bent to a 90° angle from the connector and sealed with acrylic dental cement. Electrical continuity was ensured in all instances using a voltmeter. The ends of the wires were cut at an angle so that one was marginally below the other. For monopolar-recording electrodes consisted of one piece of Teflon coated tungsten wire (112 μm diameter, Advent Research Materials) soldered to a single pin socket connector.

Co-ordinates for the electrodes were measured from a zero point at the intersection of the midline plate and bregma sutures. The recording electrode position was measured at 3.4 mm posterior to the bregma and 2.5 mm lateral to the midline. The stimulating electrode position was measured at 4.2 mm posterior to the bregma and 3.8 mm lateral to the midline. A screw reference electrode was positioned 8 mm anterior of the bregma and 1 mm lateral to the midline. A screw ground electrode was positioned 7 mm posterior to the bregma and 5 mm lateral of the midline. Both were located on the opposite half of the skull from the electrodes and cannula. Co-ordinates for cannula and electrode placements were taken from the rat brain atlas (Paxinos et al., 1998). The ground and reference electrodes were constructed by soldering a single pin socket connector to a cleaned hydrochloric acid coated stainless steel screw (Bilaney, Germany).

Holes for electrode and cannula implantation sites were made using a dental drill with diameter of 1.0 and 1.5 mm, respectively. Care was taken not to damage the underlying dura mater and cortical hemispheres with the drill bit after penetration through the skull. The ground and reference electrodes were first put in place and affixed to the skull using dental cement. The rat was transferred to the stereotaxic apparatus (ASI Instruments, USA) and the skull was fixed in place. The stimulating and recording electrodes were positioned and lowered into the Schaffer collateral pathway and CA1 regions, respectively (Figure 2.1). Once the desired response was obtained, the

electrodes were fixed with cyanoacrylate, and then the whole assembly was sealed with dental cement.

2.2.3 Intra-cerebroventricular cannula implantation

Cannulae were constructed to deliver solutions to the brain via the ventricles. An internal cannula was assembled using a stainless steel hypodermic needle (22 gauge, 0.7 mm outer diameter), which was cut to a length of 15.5 mm. The beveled end of the needle was ground down to reduce the angle of the exposed tip. An internal plug (28 gauge, 0.36 mm diameter) was fitted from stainless steel wire, to prevent blockages when not in use.

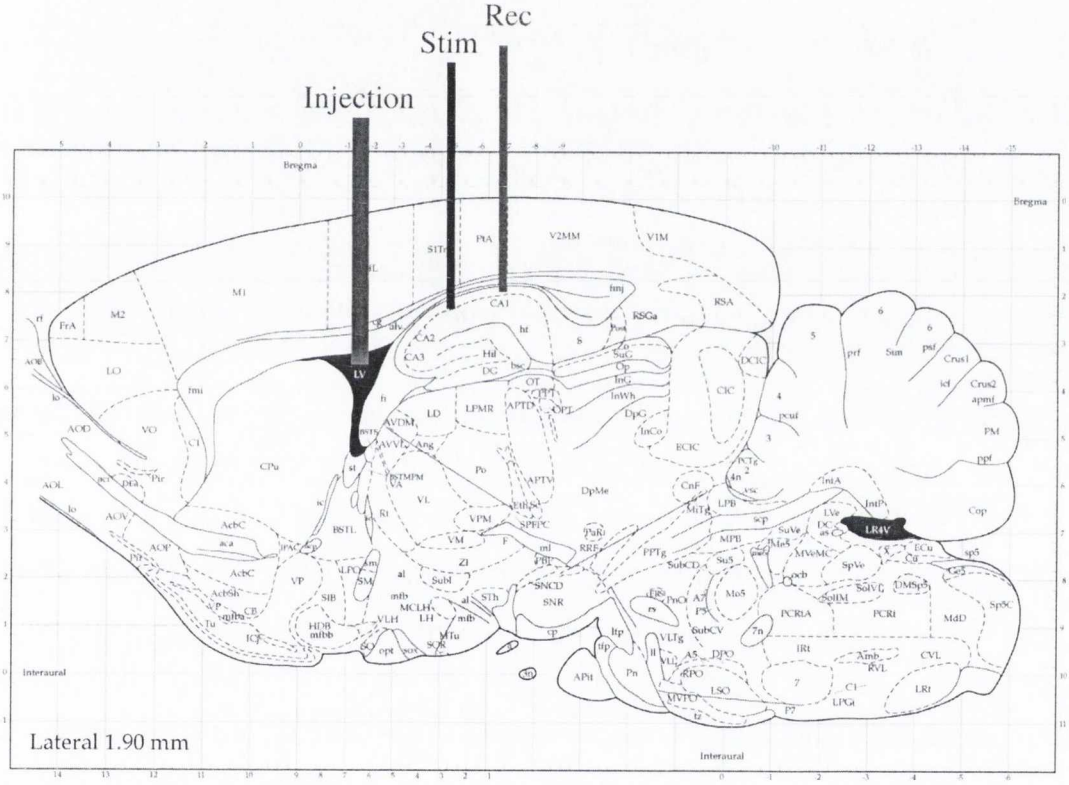


Figure 2.1 – Position of the cannula, recording and stimulating electrodes in the rat hippocampus. The diagram shows a transverse section of the dorsal hippocampus of the rat (not to scale). The recording electrode terminates in the CA1 stratum radiatum region, and the stimulating electrode terminates in the Schaffer collateral/commissural fibres. A cannula was positioned in the ventricle, located anterior to the electrodes, for rats receiving i.c.v injections.

Co-ordinates for the cannula were measured from a zero point at the intersection of the midline plate and bregma sutures. The cannula position was measured at 0.5 mm

posterior to the bregma and 1.3 mm lateral to the midline. The cannula was lowered to a depth of between 4.0 and 4.5 mm below the surface of the skull, into the right lateral cerebral ventricle (Figure 2.1), with the rat under anesthesia and in the stereotaxic apparatus. The cannula was placed before the electrodes were implanted. Injections were made via a Hamilton syringe injector, which was connected to the internal cannula (28 gauge, 0.36 mm outer diameter). Solution was injected in a 5μL volume over a 3-min period. The injector was removed 5 min post-injection and the stainless steel plug was reinserted. The position of the cannula was verified post-mortem by investigating the spread of dye (India ink) throughout the ventricles after i.c.v injection.

2.2.4 Recording apparatus

A Faraday cage surrounded the acute recording area. All instruments in the cage were grounded to a central point to eliminate 50 Hz noise.

2.2.5 Location of recording of electrodes during surgery

Electrode positions were constantly monitored as they were lowered through the cortical and hippocampal layers to the dendrites of the stratum radiatum in the region CA1 (Figure 2.1). This was carried out by generating a 0.2 ms duration stimulus, using a stimulus intensity adjusted to give approximately 50% of maximal EPSP response through the stimulating electrode at a frequency of 0.1 Hz (1 per 10 seconds). Evoked responses were recorded by the monopolar electrode and were displayed on a computer screen. Both the cerebral cortex and the hippocampal formation possess laminar structures. When a local depolarization such as an excitatory post-synaptic potential (EPSP) was created, an electron "sink" was set up along a vertical superficial-deep axis. A phase reversal was encountered when this dipole was crossed; indicating that this was the area generating electromotive forces and the response recorded was not from a distal site by voltage conduction. From this method it was possible to determine which layer the electrodes were in by referring to the electrophysiological criteria determined for each region of the hippocampus as defined by Leung (1980).

The bipolar stimulating electrode was lowered to a depth of 1.5 mm from the surface of the dura mater using the coarse manipulator control, and followed to the same depth by the monopolar recording electrode. Occasionally a transient cortical potential was seen. The stimulating electrode was then lowered in 10-20 μm increments until a first negative deflecting EPSP response was observed (approximately 2.4-2.8 mm below the surface). The recording electrode was then lowered by similar increments. The second potential recorded was a small positive deflecting waveform of 10 ms latency that increased in amplitude as the distance between the recording and stimulating electrodes decreased. This first potential was the evoked response recorded as the stimulating electrode penetrated the alveus and the large second potential was the evoked response observed from stimulating the stratum oriens. As the recording electrode approached the cell body layer, the amplitude of the evoked response became positive. At this point the recording electrode passed through the cell body layer and into the dendritic layer of stratum radiatum, whereby a second negative amplitude evoked response was observed.

Both electrodes were adjusted to a depth of 150-300 μm below the cell body layer in the stratum radiatum to maximize paired pulse facilitation (PPF) and EPSP amplitudes. PPF was used as a physiological measure of short-term plasticity. When a presynaptic neuron receives two stimuli in rapid succession, the postsynaptic response will commonly be larger for the second than for the first pulse — a phenomenon known as PPF. The mechanism responsible for PPF is thought to be a result of 'residual calcium', and theoretical considerations have led to the suggestion that PPF could also arise from the partial saturation of a calcium buffer (Rozov et al. 2001).

2.2.6 Temperature

Deeply anaesthetized rats lose control of the homeostatic body temperature regulatory mechanism. In all experiments using a thermal blanket regulated the body temperature of the rat. An externally controlled heating blanket maintained the temperature to approximately 37°C for the length of the experiment. As body temperature is maintained in a stable range, the possibility that temperature fluctuations give rise to alterations in EPSP amplitude can be discounted (Moser, 1993).

2.3 Excitory postsynaptic potential (EPSP) recordings

The population field EPSP was used as a measure of excitatory synaptic transmission in the dorsal hippocampus. In all experiments, test EPSPs were evoked by a single square wave pulse of current at low frequency (0.033 Hz, 0.2 ms in duration with a stimulus intensity adjusted to give 50% of maximal EPSP response) generated by a constant current isolation unit (Grass Instruments Co., USA) to the bipolar stimulating electrode embedded in the axons of Schaffer collaterals and commissural fibres. This evoked a response, which was transmitted via a pre-amplifier (gain 11) with a broadband setting of 4 Hz to 6 kHz, to an analogue-to-digital converter (MacLab/2e, Analog Digital Instruments). This was a digitized system linked to an Apple Macintosh computer (Classic II), which interfaced with the converter via a specifically written software package (Scope, version 3.8). This was customized to control both the generation of the square wave pulses and recording of the evoked potentials. Field EPSPs were displayed on-line and could be analyzed at the time of recording or at a later date.

The slope and amplitude of the field EPSP were measured simultaneously previously in our laboratory, and it was found that there is a positive linear correlation between the two (Cullen, 1997). Therefore the amplitude of the response was taken as the main indicator of excitatory synaptic transmission. An input/output curve was recorded, after electrodes were in place and the site was sealed with dental cement, to determine the maximum EPSP amplitude. The test EPSP used for baseline and subsequent measurements in the experiment was 50% of this maximum value. Unless stated otherwise, LTP was induced using high frequency stimulation (HFS) consisting of a single series of 10 trains of 20 stimuli with an inter-stimulus interval of 5ms (200 Hz) and an inter-train interval of 2 s. The stimulation intensity was raised to give an EPSP of 75% maximum during the high frequency tetanus.

Baseline measurements of field EPSP amplitude were made for 30-90 min before injection and typically for 15 min following administration at which time the HFS was applied.

2.4 Preparation of natural Aβ oligomers

2.4.1 Aβ preparation

Aβ was collected and prepared by Dr. Dominic Walsh's laboratory from 7PA2-cell CM as previously described by Walsh et al. 2005. 7PA2 cells are a Chinese hamster ovary (CHO) line that stably expresses human APP751 containing the V717F mutation (Walsh et al. 2006). The conditioned medium was pooled and aliquoted to produce a large number of identical medium samples for experiments. These aliquots were stored at –80°C until use.

2.4.2 Immunoprecipitation (IP)

Dr. Walsh's laboratory performed immunoprecipitation of Aβ from 8 ml of CM prepared as above. Samples were precleared with protein A sepharose for 30 min. The CM was then immunoprecipitated overnight with a 1:75 dilution of a polyclonal anti-Aβ antibody R1282 (Walsh et al. 2006). The beads were washed with STEN buffers. The samples were then resuspended in 2x Tricine sample buffer, boiled, and the supernatant was frozen at –80°C or loaded directly onto Tricine SDS-PAGE gels.

2.4.3 Size-exclusion chromatography and ion exchange purification

To physically separate natural Aβ oligomers using size exclusion chromatography, 7PA2 CM was run by Dr. Walsh's laboratory on two Superdex 75 prep grade 10 x 300 mm columns (100 ml volume) arranged in series (Amersham Biosciences AB, Uppsala, Sweden). One millilitre of 10x concentrated CM was injected onto the columns and eluted with 50 mM ammonium acetate pH 8.5. An AKTA fast protein liquid chromatograph (GE Healthcare Biosciences AB, Uppsala,Sweden) was used to collect 1 ml fractions, which were stored at –20°C until lyophilized. Blots of CHO-control CM (not shown) were similar to previously reported results (Walsh et al., 2005). Each lyophilised aliquot was resuspended in 20-ul-2x-sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The remaining volume was immediately frozen and stored at -80°C pending use.

To physically separate the natural oligomers using ion exchange chromatography, the above suspension was then applied to a MonoQ 4.6/100 PE anion-exchange column (GE) and equilibrated as follows:

1. 5 column volumes (CV = 1.7 ml) of binding buffer (=20 mM [AmAc] pH 8.5).
2. 10 column volumes of elution buffer (= 1 M NaCl, 20 mM AmAc pH 8.5).
3. 5 column volumes (CV = 1.7 ml) of binding buffer (=20 mM AmAc pH 8.5) or until baseline are stable.

CM concentrate (equivalent to up to 100 ml 7PA2 CM) was diluted 1:10 using binding buffer and loaded onto the column using a 50 ml superloop. The run was carried out at a flowrate of 2ml/min. Unbound material was washed off the column with binding buffer until the absorption at 280 nm reached baseline (typically about 15-20 CV). Elution was carried out using a linear 0-100% gradient of elution buffer over 30 ml with 1 ml fractions collected. Fractions were immediately kept on ice and diluted 1:1 in ice cold binding buffer to use directly for IP or frozen down at -80 °C pending analysis.

2.4.4 4-step purification protocol of 7PA2 CM

Dr. Walsh's laboratory developed a 4-step purification protocol to retrieve SDS-stable Aβ-dimers from 7PA2 CM. This is briefly described herewith:

1. Remove the large amount of contaminating APPs from the CM.
2. Use immunoaffinity to enrich Aβ species with either polyclonal or monoclonal Aβ antibodies.
3. Use peptide ligand (a fragment of Aβ) as an additional affinity-binding reagent.
4. Separate the resultant Aβ assemblies by size on a superdex 75 SEC column.

2.4.5 Human brain derived Aβ dimers

Frozen samples of frontal (Brodmann area 8/9) and temporal (Brodmann area 21/22) cortices weighing ~0.5 g were dissected to produce 0.2 g aliquots and used to prepare homogenates. Brain tissue (0.2 g) was homogenized with 25 strokes of a Dounce homogenizer (Fisher, Ottawa, Canada) in 1 ml of freshly prepared ice-cold Tris-buffered saline (TBS). Homogenates were then centrifuged at 91,000 g and 4°C in a

TLA-55 rotor (Beckman Coultour, Fullerton, CA, USA) for 78 min. The supernatant referred to as the TBS extract was divided into 300 µl aliquots and stored at –80°C. The pellet was re-homogenized (1:5 w/v) in TBS containing 1% Triton-X 100 (TBS-TX) plus inhibitors, centrifuged as before, the supernatant removed, aliquoted and stored. The pellet was re-suspended in 88% formic acid (1:0.5 w/v) with gentle agitation overnight at 4°C. Next day, the formic acid extracts were aliquoted and transferred to –80°C pending analysis. Extracts of human brain were immunoprecipitated with a polyclonal anti-Aβ antibody (AW8), and analyzed by Western blot using C-terminal mAβ antibodies (2G3 and 21F12). N-terminal Aβ antibodies (3D6 and 6E10) were used to confirm that the bands appearing on the blot as monomer (4.1 kDa), dimer (7.5 kDa)—and in a few cases, trimer (12.1 kDa) and tetramer (17.8 kDa)—were, in fact, full-length Aβ species.

2.4.6 Western blots

Samples were electrophoresed on 10–20% Tricine gels (Invitrogen or Bio-Rad), and the proteins transferred to 0.2 µm Optitran nitrocellulose. The membranes were boiled in water or phosphate-buffered saline (to enhance the exposure of Aβ epitopes) and blocked for 1 h in 50% Odyssey blocking buffer diluted in PBS. Blots were probed with the monoclonal antibody 2G3 (Elan), which is specific for Aβ peptides ending at residue 40, or with 6E10 (Signet), which recognizes amino acids 4–8 near the N-terminus of Aβ. Immunoreactive bands were detected and quantified using a Licor Odyssey imaging system (Figure 2.2).

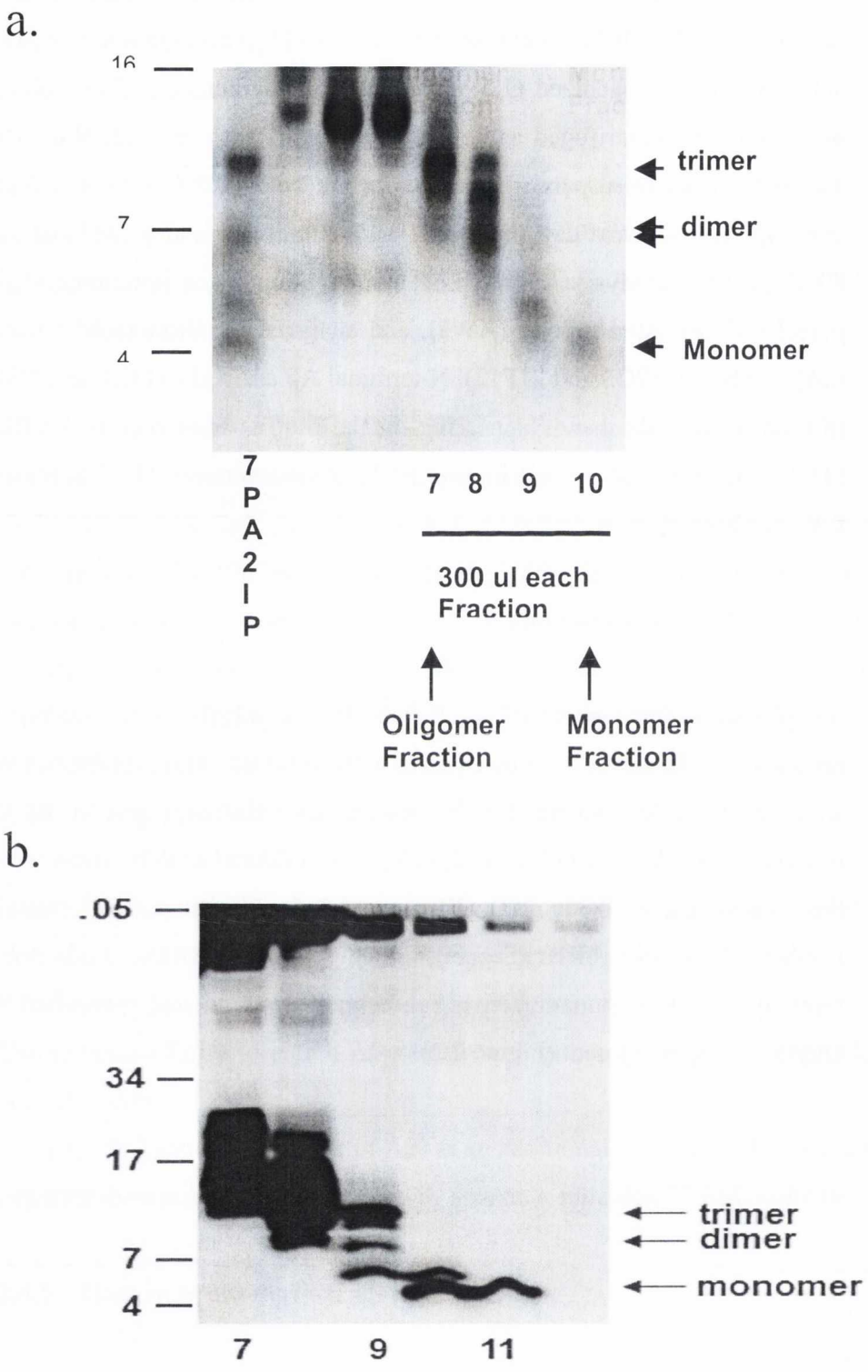


Figure 2.2 – Western blots of a) size exclusion chromatography separated (SEC) 7PA2 natural oligomers and monomers (lanes 8 and 10, respectively) and b) sequential SEC and ion exchange separated 7PA2 natural oligomers and monomers (lanes 9 and 11, respectively) (Courtesy of Professor D. Walsh, UCD).

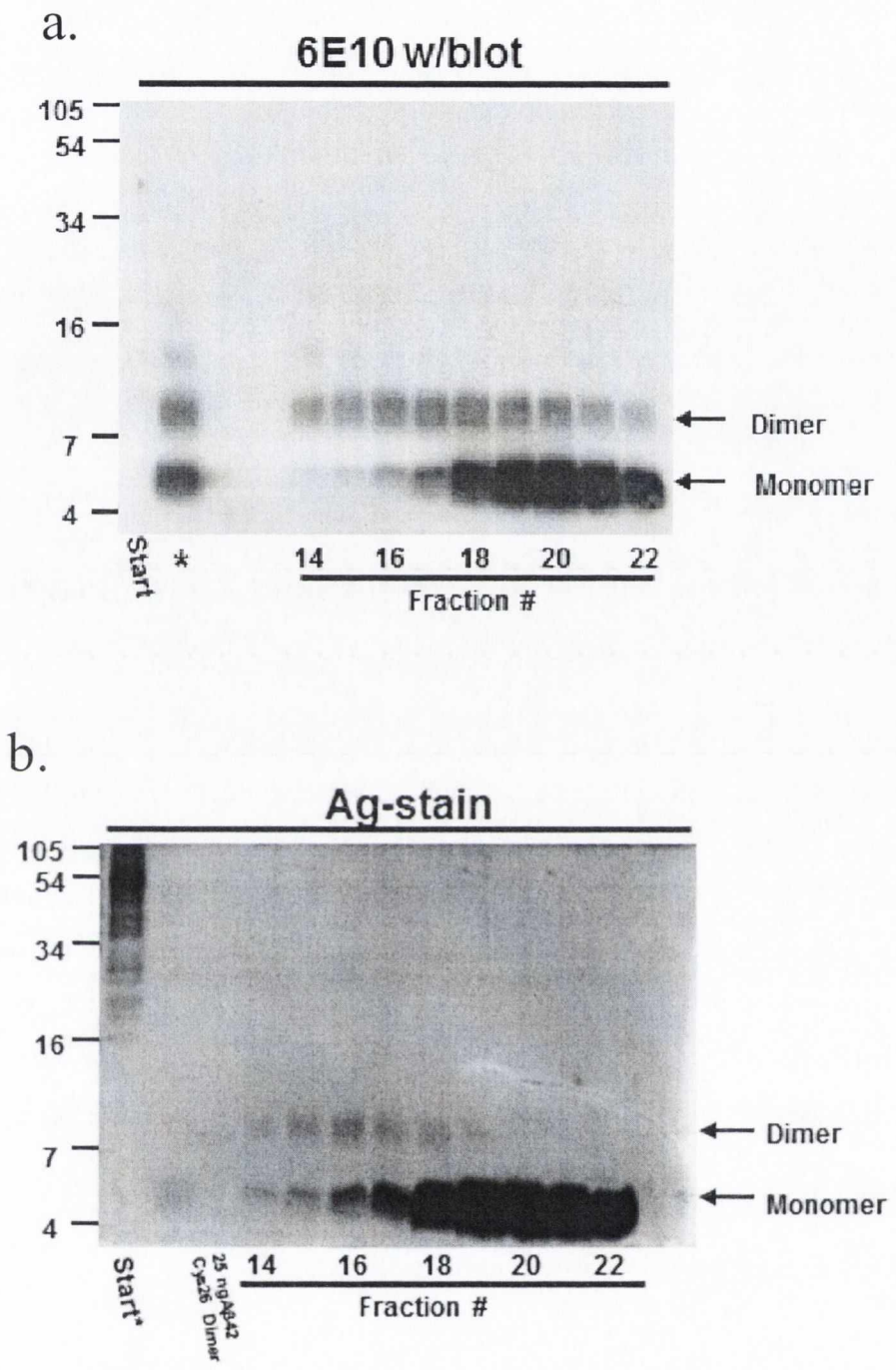


Figure 2.3 – a) Western blot of 4-step separated cell-derived dimers and monomers and b) silver-staining of preparation indicates all other proteins of the same size were removed (Courtesy of Professor D. Walsh, UCD).

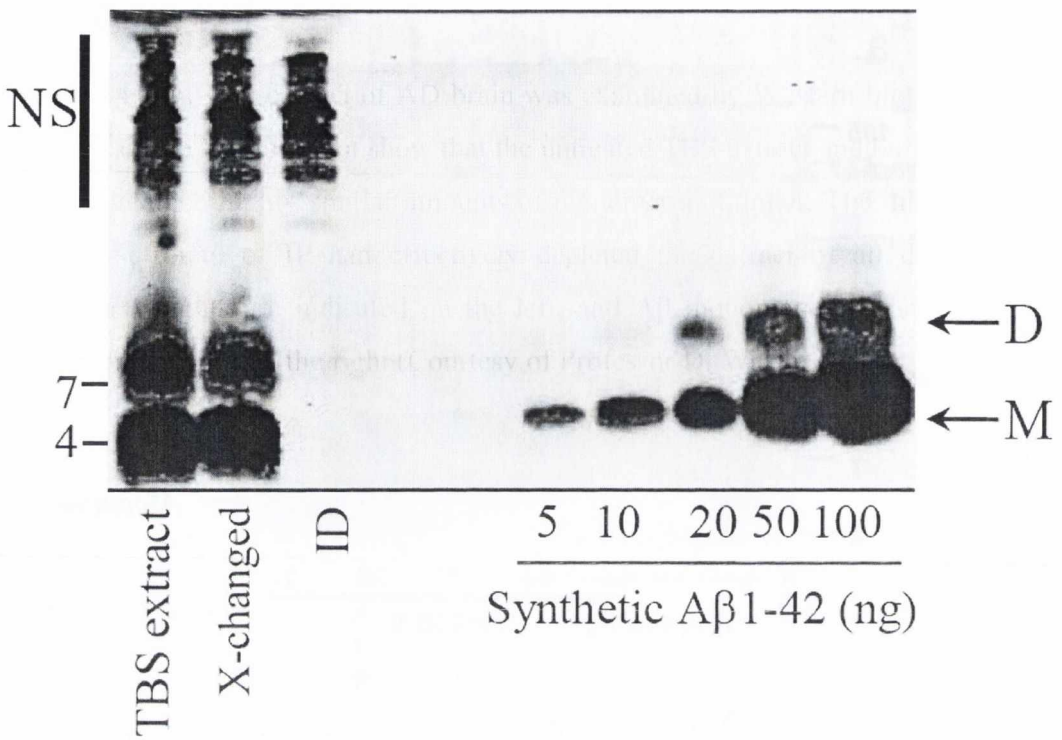


Figure 2.4 – A TBS extract of AD brain was examined by Western blotting. The first two lanes of the Western blot show that the untreated TBS extract and buffer-exchanged extract contained highly similar amounts of monomer and dimer. The third lane shows that a first round of IP had effectively depleted the extract of all detectable Aβ. Molecular weights are indicated on the left, and Aβ monomers (M) and dimers (D) labeled with arrows on the right (Courtesy of Professor D. Walsh, UCD).

2.5 Preparation of soluble synthetic Aβ

$A\beta_{1\text{-}42}$ peptide powder was obtained from David Teplow's laboratory (University of California, LA, USA). A nominally 100μM stock was prepared in a low concentration (≈ 0.1%) of ammonium hydroxide (NH_4OH). This solution was ultra-centrifuged at 100,000**g** for 3 h at 4°C. Supernatant was collected from the tube, and stored at -80°C with a final concentration of 64μM. Peptides were diluted with water for injection (WFI) to obtain doses of 160 and 80 pmol for experiments.

2.6 Preparation of the anti-Aβ oligomer antibody A11

The anti-Aβ oligomer antibody A11 (2.0 mg/mL) was obtained from Professor Charles Glabe's laboratory (University of California, USA). It was created as described by Kayed et al. 2006. Oligomer antigens were prepared by stirring 2 mg/ml $A\beta_{1-42}$ peptide in 50% HFIP/H_2O, 0.02% sodium azide for 7 days. Afterwards, the HFIP was evaporated under a stream of nitrogen and the sample was stirred for an additional 7 days and dialyzed against PBS. The resulting oligomers were checked by electron microscopy (EM) and the purity was confirmed by the absence of fibrils using anti-fibril antibody. The antigens were each used to immunize two New Zealand white rabbits according to protocols approved by IACUC. Each rabbit immunized with 500 µl of antigen in complete Freund's adjuvant (CFA), and then boosted twice at four-week intervals with 500 µl of antigen in incomplete Freund's adjuvant (IFA).

2.7 Preparation of the anti-Aβ fibril antibodies WO1/WO2

The anti-Aβ oligomer antibodies WO1 (0.6 mg/mL) and WO2 (1.6 mg/mL) were obtained from Dr Brian O'Nuallain (University College Dublin, Ireland). They were created as described by O'Nuallain and Wetzel (2001). Five standard, female BALB/c mice were immunized with 50 µg/mouse/injection with sonicated $A\beta_{1-40}$ fibrils. Two additional injections were given at 2-wk intervals. Bleeds were taken 1 wk after each injection and screened by using a modification of the microplate assay described below. After the third injection, two mice were killed and their spleens used to generate hybridoma cells. Initial screening of clones was performed by testing the ability of membranes containing uniformly deposited monomeric or fibrillar Aβ to bind Abs from an array of clonal supernatants.

2.8 Preparation of the anti-PrPc antibody fragments D13/R1

The anti-PrP antibody fragments D13 (1.1 mg/mL) and R1 (1.0 mg/mL) were obtained from Dr Mike Scott (University College Dublin, Ireland). They were created as described by Williamson et al. (1998). Antibody libraries were constructed from Prnp0/0 mice immunized either with prion rods containing Mouse PrP 27-30 or with disaggregated PrP 27-30 incorporated into liposomes. Mice immunized with prion rods

received an immunization and three boosts. Animals immunized with PrP 27-30 in liposomes were divided into two groups and received both an immunization and two boosts (long immunization) or, in an attempt to increase the diversity of the antibody response, an immunization and a single boost (short immunization). For each mouse, PrP-specific reactivity in all four subclasses of serum immunoglobulin G (IgG) was determined by enzyme-linked immunosorbent assay (ELISA) against mouse PrP 27-30 treated with the denaturant guanidium thiocyanate (GdnSCN). Mice immunized with prion rods generated PrP-specific serum antibody titers predominantly in the IgG1 and IgG2b subclasses, whereas mice immunized with PrP 27-30 liposomes produced a strong PrP-specific response in all IgG subclasses.

2.9 Preparation of the $PrP_{106-126}$ peptide

The human prion protein peptide $PrP_{106-126}$ (catalog number H-1566) was purchased from Bachem (Weil am Rhein, Germany). The lypholized peptide was dissolved in de-ionized water. A working stock concentration of ≈ 0.5 mg/mL was used. A dose of 0.05 nmol was selected as it has been described as the lowest effective dose to inhibit LTP, in unpublished work from our laboratory (Cullen, 2002).

2.10 Preparation of pre-aggregated Aβ

$A\beta_{1-42}$ was dissolved in MilliQ water and incubated overnight at 37°C. Fibrillar material was collected after centrifugation and diluted to a final concentration of 100 uM. Fibrillar peptide purity was calculated at ≥ 95%.

2.11 Preparation of 'washed' pre-aggregated Aβ

Pre-aggregated $A\beta_{1-42}$ material was suspended in SDS and centrifuged at 175,000g for 30 min with a TLA100.2 Beckman TL 100 centrifuge. The supernatant was collected and the remaining pellet was resuspended in 150 μL SDS, boiled at 100°C for 5 min, and centrifuged at 10,000g for 5 min. The supernatant was collected and the remaining pellet was resuspended in 150 μL TBS, and centrifuged at 500g for 5 min. Again, the supernatant was collected and the remaining pellet was resuspended in 150 μL TBS,

and centrifuged at 500g for 5 min. The pellet was resuspended using formic acid, aliquoted and stored in -20°C.

2.12 Preparation of the control antibodies

Control antibodies were obtained from R&D Systems (USA) and prepared per instructions. For A11 control we used normal rabbit polyclonal IgG rabbit antibody (serum was obtained from naive non-immunized animals) (catalog number AB-105-C). The lypholized rabbit IgG was dissolved in sterile PBS, pH 7.4. A working stock concentration of ≈ 2.0 mg/mL was used. For WO2 control we used IgMκ from murine myeloma (serum was obtained from naive non-immunized animals) (Sigma Aldrich, Germany; catalog number M1520). The lypholized murine IgM was dissolved in de-ionized water. A working stock concentration of ≈ 1.6 mg/mL was used.

2.13 Injection paradigms

Two injection paradigms were used for our experiments. In the experiments with the pan-oligomer antibody (A11) and the (proto)-fibril antibodies (WO1, WO2), the soluble and insoluble Aβ assemblies and antibodies were incubated together at room temperature (~ 23°C) for 1 h prior to co-injection. In the experiments with the anti-PrP antibody fragments (D13 and R1), the Fabs were injected 30 min prior to injection of the peptide.

Experiments	At -60 min	At -30 min	At -15 min	At 0 min
A11, WO1, WO2 and control antibodies	Pre-incubate antibody and peptide		Inject combination of antibody and Aβ peptide	Give HFS
Antibody fragments D13 and R1		Inject D13 or R1	Inject peptide	Give HFS

2.14 Data Analysis and Statistics

The baseline EPSP amplitude was calculated by averaging 60 responses evoked by stimulating the stratum radiatum over 30 min, twice per min (a rate of 0.033 Hz) before delivery of the drug(s) to be tested. This value was used for the calculation of the percentage increase or decrease seen in all subsequent recordings.

Statistical significance of within group comparisons was calculated using a student's t-test that compared the 10 min epochs prior to HFS and at 3 h post-HFS. For between groups multiple comparisons, a 1-way ANOVA followed by post-hoc test (Tukey) for the 10 min epochs at the end of the 3 h post-HFS period. Unpaired Student tests were carried out when there were only two groups to compare. For groups of n=3 Wilcoxon and Mann Whitney U tests were used to compare within and between group differences, respectively. Statistical significance was calculated at the 95% level (two-tailed). Unless otherwise stated the values are expressed as mean (± standard errors of the mean [SEM]) for EPSP amplitude. Statistical results and graphs were obtained using Prism Graph Pad v5b (Graphpad Software Inc., 2003). Percentage potentiation (i.e. the level of LTP) was calculated using absolute increase in EPSP baseline (taken as a 100%).

2.15 Chemicals and drugs

1. $A\beta_{1-42}$ peptide powder – Dr D. Teplow (University of California, LA, USA).
2. IgMκ - Sigma Aldrich, Germany; catalog number M1520.
3. Normal rabbit polyclonal IgG - R&D Systems (USA); catalog number AB105C.
4. WO1 and WO2 antibodies - Dr B. O'Nuallain (University College Dublin, IRL).
5. Human prion protein peptide $PrP_{106-126}$ - Bachem (Weil am Rhein, Germany); catalog number H-1566.
6. Anti-PrP antibody fragments D13 and R1 - Dr M. Scott (UCD, Ireland).
7. Anti-Aβ oligomer antibody A11 - Professor C. Glabe (U of California, USA).
8. Pre-aggregated Aβ – Dr L. Fulop (Szegedi University, Hungary).
9. Washed Aβ fibrils – Dr. D Walsh (University College Dublin, IRL).
10. Cell-derived Aβ oligomers - Dr. D Walsh (University College Dublin, IRL).
11. Norocaine - Lidocaine 20% and adrenaline <1%; Norbrook Laboratories, UK.
12. Urethane – U2500-500G Sigma

Chapter 3 - Results

3.1 – Effects of purified Aβ monomers and oligomers on hippocampal LTP in vivo

Previous studies (both *in vitro* and *in vivo*) show that soluble, oligomeric forms of Aβ have potent neurotoxic activities and may also be the proximal effectors of the neuronal injury and death occurring in AD (Walsh et al. 2002).

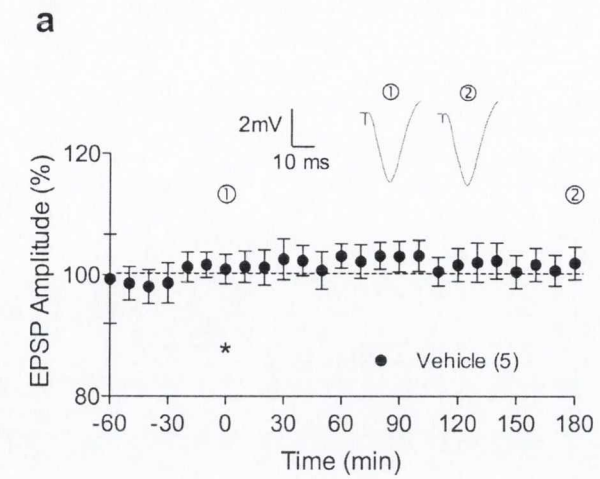
3.1.1 2-step versus 1-step purification of 7PA2 conditioned medium: effects on baseline excitatory synaptic transmission

We determined if cell-derived Aβ monomers and oligomers from two different purification methods had an effect on baseline excitatory transmission at CA3 to CA1 synapses in the dorsal hippocampus of the anaesthetized rat. Aβ monomers and oligomers in the cultured medium (CM) from a cell line that expresses APP, termed 7PA2 cells, were isolated using size-exclusion chromatography (SEC). Overall there was no significant difference between groups in the amplitude of EPSP (Figure 3.1.1[f]).

Figure 3.1.1(a) shows the effects of acute i.c.v injection of 5μL of vehicle (using water for injection 'WFI'), at "time zero". Vehicle had no effect on baseline measured at 3 h after injection (102 ± 2%, n = 5; $p > 0.05$, compared with pre-injection baseline). Figure 3.1.1(b) shows the effects of acute i.c.v injection of 5μL natural Aβ monomers purified by SEC alone. These natural Aβ monomers had no effect on baseline (97 ± 3%, n = 4; $p > 0.05$ compared with pre-injection baseline). Figure 3.1.1(c) shows the results of both acute i.c.v injection of 5μL of natural Aβ monomers purified by SEC + ion exchange (IE). These natural Aβ monomers had no effect on baseline (101 ± 3%, n = 4; $p > 0.05$ compared with baseline, $p > 0.05$ compared with vehicle injected controls). Results were not statistically different from the SEC alone treatment ($p > 0.05$).

We also examined the effects of cell-derived Aβ oligomers on baseline synaptic transmission. Figure 3.1.1(d) shows the effects of acute i.c.v injection of 5μL of natural Aβ oligomers purified by SEC alone. These natural Aβ oligomers had no effect on baseline at 3 h post-injection (98 ± 2%, n = 5; $p > 0.05$ compared with pre-injection

baseline; $p > 0.05$ for 1-way ANOVA with repeated measures for comparing 4 x 10 min epochs (10 min prior to baseline, 50-60, 140-150 and 170-180 min). Figure 3.1.1(e) shows the effects of acute i.c.v injection of 5μL of natural Aβ oligomers purified by SEC + IE, at "time zero". These natural Aβ oligomers had no effect on baseline (93 ± 4%, $n = 5$; $p > 0.05$ compared with baseline; $p > 0.05$ for 1-way ANOVA with repeated measures for comparing 4 x 10 min epochs (10 min prior to baseline, 50-60, 140-150 and 170-180 min). Results were not statistically different from the SEC alone treatment ($p > 0.05$). 1-step SEC oligomer and 1-step SEC monomer groups were not statistically different ($p > 0.05$) from each other. 2-step SEC oligomer and 2-step SEC monomer groups were not statistically different ($p > 0.05$) from each other.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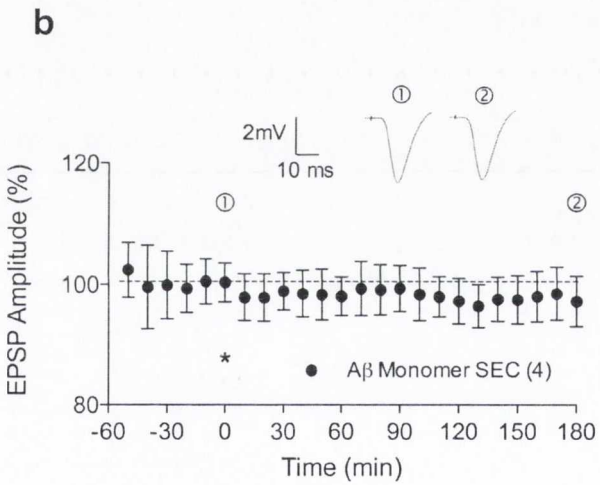
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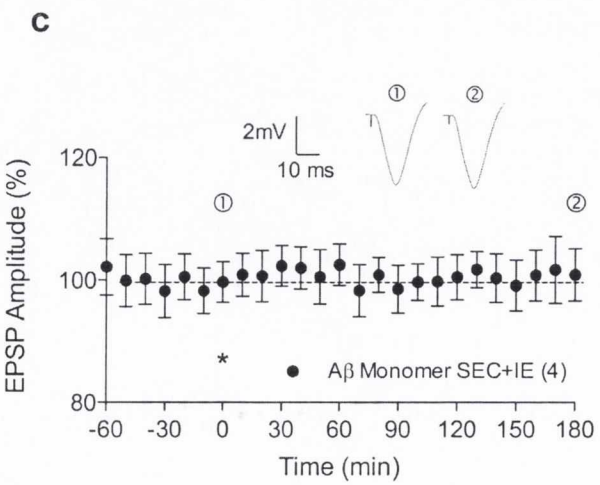
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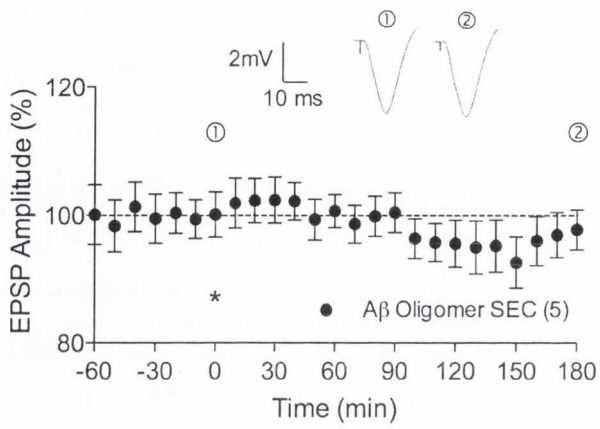
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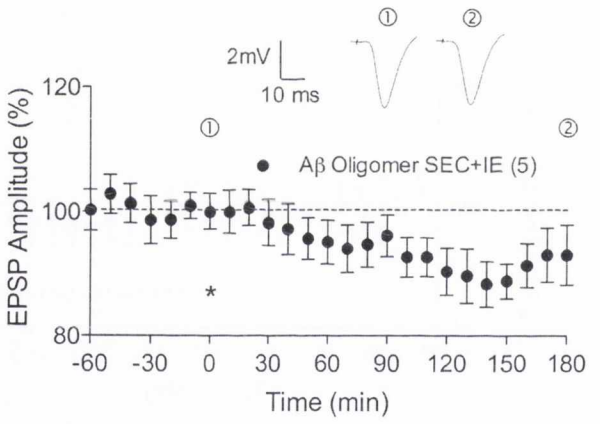
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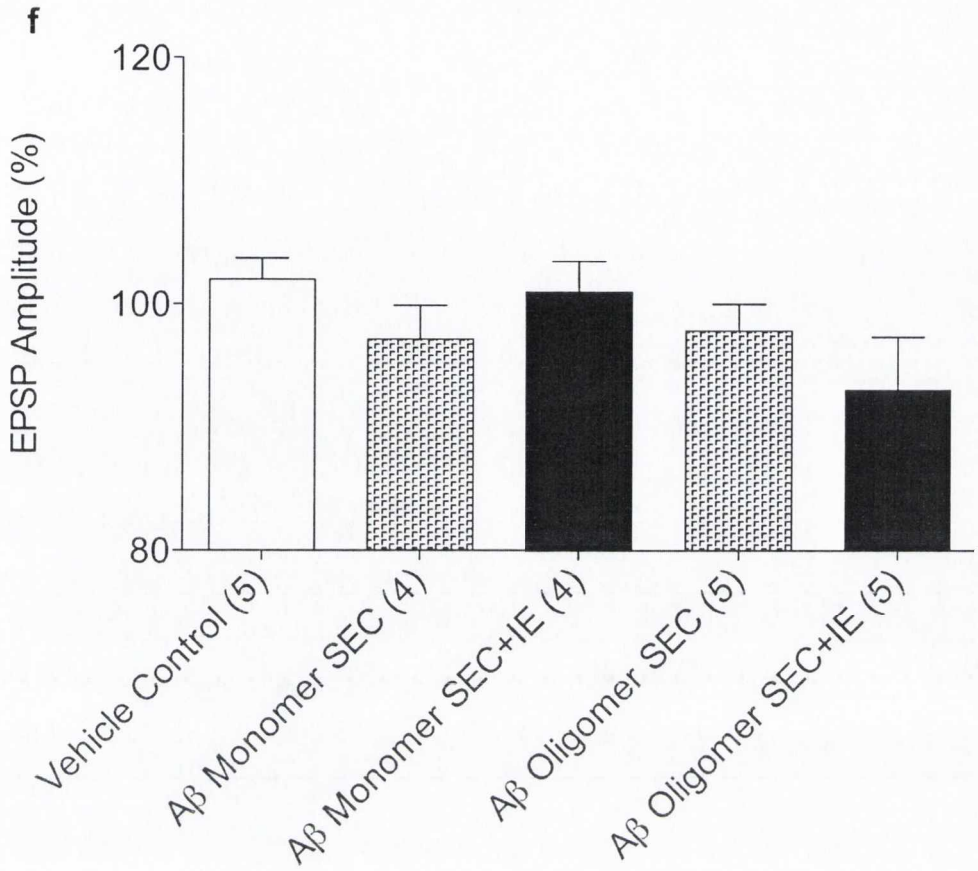


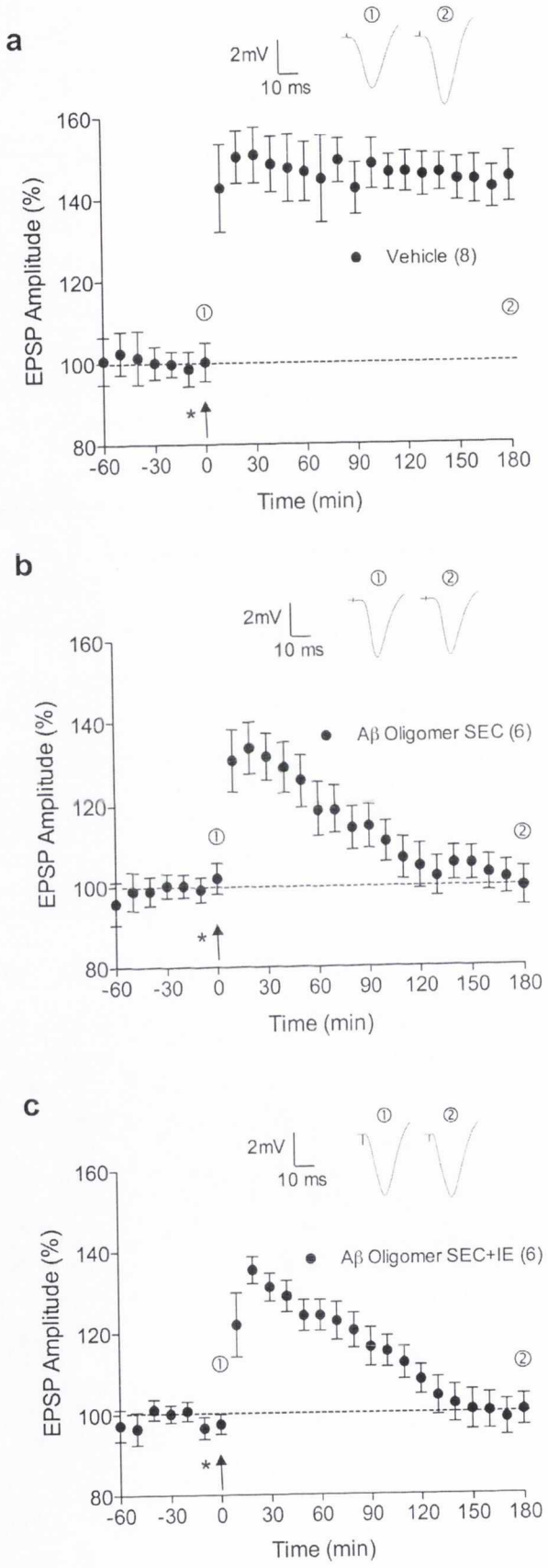
Figure 3.1.1 - 2-step versus 1-step purification of 7PA2 conditioned medium: purified natural Aβ monomers and oligomers had no effect on the baseline amplitude of excitatory synaptic transmission in the CA1 area of the dorsal hippocamus of the anaesthetized rat. **a**, A vehicle injection of 5 μL did not effect baseline at 3 h ($n=5$, $p > 0.05$, compared with pre-injection). **b**, Natural monomers purified with SEC alone had no effect on baseline ($n=4$, $p > 0.05$). **c**, Natural monomers purified with SEC+IE also had no effect on baseline ($n=4$, $p > 0.05$). **d**, Natural oligomers purified with SEC alone had no significant effect on baseline ($n=5$, $p > 0.05$) 3 h after injection. **e**, Natural oligomers purified with SEC+IE treatment also had no significant effect on baseline ($n=5$, $p > 0.05$). All animals received an injection at 0 mins indicated by a star (*). Insets show representative traces of field EPSPs recorded at times 0 (①) and 180 min (②). **f**, Summary of the effects of 2-step vs 1-step separation treatment on baseline synaptic transmission at 3 h. There was no statistically significant difference between groups ($p > 0.05$, posthoc intergroup comparisons) at 3 h.

3.1.2 2-step versus 1-step purification of cell-derived 7PA2: effects on LTP

Previous studies from Walsh et al. (2002) have provided evidence that a naturally secreted soluble non-fibrillar form of cell-derived Aβ can perturb LTP in the rat. To determine if cell-derived Aβ monomers and oligomers from the two different purification methods were differentially active *in vivo*, we examined their effects on the magnitude of LTP of excitatory synaptic transmission. Figure 3.1.2(a) shows the results of acute i.c.v injection of 5μL of vehicle (WFI), 15 min prior to HFS delivery. In vehicle controls HFS triggered LTP (144 ± 5%, at 3 h post-HFS $n = 8$, $p < 0.05$ compared to 99 ± 4% pre-HFS baseline). Figure 3.1.2(b) shows the results of acute i.c.v injection of 5μL of natural Aβ oligomers purified by SEC alone, 15 min prior to HFS delivery. Natural Aβ oligomers completely inhibited LTP at 3 h post-HFS (100 ± 5%, $n = 6$; $p > 0.05$ compared with 102 ± 4% pre-HFS baseline; $p < 0.05$ compared with vehicle injected controls at 3 h). Figure 3.1.2(c) shows the effects of acute i.c.v injection of 5μL, 15 min prior to HFS delivery, of natural Aβ oligomers purified by SEC + IE. These natural Aβ oligomers also completely inhibited LTP at 3 h (101 ± 3%, $n = 6$; $p > 0.05$ compared with 98 ± 3 pre-HFS baseline; $p < 0.05$ compared with the vehicle injected controls at 3 h). There was no statistically significant difference from natural Aβ oligomers enriched using SEC alone ($p > 0.05$).

To determine whether cell-derived Aβ monomers from two different purification methods have similar activity *in vivo*, we examined their effects in our rat model. Figure 3.1.3(d) shows the results of acute i.c.v injection of 5μL of natural Aβ monomers purified by SEC alone, 15 min prior to HFS delivery. Natural Aβ monomers purified with SEC alone displayed LTP for at least 3 h (143 ± 8%, $n = 5$; $p < 0.05$ compared with pre-HFS baseline 101 ± 5%; $p > 0.05$ compared with vehicle injected controls). Figure 3.1.3(b) shows the results of acute i.c.v injection of 5μL of natural Aβ monomers purified by SEC + IE), 15 min prior to HFS delivery. These natural Aβ monomers displayed LTP for at least 3 h (129 ± 4%, $n = 5$; $p < 0.05$ compared with 100 ± 4% pre-HFS baseline; $p > 0.05$ compared with vehicle injected controls). This group was not statistically different from Aβ monomers prepared with SEC alone ($p > 0.05$). 1-step SEC oligomer and 1-step SEC monomer groups were statistically different ($p < 0.05$).

2-step SEC oligomer and 2-step SEC monomer groups were statistically different ($p < 0.05$).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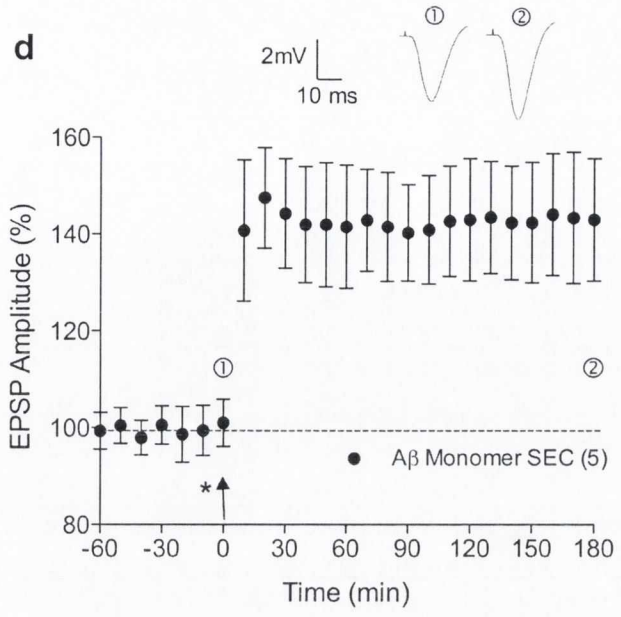
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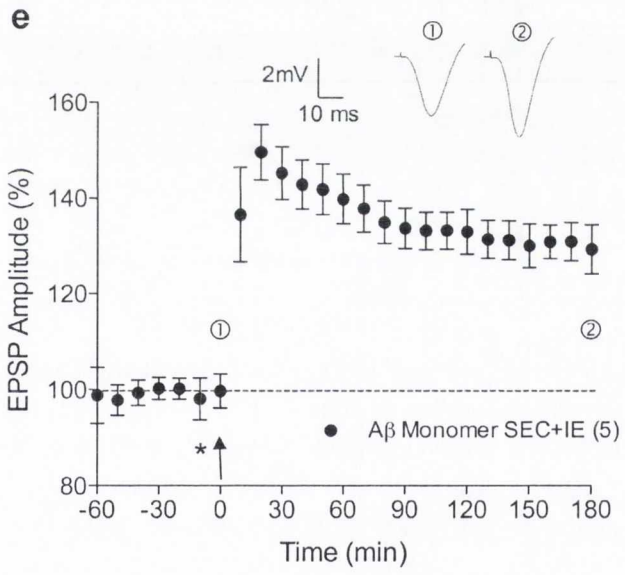
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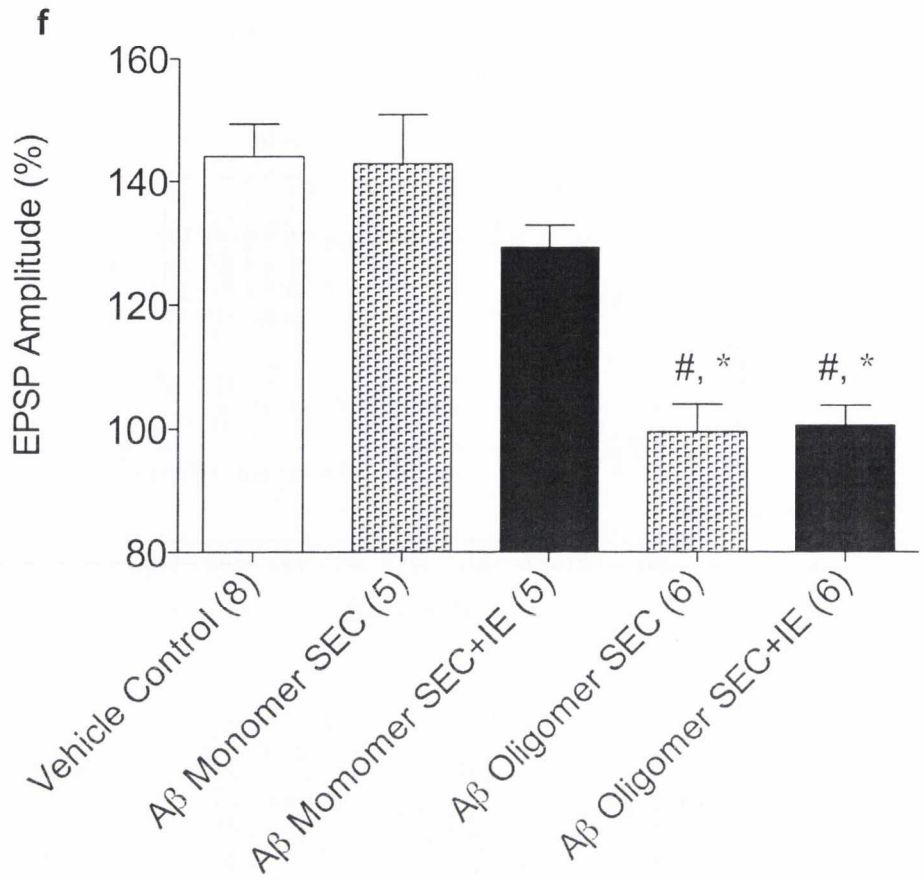


Figure 3.1.2 - 2-step versus 1-step purification of 7PA2 conditioned medium: purified natural Aβ oligomers, but not monomers, block hippocampal LTP *in vivo*. **a**, A high frequency stimulation (HFS) applied (↑) 15 min after vehicle injection (*) of 5 μL of WFI induced significant LTP which persisted for at least 3 h ($n=8$, $p < 0.05$, compared with baseline at 3 h post- HFS). **b**, Natural oligomers purified with SEC treatment blocked hippocampal LTP ($n=6$, $p > 0.05$). **c**, Natural oligomers purified with SEC and IE treatment also blocked hippocampal LTP ($n=6$, $p > 0.05$). **d**, Natural monomers purified with SEC treatment alone did not prevent HFS-induced LTP ($n=5$, $p < 0.05$). **e**, Natural monomers purified with SEC+IE treatment also did not prevent HFS-induced LTP ($n=5$, $p < 0.05$). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **f,** Summary of posthoc intergroup comparisons of 2-step vs 1-step purification treatment activity at 3 h (posthoc intergroup comparisons). There was a statistically significant difference between both natural Aβ oligomer groups and WFI group (#, $p < 0.05$), and both natural Aβ oligomer groups and their respective monomer groups at 3 h (*, $p < 0.05$).

3.1.3 4-step purification of 7PA2 conditioned medium: effects on LTP

Recently, Klyubin et al. (2008) showed that in vivo administration of Aβ dimer-containing human CSF disrupted LTP. To determine if cell-derived Aβ monomers and dimers from a 4-step purification method were differentially active *in vivo*, we examined their effects. Aβ dimers in the CM from 7PA2 cells were isolated using a 4-step purification protocol. 3.1.3(a) shows the results of acute i.c.v injection of 5μL of cell-derived monomers, 10 min prior to HFS delivery. High frequency stimulation induced robust LTP in animals injected with natural Aβ monomers purified with the 4-step method (135 ± 4%, n = 6; $p < 0.05$ compared with baseline 101 ± 3%). Figure 3.1.3(b) shows the effects of acute i.c.v injection of 5μL of natural Aβ dimers (≈250 fmol) purified by the same 4-step method, 10 min prior to HFS delivery. Cell-derived Aβ dimers completely inhibited LTP (105 ± 9%, n = 5; $p > 0.05$ compared with baseline 99 ± 5%). This group was statistically different from animals injected with the natural Aβ monomers ($p < 0.05$).

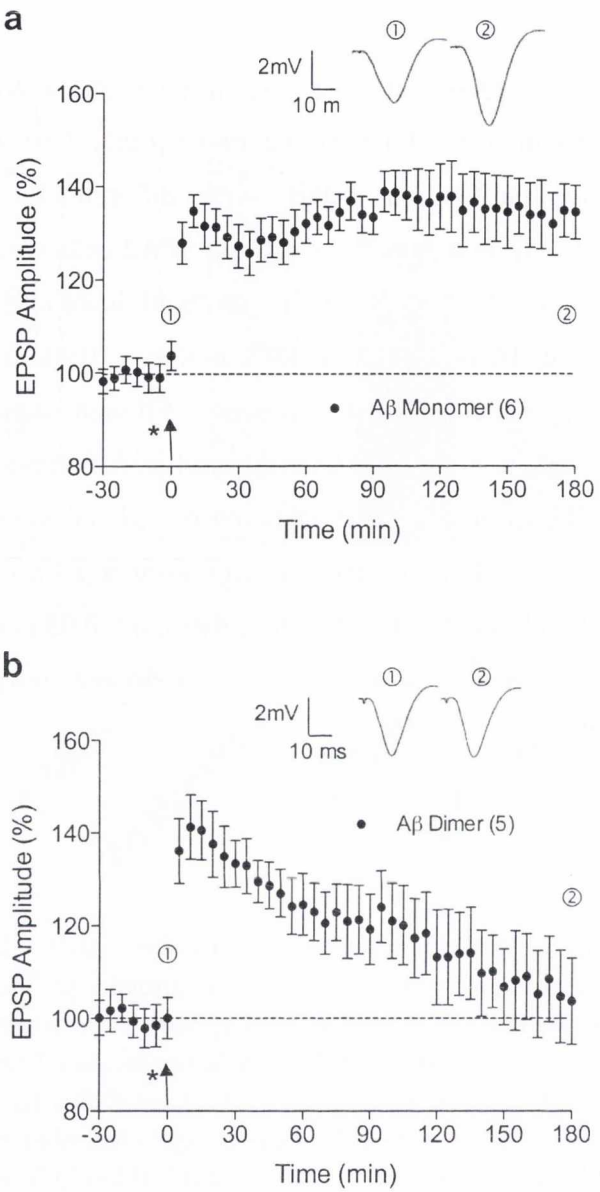
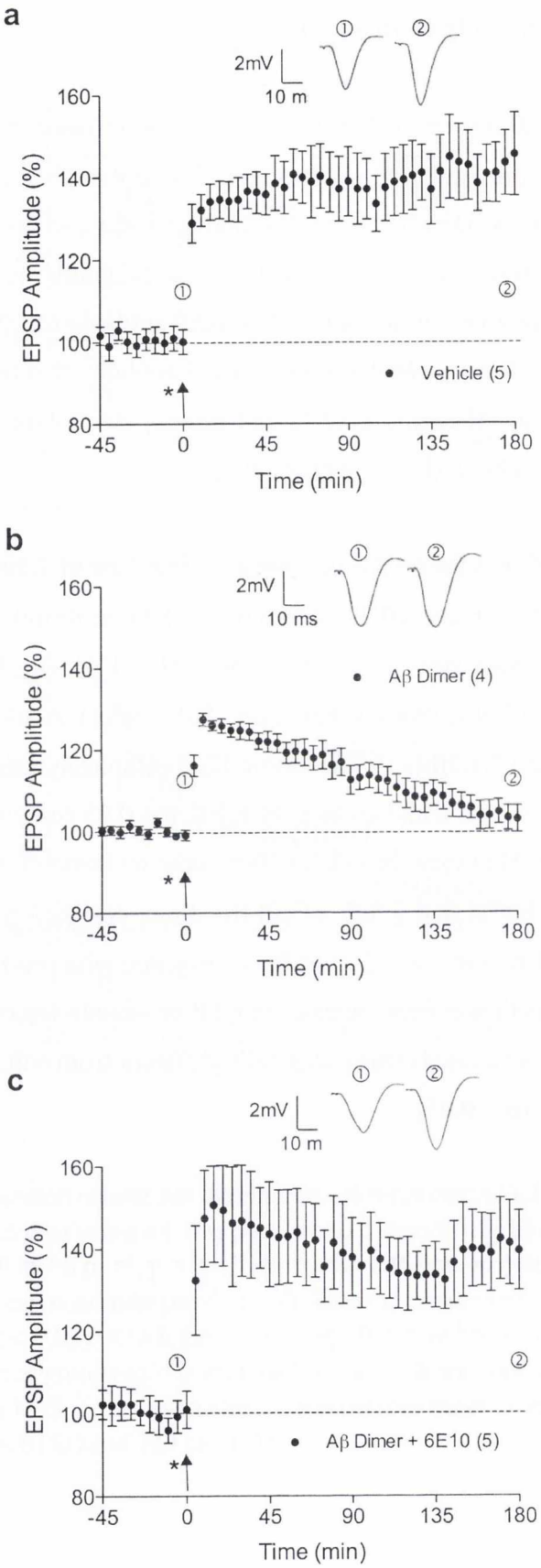


Figure 3.1.3 - Purified natural Aβ dimers blocked hippocampal LTP *in vivo*. **a**, Natural dimers purified using a 4-step purification protocol fully blocked hippocampal LTP at 3 h (n=5, $p > 0.05$, compared with baseline). **b**, In animals injected with natural monomers purified with the same protocol robust LTP was observed (n=6, $p < 0.05$). At 3 h there was a significant difference between the dimer and monomer groups ($p < 0.05$, t-test). All animals received an injection (*) at 10 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②).

3.1.4 4-step purification of conditioned medium: effects of the anti- Aβ antibody 6E10 on LTP

Klyubin et al. (2008) reported that acute systemic infusion of an anti-Aβ monoclonal antibody (4G8) prevented this disruption of synaptic plasticity by human CSF that contains Aβ dimers. Here we used Aβ dimers in the CM from 7PA2 cells that were isolated using a modified 5-step purification protocol similar to that studied in 3.1.3. We determined whether i.c.v infusion of the anti-Aβ antibody 6E10 prevented the inhibition of LTP, by these dimers. 6E10 is a monoclonal antibody that recognizes Aβ residues 1–16 and which can efficiently bind to and directly neutralize Aβ oligomers after i.c.v injection in the rat brain (Klyubin et al., 2005).

Figure 3.1.3(a) shows the results of acute i.c.v injection of 7.5μL of vehicle controls, 10 min prior to HFS delivery. HFS-induced robust LTP in vehicle-injected controls (146 ± 10%, n = 5, $p < 0.05$ compared to baseline, 101 ± 1%). Aβ dimers (≈250 fmol) were diluted in buffer (2.5μL dimer solution and 5μL buffer). Acute i.c.v injection of 7.5μL Aβ dimers (Figure 3.1.3[b]) 10 min before HFS completely inhibited LTP (102 ± 5%, n = 4; $p > 0.05$ compared with baseline, 99 ± 1%; $p < 0.05$ compared with vehicle injected controls at 3 h). However, in animals that were co-injected with 6E10 (2.5μL dimer solution, 2.5μL buffer and 2.5μL 6E10) the dimer-enriched fraction of CM no longer inhibited LTP (140 ± 8%, n = 5; $p < 0.05$ compared with pre-HFS baseline 100 ± 1%), the level of potentiation being similar to LTP in vehicle injected control animals ($p > 0.05$). LTP in these animals was statistically different from animals injected with natural Aβ dimers alone ($p < 0.05$).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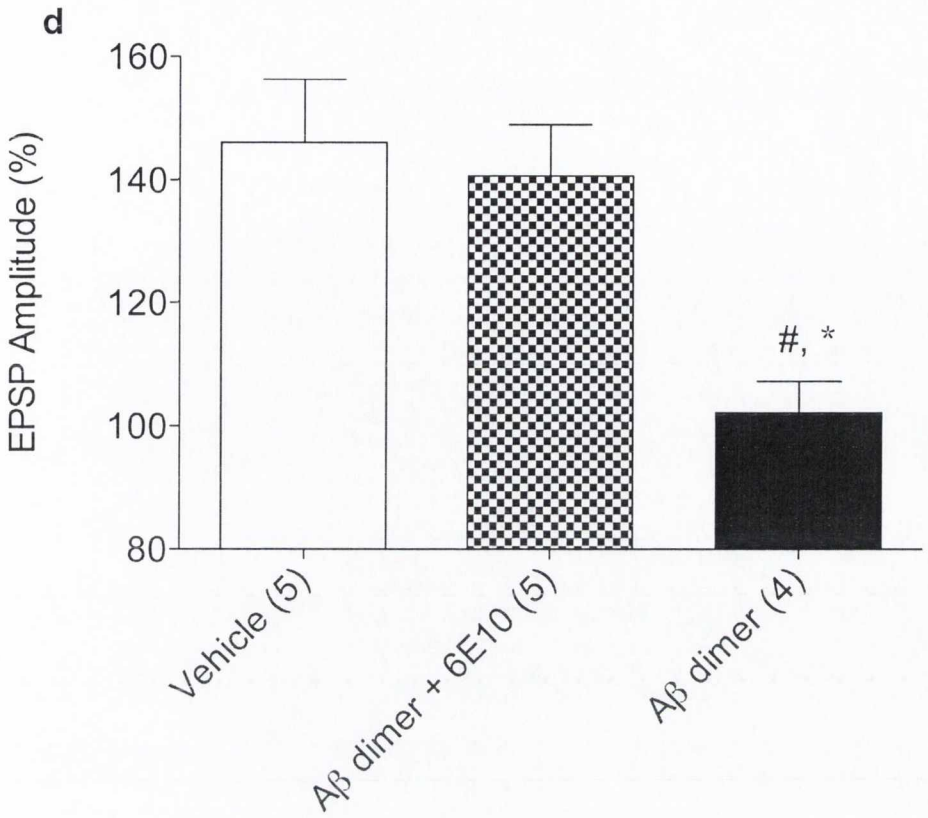


Figure 3.1.4 - The effect of the anti-Aβ monoclonal antibody 6E10 on the inhibition of LTP by highly purified cell-derived Aβ dimers . **a**, In vehicle injected rats (7.5 μL of WFI i.c.v) HFS induced LTP lasting at least 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Cell-derived dimers blocked hippocampal LTP (n=4, $p > 0.05$). **c**, Cell-derived dimers coinjected with antibody 6E10 failed to block hippocampal LTP (n=5, $p < 0.05$). All animals received an injection (*) at 10 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d,** Summary of the effects of anti-Aβ antibody 6E10 on the inhibition of LTP by highly purified cell-derived dimer activity. Statistically significant difference between from vehicle (#) or Aβ dimer + 6E10 (*) groups ($p < 0.05$, posthoc intergroup comparisons) at 3 h post- HFS.

3.2 – The *in vivo* effects of the pan-oligomer antibody A11 on the inhibition of LTP by Aβ and the prion protein peptide $PrP_{106-126}$

The antibody A11 has been reported to be selective for prefibrillar Aβ oligomers (Kayed et al., 2003), recognizing a generic epitope common to prefibrillar oligomers and not fibrils, monomers or natively folded precursor proteins. In this section we examined the ability of A11 to prevent the inhibition of LTP by $A\beta_{1-42}$ and $PrP_{106-126}$.

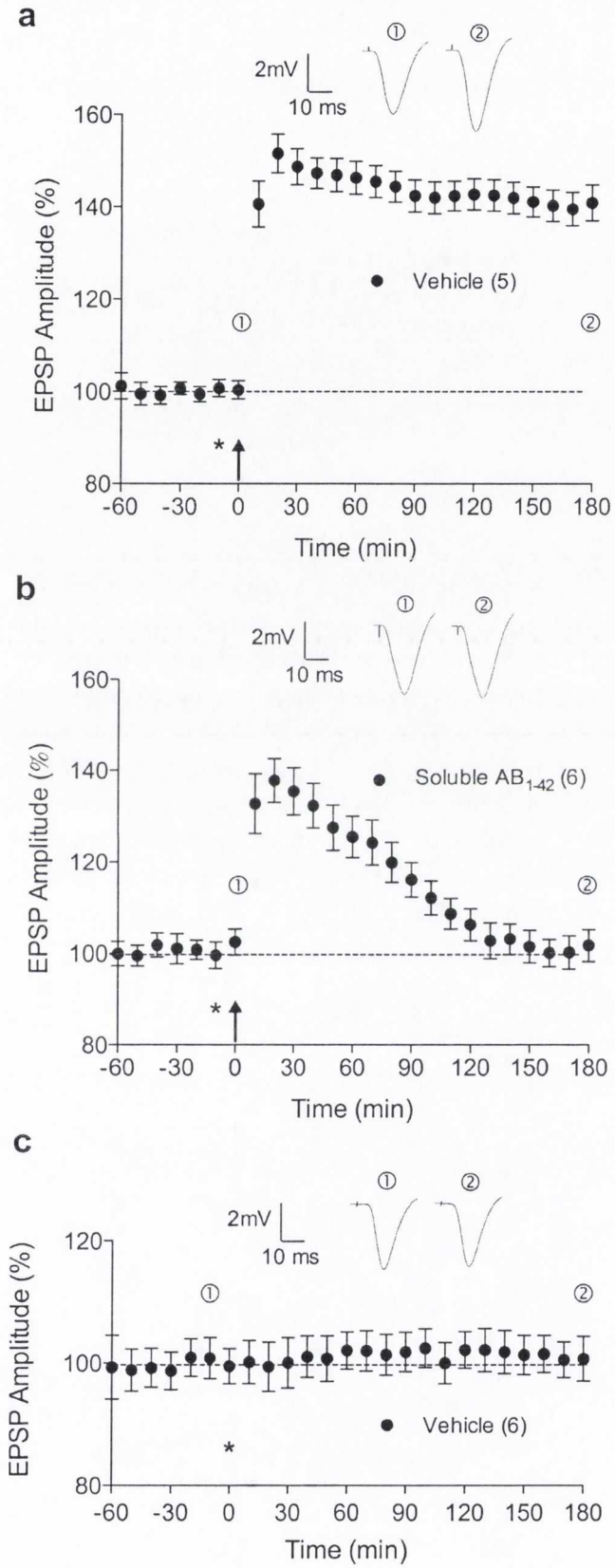
3.2.1 The effects of soluble synthetic amyloid beta ($A\beta_{1-42}$) on hippocampal baseline synaptic transmission and LTP

Synthetic soluble $A\beta_{1-42}$ strongly inhibits LTP (Cullen et al., 1998; Lambert et al., 1998; Origlia et al., 2008). The present studies were designed to determine the magnitude and time course of the effects of soluble $A\beta_{1-42}$ on baseline excitatory synaptic transmission and LTP as a preliminary to mechanistic studies.

I.c.v injection of soluble $A\beta_{1-42}$ (80 pmol) strongly inhibited LTP and did not effect baseline transmission. Figure 3.2.1(a) shows the effect of HFS in animals given an acute i.c.v injection of 5μL of WFI 15 min previously. In these animals LTP was stable for at least 3 h post-HFS (141 ± 4%, at 3 h post-HFS, n = 5; $p < 0.05$ compared with baseline 100 ± 2). Figure 3.2.1(b) shows the effect of acute i.c.v injection of 5μL of synthetic soluble $A\beta_{1-42}$ (80 pmol) 15 min prior to HFS delivery. Synthetic soluble Aβ completely inhibited LTP (102 ± 3, n = 6; $p > 0.05$ compared with baseline 102 ± 3%; $p < 0.05$ compared with vehicle injected controls at 3 h, 141 ± 4%, n = 5).

To investigate the effects of i.c.v injection of soluble synthetic $A\beta_{1-42}$ (80 pmol) on baseline synaptic transmission, in keeping with the HFS protocols, we administered solutions at "time zero"; i.e. when HFS would have been delivered. Figure 3.2.1(d) shows the results of acute i.c.v injection of 5μL of WFI. Vehicle injection had no effect on baseline (101 ± 4%, mean ± (SEM) percentage at baseline at 3 h post-injection, n = 6; $p > 0.05$ compared with pre-injection baseline 100 ± 3%). To determine if soluble synthetic Aβ had an effect on baseline at the dose used to inhibit LTP we administered

an acute i.c.v injection of 5μL of synthetic $A\beta_{1-42}$ (80 pmol) at "time zero" (Fig 3.2.1[c]). Soluble synthetic Aβ had no significant effect on baseline at 3 h after injection (96 ± 5%, n = 5; $p > 0.05$ compared with baseline 102 ± 4%, $p > 0.05$ compared with vehicle injected; $p > 0.05$ for 1-way ANOVA with repeated measures for comparing 4 x 10 min epochs (10 min prior to baseline, 50-60, 110-120 and 170-180 min).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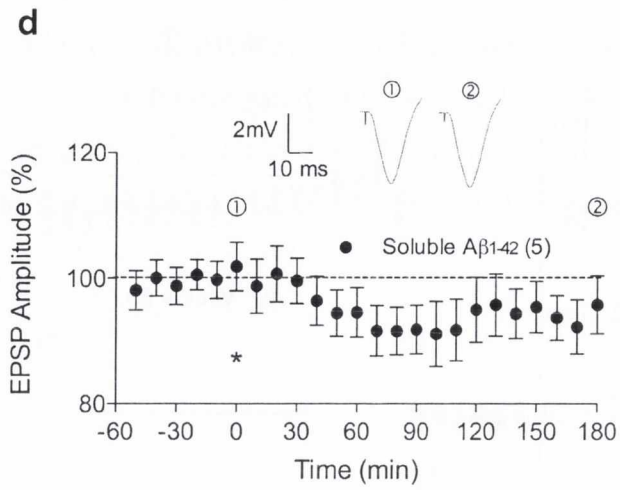


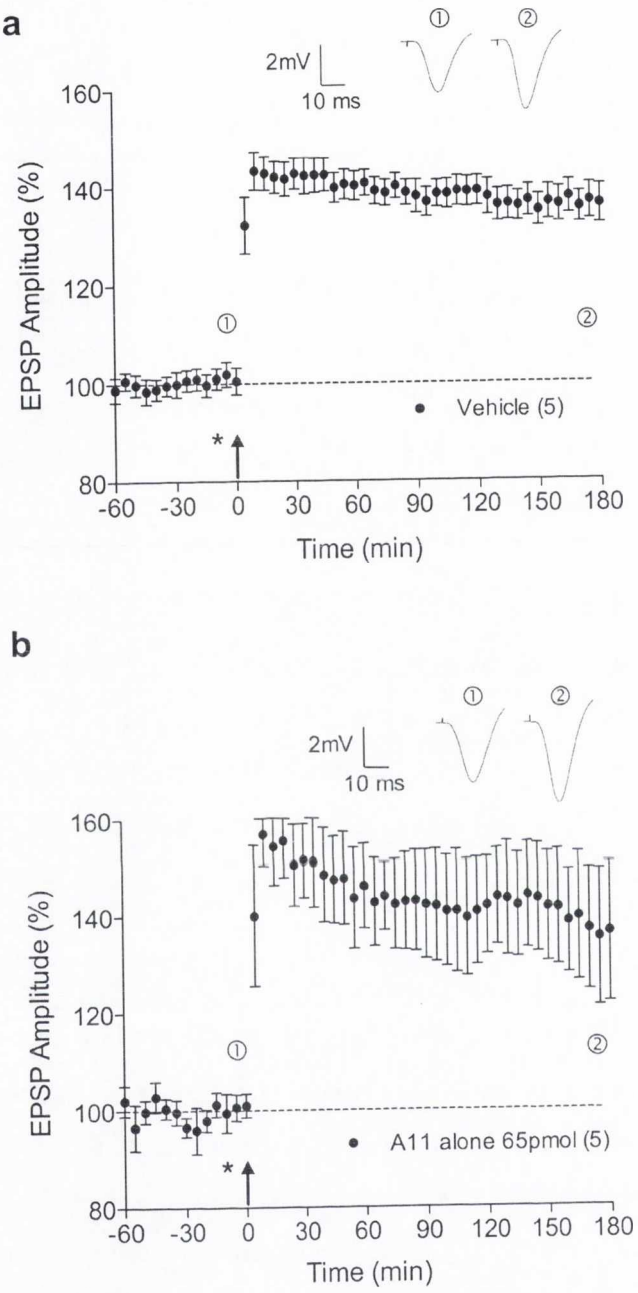
Figure 3.2.1 - Soluble synthetic Aβ1-42 blocked hippocampal LTP using a dose that did not affect baseline excitatory synaptic transmission *in vivo*. **a**, Vehicle (WFI) injected control experiments showed stable potentiation for 3 h post- HFS ($n=5$, $p < 0.05$, compared with baseline). **b**, Synthetic soluble Aβ inhibited LTP at 3 h ($n=6$, $p > 0.05$ compared with baseline). At 3 h there was a statistically significant difference between the WFI and synthetic Aβ groups ($p < 0.05$, t-test). **c**, A vehicle injection of 5 μL of WFI had no significant effect on baseline at 3 h post-injection ($n = 6$, $p > 0.05$). **d**, Synthetic soluble Aβ had no significant effect on hippocampal baseline ($n = 5$, $p > 0.05$). There was no statistically significant difference between the WFI and Aβ groups at 3 h post-injection ($p > 0.05$, t-test). All animals received a 5μL i.c.v injection (*) at time 0.

3.2.2 The prevention of $A\beta_{1-42}$ mediated inhibition of LTP by A11

In this section the effects of i.c.v co-injection of soluble Aβ and the antibody A11 on LTP were investigated in our rat model. The dose of the antibody A11 to be studied with Aβ had no effect on LTP when given alone. Figure 3.2.2(a) shows the effect of acute i.c.v injection of 5μL of WFI 15 min prior to HFS delivery. Animals given a control injection displayed LTP for at least 3 h post-HFS (137 ± 4%, n = 5; $p < 0.05$ compared with baseline 101 ± 3%). Animals receiving a co-injection of 5μL of A11 (65 pmol) + WFI 15 min prior to HFS delivery (Figure 3.2.2[b]) showed LTP for at least 3 h post-HFS (136 ± 14, n = 6; $p < 0.05$ compared with baseline 101 ± 3%; $p > 0.05$ compared with vehicle injected controls at 3 h).

To determine if the anti- Aβ oligomer antibody A11 co-injected with soluble synthetic Aβ can prevent the inhibition of LTP we administered this combination i.c.v prior to HFS. As a positive control we tested the effects of acute 5μL i.c.v injection of soluble synthetic $A\beta_{1-42}$ (40 pmol), 15 min prior to HFS delivery (Figure 3.2.2(d)). This dose of soluble synthetic Aβ completely inhibited LTP (102 ± 2, n = 6; $p > 0.05$ compared with baseline 100 ± 1%; $p < 0.05$ compared with separate group of vehicle injected controls at 3 h).

Figure 3.2.2(e) shows the effects of acute 5μL i.c.v co- injection of synthetic Aβ + A11 antibody, 15 min prior to HFS delivery. Injection was antibody A11 (50 pmol) + synthetic Aβ (40 pmol). A11 antibody prevented the inhibition of LTP at this dilution (137 ± 1, n = 5; $p < 0.05$ compared with baseline 101 ± 2%; $p > 0.05$ compared with vehicle injected controls; $p < 0.05$ compared with the soluble synthetic Aβ + WFI treatment at 3 h). In vehicle injected controls (Figure 3.2.2[c]) LTP lasted at least 3 h (130 ± 2%, n = 5; $p < 0.05$ compared with baseline 100 ± 1%).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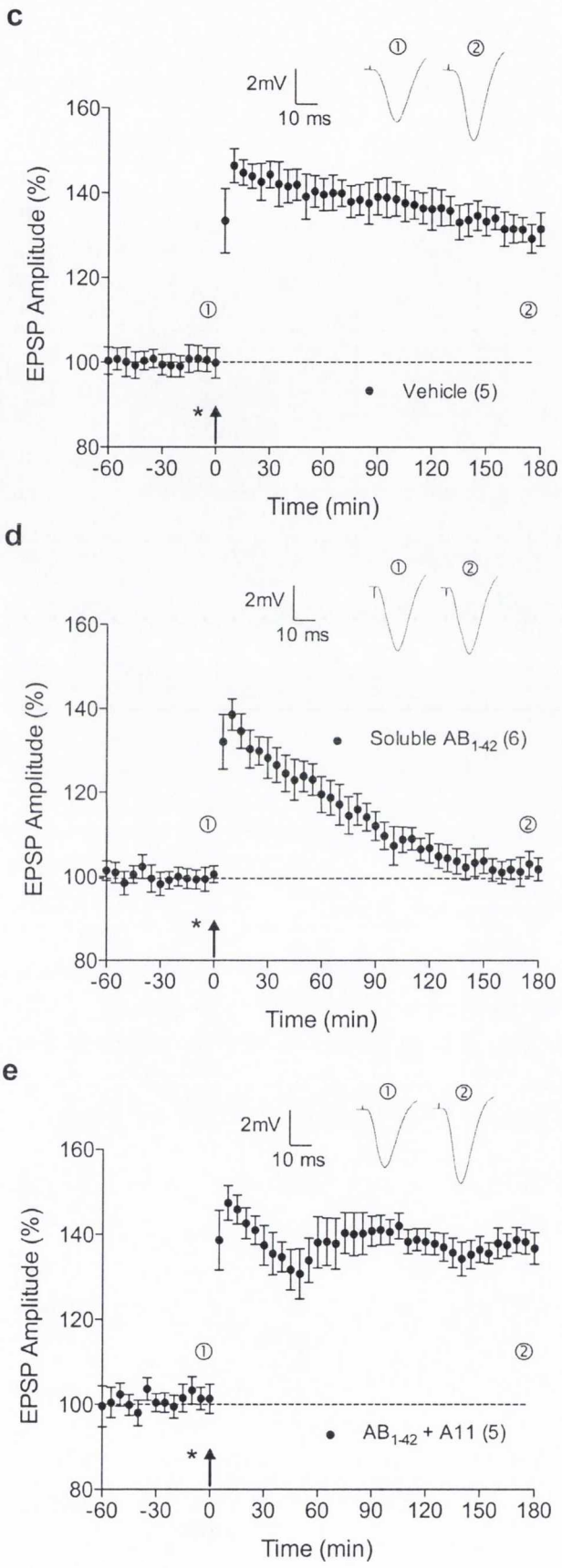
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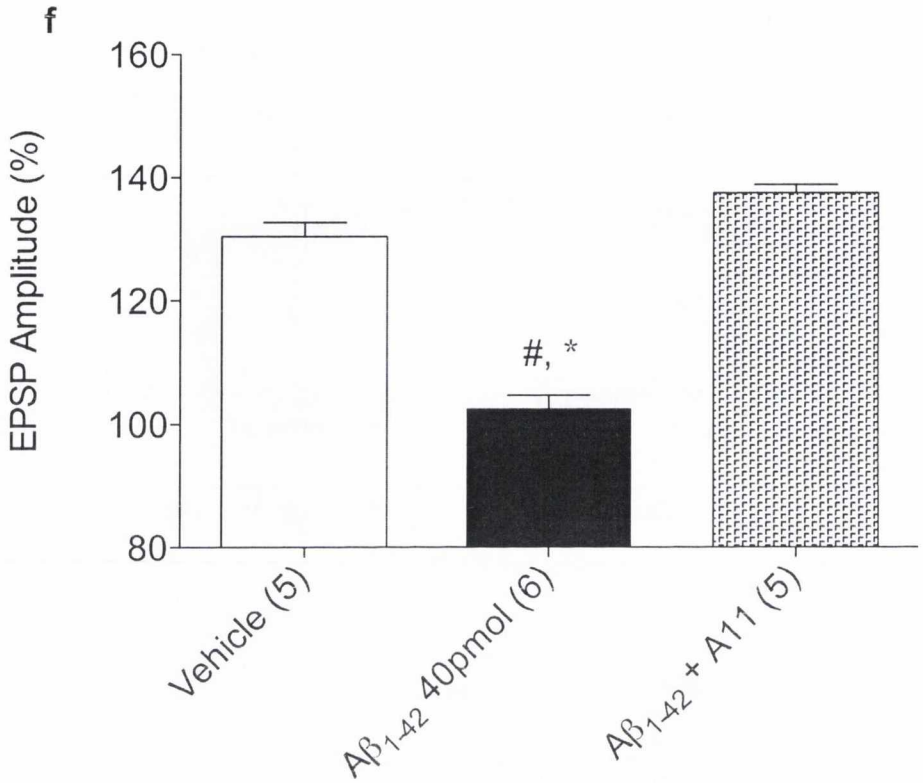
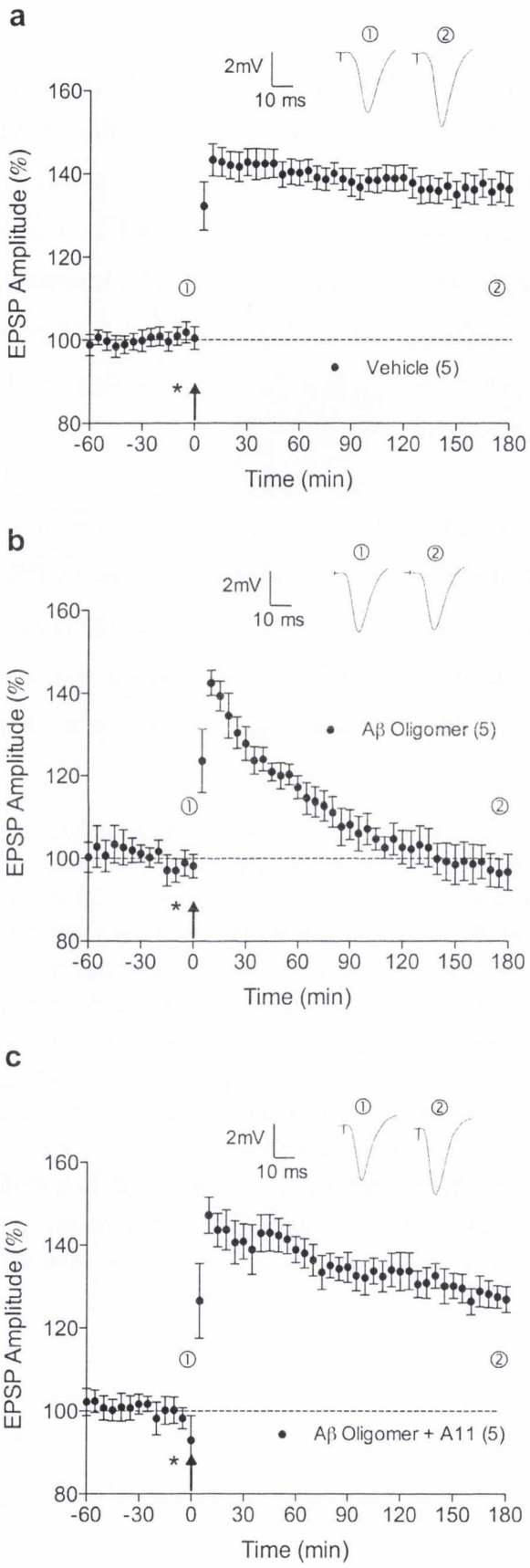


Figure 3.2.2 - The pan-oligomeric antibody A11 prevented the inhibition of hippocampal LTP by soluble synthetic Aβ1-42 *in vivo*. **a**, Vehicle controls exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, A11-injected animals exhibited LTP for 3 h (n=5, $p < 0.05$). There was no statistically significant difference between the vehicle and synthetic A11-injected group ($p > 0.05$, t-test). **c**, A separate group of vehicle controls exhibited LTP for 3 h (n=6, $p < 0.05$). **d**, $A\beta_{1-42}$ blocked hippocampal LTP (n=6, $p > 0.05$). **e**, Synthetic Aβ protein failed to inhibit LTP in animals pre-incubated with A11 (n=5, $p < 0.05$, compared with baseline). All animals received an injection (*) at 10 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **f**, Summary of the effects of pre-incubation of $A\beta_{1-42}$ with A11 (posthoc intergroup comparisons). There was a statistically significant difference from vehicle (#, $p < 0.05$) and synthetic Aβ + A11 group (* , $p < 0.05$).

3.2.3 Prevention of the effects of cell-derived Aβ oligomers (7PA2) by A11

Cell derived Aβ strongly inhibits LTP (Walsh et al., 2002; Klyubin et al., 2004, 2005; and Section 3.1.2 Results). To determine if co-injection of A11 (2.0 mg/ml) prevented the inhibition of LTP by natural Aβ oligomers we administered this combination i.c.v prior to HFS. First we tested the effects of acute 5μL i.c.v injection of natural oligomer, 15 min prior to HFS delivery (Figure 3.2.3[b]). This injection of natural oligomer (≈ 25 fmol) completely inhibited LTP (97 ± 4, n = 5; $p > 0.05$ compared with baseline 96 ± 4%; $p < 0.05$ compared with vehicle injected controls (Figure 3.2.4[a]), 137 ± 5%, n = 5; $p < 0.05$ compared with baseline 101 ± 3%).

Figure 3.2.3(c) shows the effects of acute 5μL i.c.v co- injection of antibody A11 (50 pmol) + natural oligomer (≈ 25 fmol), 15 min prior to HFS delivery. A11 antibody prevented the inhibition of LTP (127 ± 1, n = 5; $p < 0.05$ compared with baseline 99 ± 5%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the natural Aβ + WFI group at 3 h ($p < 0.05$).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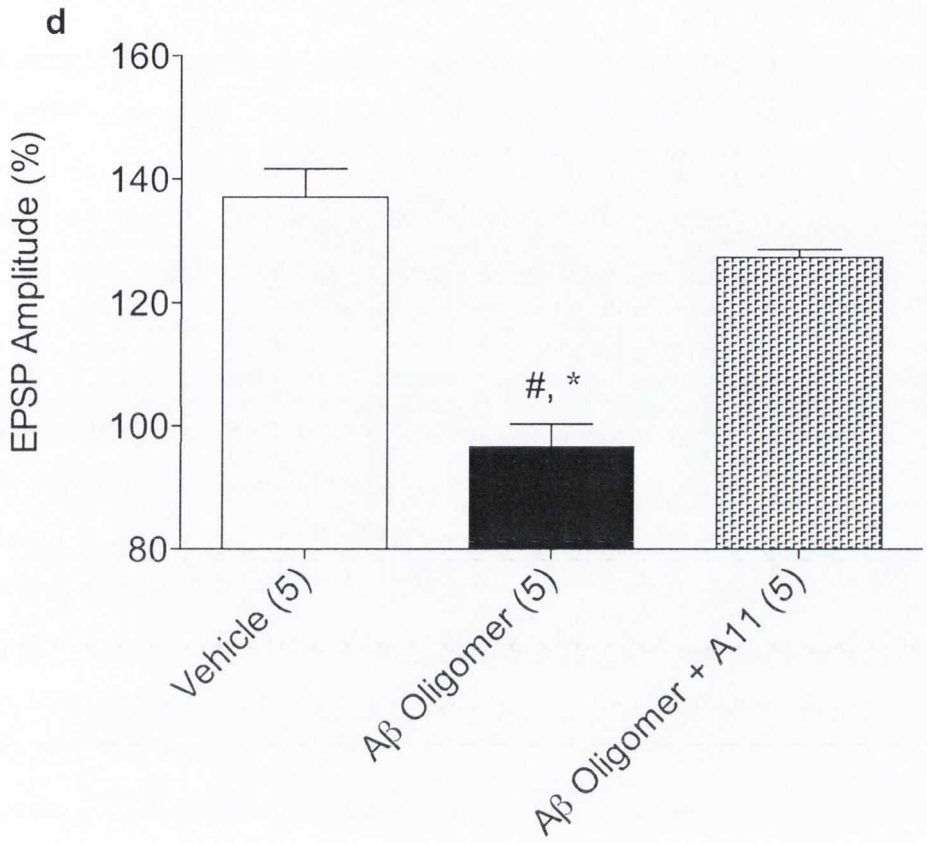


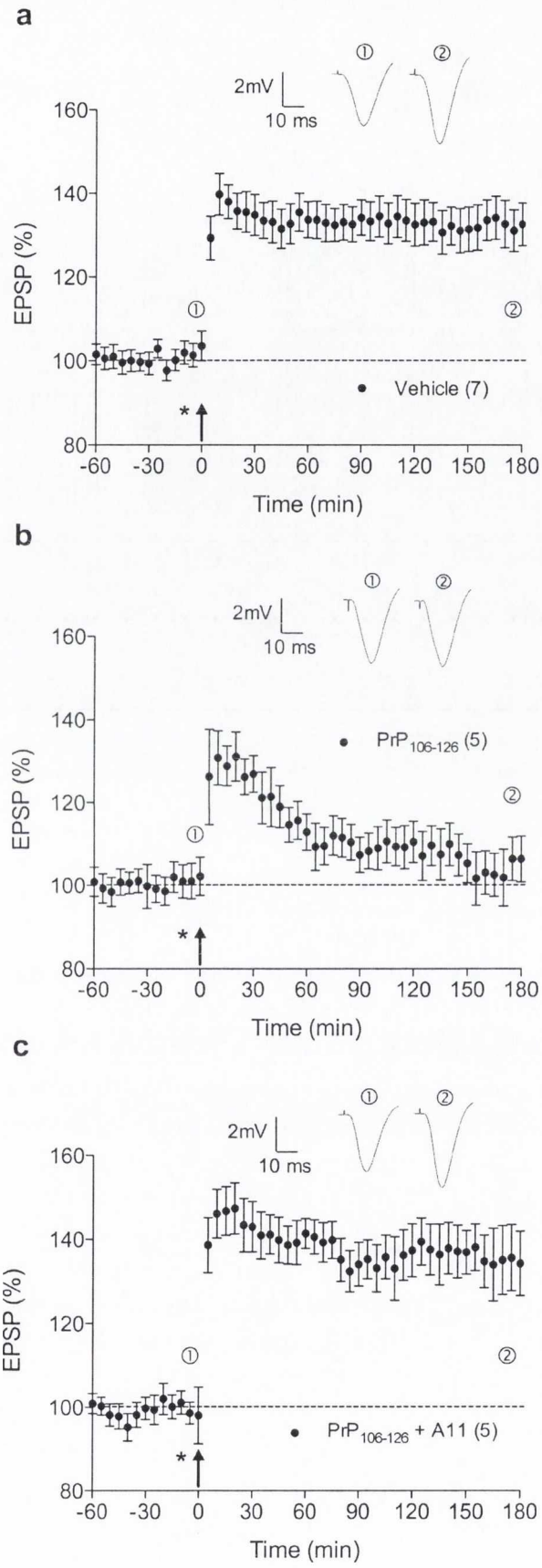
Figure 3.2.3 - The antibody A11 prevented inhibition of hippocampal LTP by cell derived Aβ oligomers *in vivo*. **a**, Vehicle injected controls exhibited LTP at 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Natural oligomers purified with SEC treatment inhibited hippocampal LTP (n=6, $p > 0.05$). **c,** Natural oligomers failed to inhibit LTP in animals co-injected with A11 (50 pmol) (n=5, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d,** Summary of the effects of pre-incubation of natural oligomers with A11 3 h post- HFS. There was a statistically significant difference compared with Aβ oligomer + A11 group (* , $p < 0.05$) and the vehicle group (#, $p < 0.05$).

3.2.4 A11 prevents the inhibition of LTP by cellular prion protein peptide

The antibody A11 binds not only to oligomers of Aβ, but also amyloidogenic proteins with diverse amino acid sequences, such as oligomeric aggregates formed by α-synuclein, islet amyloid polypeptide, polyglutamine, lysozyme, insulin and prion protein (Kayed and Glabe, 2006). The prion protein peptide ($PrP_{106-126}$) has been shown in our lab to potently inhibit LTP (W.K. Cullen- unpublished data).

To determine if co-injection of A11 prevented the inhibition of LTP by the peptide $PrP_{106-126}$ we administered this combination i.c.v prior to HFS. In animals given vehicle injection 15 min prior to HFS (Figure 3.2.4[a]) LTP presented for at least 3 h post-HFS (132 ± 5%, n = 7; $p < 0.05$ compared with baseline 103 ± 2%). We tested the effects of acute 5μL i.c.v injection of $PrP_{106-126}$ (50 pmol), 15 min prior to HFS delivery (Figure 3.2.4[b]). This dose of $PrP_{106-126}$ completely inhibited LTP (106 ± 4, n = 5; $p > 0.05$ compared with baseline 102 ± 3%; $p < 0.05$ compared with vehicle injected controls). Figure 3.2.4(c) shows the effects of acute 5μL i.c.v co- injection of $PrP_{106-126}$ (50 pmol) + A11 (50 pmol) antibody, 60 min after co-incubation and 15 min prior to HFS delivery. A11 antibody prevented the inhibition of LTP (135 ± 8, n = 5; $p < 0.05$ compared with baseline 98 ± 1%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the $PrP_{106-126}$ alone group at 3 h ($p < 0.05$).

To determine if co-injection of a control polyclonal antibody (IgG isotype) prevented the inhibition of LTP by the peptide $PrP_{106-126}$ (50 pmol) we administered this combination i.c.v prior to HFS. The results in figure 3.2.4(d) show that the control antibody (50 pmol) did not prevent the inhibition of LTP by $PrP_{106-126}$ (101 ± 4, n = 5; $p > 0.05$ compared with baseline 100 ± 1%; $p < 0.05$ compared with WFI vehicle injected controls at 3 h; $p > 0.05$ compared with $PrP_{106-126}$ alone group at 3 h). Animals injected with the control antibody (50 pmol) on its own (Figure 3.2.4[e]) showed LTP that lasted for at least 3 h (131 ± 5%, n = 5; $p < 0.05$ compared with baseline 103 ± 1%, $p > 0.05$ compared with WFI vehicle injected controls at 3 h).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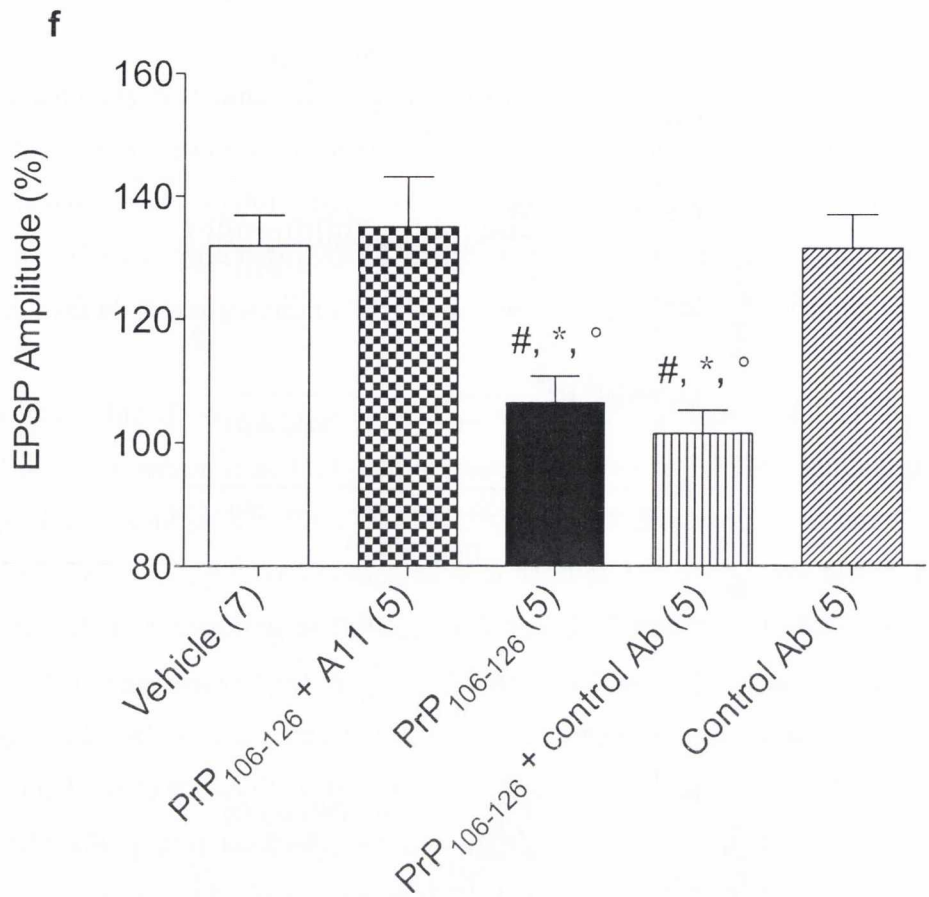


Figure 3.2.4 - Inhibition of hippocampal LTP by the prion protein peptide ($PrP_{106-126}$) *in vivo* was prevented by the antibody A11. **a**, Vehicle injected controls displayed LTP at 3 h (n=7, $p < 0.05$, compared with baseline). **b,** $PrP_{106-126}$ peptide completely inhibited hippocampal LTP at 3 h (n=5, $p > 0.05$). **c,** $PrP_{106-126}$ failed to inhibit LTP in animals pre-incubated with A11 (n=5, $p < 0.05$). **d,** The prion protein peptide $PrP_{106-126}$ preincubated with a polyclonal IgG control antibody fully inhibited hippocampal LTP *in vivo* (n=5, $p > 0.05$). **e**, The polyclonal control antibody injected animals display LTP at 3 h (n=5, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **f,** Summary of the effects of pre-incubation of A11 and $PrP_{106-126}$ at 3 h post- HFS. There was a statistically significant difference compared with the $PrP_{106-126}$ + A11(# , $p < 0.05$), the vehicle (* , $p < 0.05$) and the control Ab (°, $p < 0.05$).

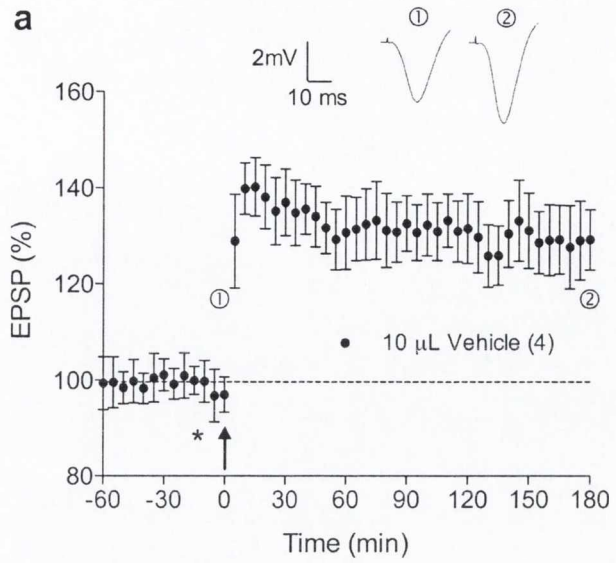
3.2.5 The effects of pre-aggregated $A\beta_{1-42}$ on baseline transmission & LTP

Hartley et al. (1999) reported that sub-micromolar concentrations of Aβ protofibrils acutely increased the electrical activity of cortical neurons, whereas LMW Aβ at the same concentrations had no significant effect. O'Nuallain et al. (2010) recently reported that Aβ dimers rapidly form stable synaptotoxic protofibrils that inhibit *in vitro* LTP. In the present study we tested pre-aggregated Aβ that is thought to contain both large insoluble fibrils and smaller protofibrils.

The effects of i.c.v injection of 5 and 10μL pre-aggregated synthetic $A\beta_{1-42}$ on LTP were investigated. For comparison, we used vehicle-injected controls (WFI). Figures 3.3.4(a)(c) show the effect of HFS 15 min after acute i.c.v vehicle injection (10 or 5μL). 10μL-vehicle injected animals display robust LTP for 3 h (129 ± 6%, at 3 h post-HFS, n = 4; $p < 0.05$ compared with baseline 97 ± 4%), as did animals injected with 5μL (134 ± 3%, at 3 h post-HFS, n = 4; $p < 0.05$ compared with baseline 100 ± 2%). To determine if pre-aggregated Aβ affected LTP, we examined the effects of acutely injecting 10μL (400 pmol) and 5μL (200 pmol) (Figures 3.3.4[b][d], respectively). 10μL of pre-aggregated Aβ 15 min prior to HFS delivery completely inhibited LTP (96 ± 1, n = 4; $p > 0.05$ compared with baseline 100 ± 3%; $p < 0.05$ compared with vehicle injected controls at 3 h), as did 5μL of pre-aggregated Aβ (103 ± 4, n = 5; $p > 0.05$ compared with baseline 104 ± 2%; $p < 0.05$ compared with vehicle injected controls at 3 h).

Next, the effects of i.c.v injection of pre-aggregated Aβ preparations on baseline synaptic transmission were investigated. Figure 3.3.4(g) shows the results of acute i.c.v injection of 10μL of WFI. Vehicle injection had no effect on baseline (102 ± 2%, 3 h post-injection, n = 5; $p > 0.05$ compared with pre-injection baseline 101 ± 2%). To determine if pre-aggregated Aβ had an effect on baseline at the dose used to inhibit LTP we administered an acute i.c.v injection of 5μL of pre-aggregated Aβ (200 pmol) at time 0 min (Fig 3.3.4[h]). Pre-aggregated Aβ had no significant effect on baseline at 3 h after injection (97 ± 2%, n = 4; $p > 0.05$ compared with baseline 101 ± 1%, $p > 0.05$ compared with vehicle injected).

As the pre-aggregated Aβ proved to be active in our model, we wanted to determine the effects of 'washing' the material in order to remove soluble Aβ species- leaving relatively pure fibrillar material. Animals given a 5μL vehicle injection (Figure 3.3.4[f]) displayed LTP that lasted at least 3 h (137 ± 3%, n = 5; $p < 0.05$ compared with baseline 101 ± 1%). We tested the effects of acute 5μL i.c.v injection of 'washed' pre-aggregated Aβ, 15 min prior to HFS delivery (Figure 3.3.4[e]). A 5μL neat injection of 'washed' pre-aggregated Aβ (420 pmol starting material) had no effect on hippocampal LTP *in vivo* (133 ± 5, n = 5; $p < 0.05$ compared with baseline 104 ± 2%; $p > 0.05$ compared with vehicle injected controls).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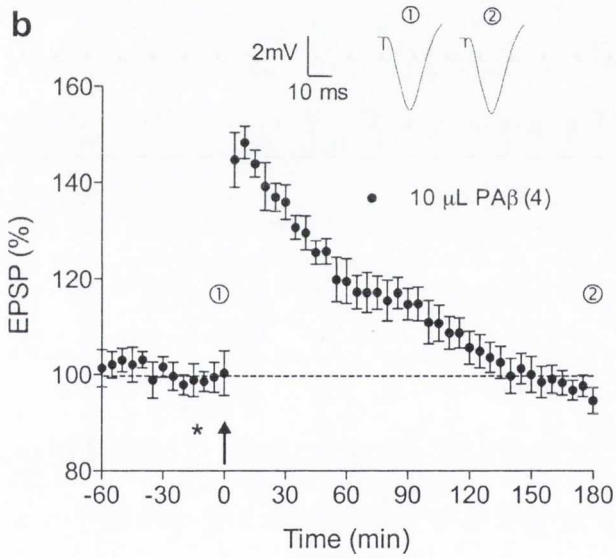
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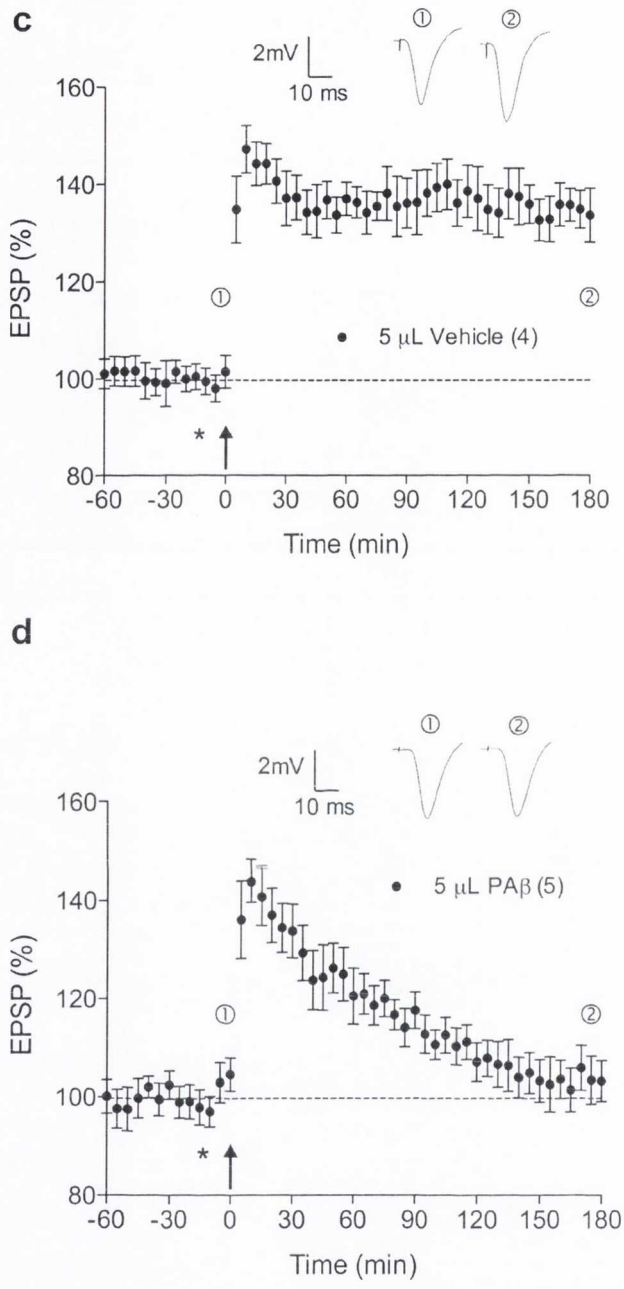
Time (min)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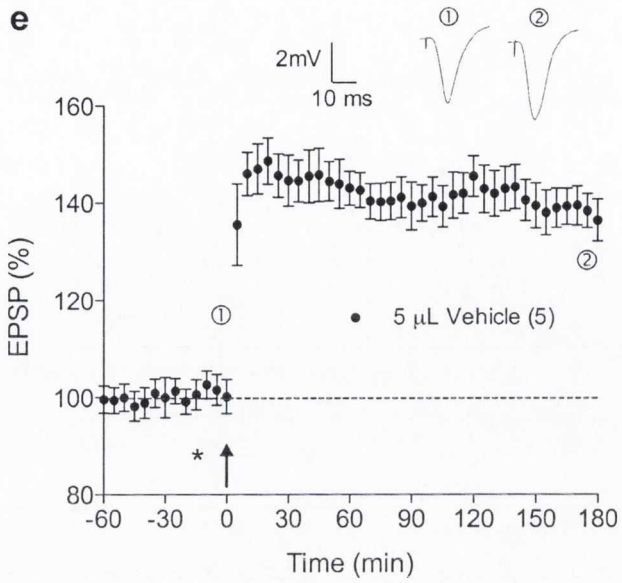
Time (min)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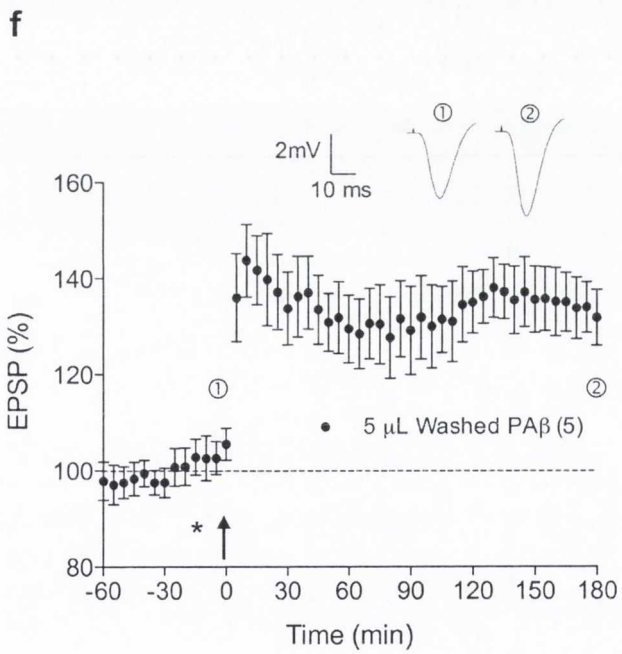
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5 μL Washed PAβ (5)
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Time (min)

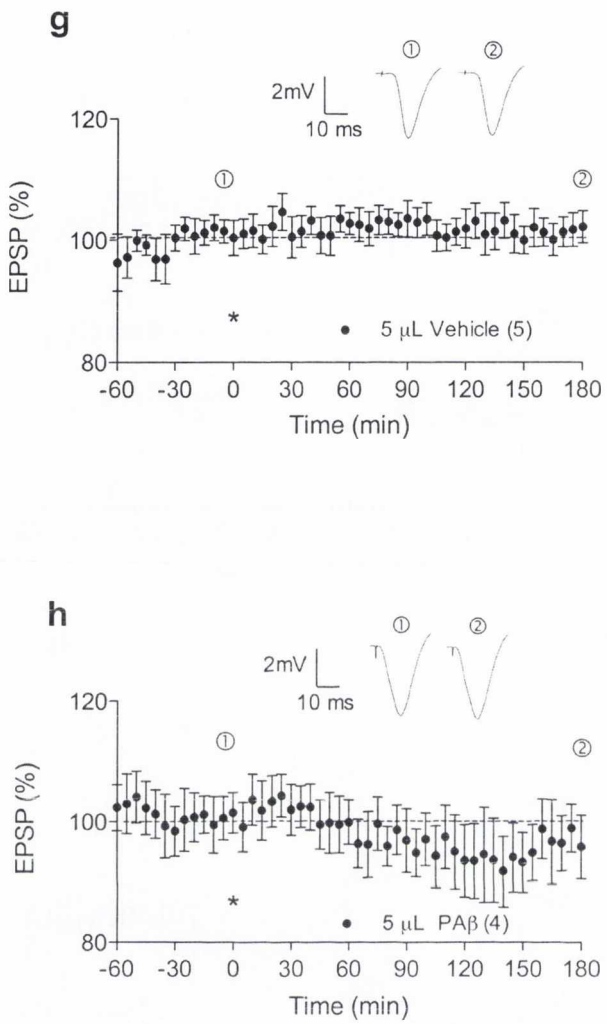
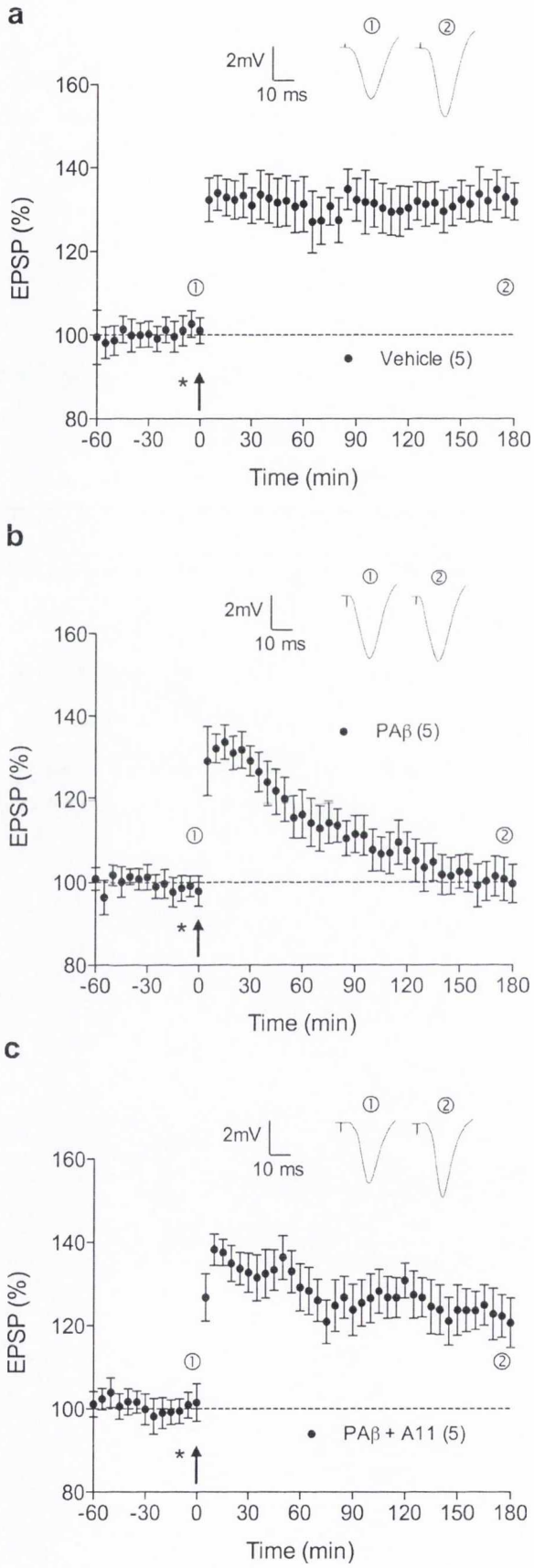


Figure 3.2.5 - Effects of preaggregated Aβ on hippocampal baseline excitatory synaptic transmission and LTP *in vivo*. **a**, Vehicle controls (10 μL) exhibited LTP for 3 h (n=4, $p < 0.05$, compared with baseline). **b**, Preaggregated Aβ (PAβ) (10 μL) completely blocked hippocampal LTP (n=4, $p > 0.05$). There was a statistically significant difference between the vehicle and PAβ groups ($p < 0.05$, t-test). **c**, Vehicle controls (5 μL) exhibited LTP for 3 h (n=4, $p < 0.05$). **d**, PAβ (5 μL) completely blocked hippocampal LTP (n=4, $p > 0.05$). There was a statistically significant difference between the vehicle and PAβ groups ($p < 0.05$, t-test). **e**, Washed preaggregated Aβ (5 μL) blocked hippocampal LTP (n=5, $p > 0.05$). **f**, Vehicle controls (5 μL) exhibited LTP for 3 h (n=5, $p < 0.05$). There was no statistically significant difference between the vehicle and washed PAβgroups ($p > 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). **g**, **h**, Vehicle controls (n=5, $p < 0.05$) and PAβ had no effect on hippocampal baseline (n=4, $p > 0.05$). There was no statistically significant difference between these groups ($p > 0.05$). All animals received an injection (*) at time 0. Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②).

3.2.6 A11 partly prevents the inhibition of LTP by pre-aggregated $A\beta_{1-42}$

As the pre-aggregated $A\beta_{1-42}$ was found to be active in the previous study and is thought to contain both large insoluble fibrils and smaller soluble assemblies, we tested this preparation with the A11 antibody to further characterize it.

To determine if co-injection of A11 prevented the inhibition of LTP by pre-aggregated synthetic $A\beta_{1-42}$ we administered this combination i.c.v prior to HFS. Animals given vehicle injection 15 min prior to HFS (Figure 3.2.5[a]) showed LTP for at least 3 h post-HFS (132 ± 4%, n = 5; $p < 0.05$ compared with baseline 102 ± 2%). Acute i.c.v injection of 10μL pre-aggregated $A\beta_{1-42}$ (200 pmol), 15 min prior to HFS delivery (Figure 3.2.5[b]) completely blocked hippocampal LTP (100 ± 5, n = 5; $p > 0.05$ compared with baseline 99 ± 1%; $p < 0.05$ compared with vehicle injected controls). Figure 3.2.5(c) shows the effects of acute 10μL i.c.v co- injection of pre-aggregated Aβ + A11 antibody (100 pmol), 60 min after co-incubation and 15 min prior to HFS delivery. The A11 antibody partially prevented the inhibition of LTP (121 ± 5, n = 5; $p < 0.05$ compared with baseline 101 ± 2%; $p < 0.05$ compared with WFI vehicle injected controls at 3 h; $p < 0.05$ compared with the pre-aggregated Aβ group at 3 h).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PAβ + A11 (5)
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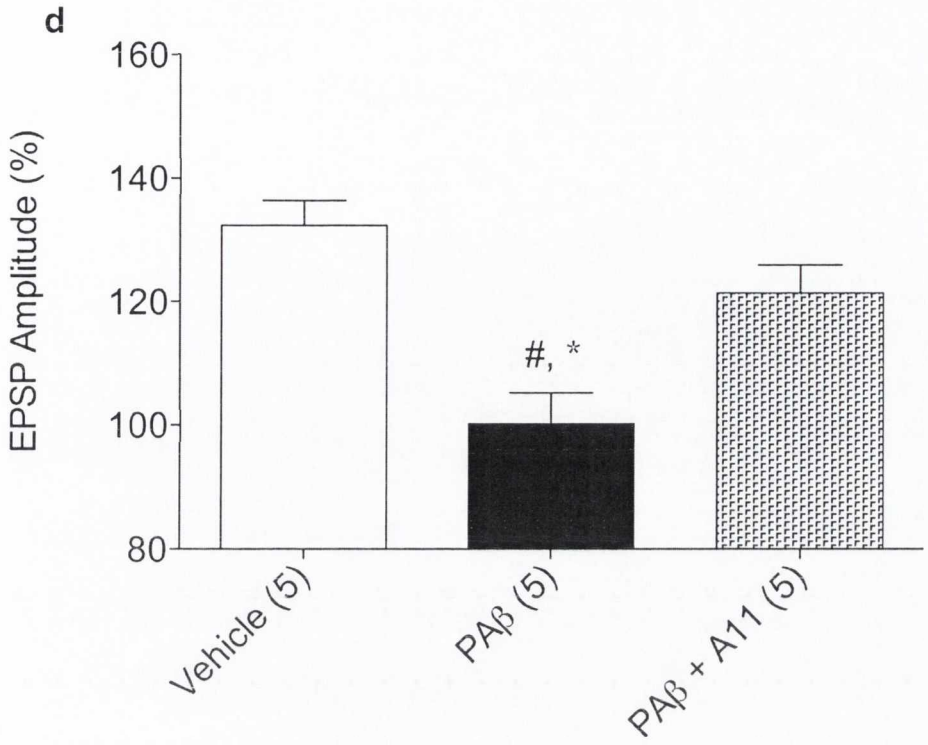


Figure 3.2.6 - The antibody A11 partly prevented inhibition of hippocampal LTP by preaggregatedl Aβ *in vivo*. **a**, Vehicle injected controls exhibited LTP at 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Preaggregated Aβ (PAβ) inhibited hippocampal LTP (n=6, $p > 0.05$). **c,** PAβ failed to inhibit LTP in animals co-injected with A11 (n=5, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d,** Summary of the effects of preinjection of A11 and preaggregated Aβ (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with preaggregated Aβ + A11 (* , $p < 0.05$) and vehicle (# , $p < 0.05$).

3.3 - The effects of the (proto)fibril antibodies WO1 and WO2 on the inhibition of hippocampal LTP by Aβ

Since we found that the pan-oligomer antibody A11 prevented the inhibition of LTP by cell-derived Aβ for comparison we also studied the effect of the pan (proto)fibril antibodies WO1 and WO2. O'Nuallain and Wetzel (2002) reported the generation and preliminary characterization of two conformation-specific mAbs, WO1 and WO2, that bind to the amyloid fibril state of the Alzheimer's peptide $A\beta_{1-40}$ and not to its monomeric state. As previously mentioned, soluble cell derived Aβ low-n oligomers strongly inhibits LTP in our rat model (Walsh et al., 2002; Klyubin et al., 2004, 2005), but a role for larger assemblies in the disruptive effects of synthetic Aβ needs further investigation.

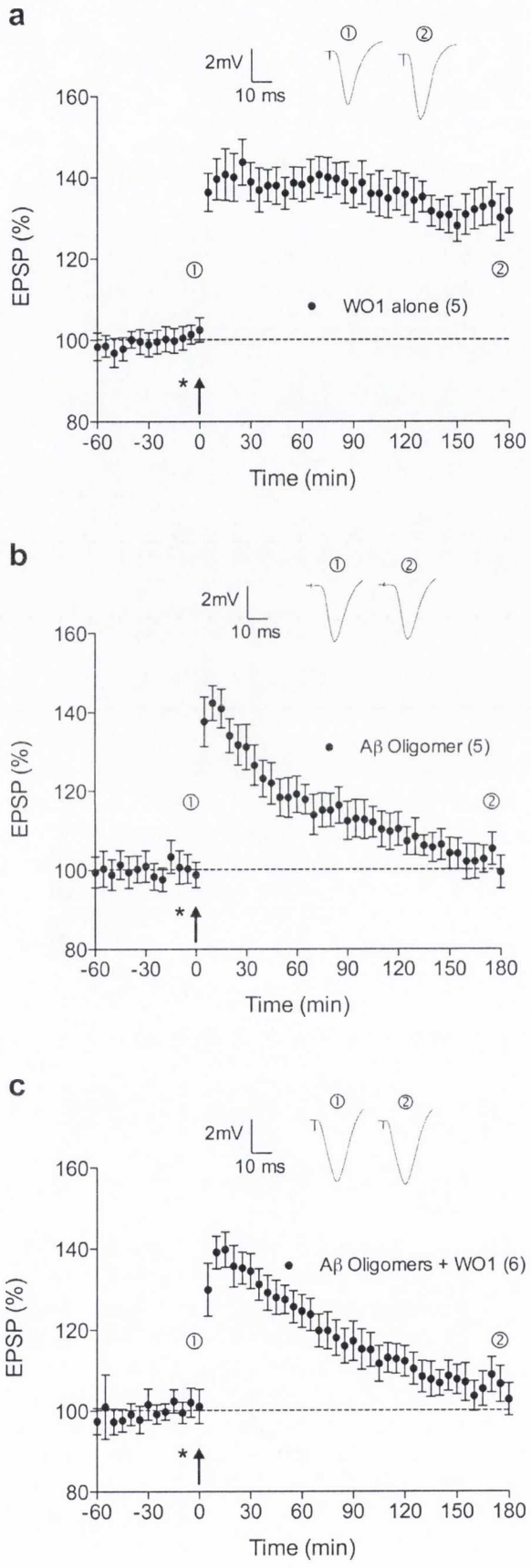
3.3.1 The effects of WO1 on the inhibition of LTP by cell-derived Aβ oligomers

Animals given vehicle injection 15 min prior to HFS (Figure 3.3.1 d) showed LTP for at least 3 h post-HFS (137 ± 4%, n = 10; $p < 0.05$ compared with baseline 102 ± 2%). Robust LTP was induced in animals injected with the dose (100 pmol) of the antibody WO1 studied in the combination studies. In animals injected with this dose, 15 min prior to HFS delivery (Figure 3.3.1[a]) LTP persisted for at least 3 h post-HFS (136 ± 4, n = 5; $p < 0.05$ compared with baseline 101 ± 3%; $p > 0.05$ compared with vehicle injected controls at 3 h).

In order to determine the effects of WO1 on the inhibition of LTP by cell-derived Aβ oligomers *in vivo,* we pre-incubated the WO1 and cell-derived Aβ oligomers together at room temperature (23°C) for 60 min prior to injection. 5μL injection of natural oligomer, 15 min prior to HFS delivery (Figure 3.3.1[b]) completely inhibited LTP (102 ± 2, n = 6; $p > 0.05$ compared with baseline 100 ± 1%; $p < 0.05$ compared with vehicle injected controls).

Figure 3.3.1(c) shows the effects of acute 5μL i.c.v co- injection of antibody WO1 (100 pmol) + natural oligomer, 15 min prior to HFS delivery. WO1 antibody did not prevent

the inhibition of LTP by the natural oligomers (105 ± 4, n = 5; $p > 0.05$ compared with baseline, 102 ± 2%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was not statistically different from the natural Aβ + WFI group at 3 h ($p > 0.05$).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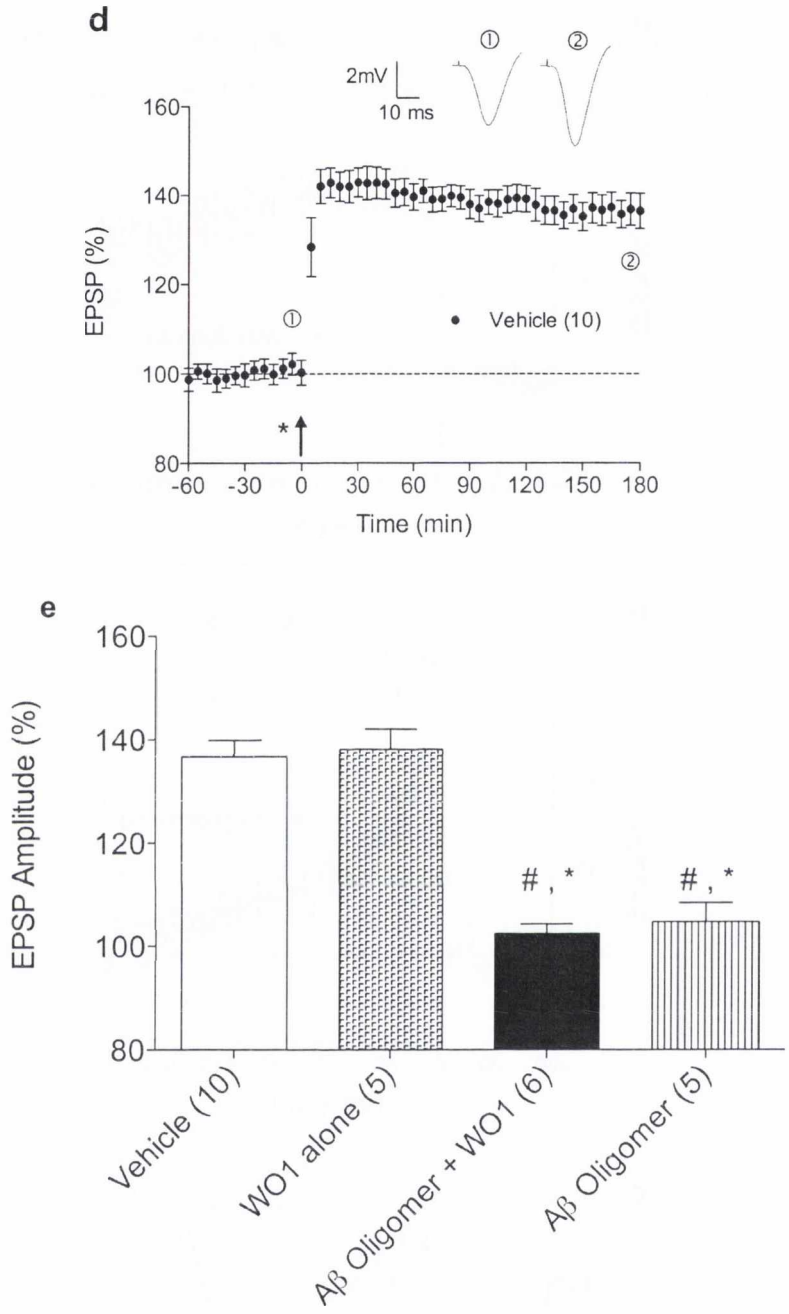
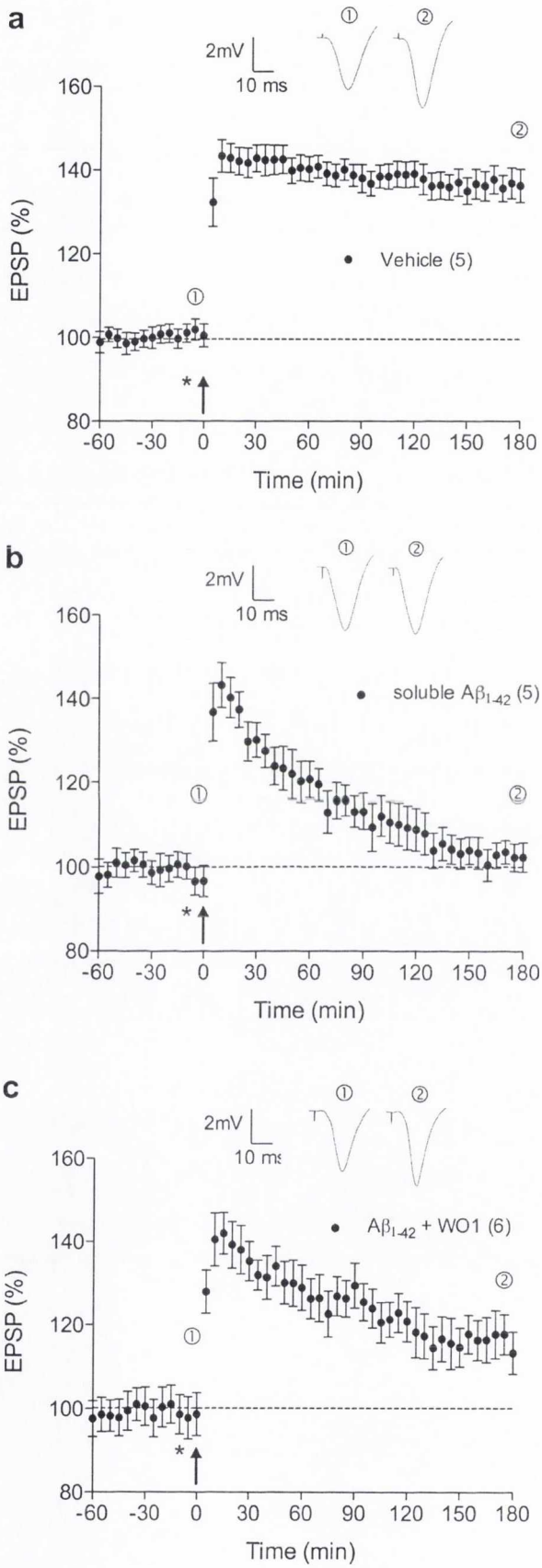


Figure 3.3.1 - The (proto)fibril antibody WO1 does not prevent the inhibition of hippocampal LTP by cell derived (7PA2) Aβ oligomers *in vivo*. **a**, Vehicle controls exhibited LTP for 3 h ($n=10$, $p < 0.05$, compared with baseline). **b**, WO1-injected animals exhibited LTP for 3 h ($n=5$, $p < 0.05$, compared with baseline). **c**, Natural oligomers purified using SEC inhibited hippocampal LTP ($n=5$, $p > 0.05$). **d**, Natural oligomers pre-incubated with WO1 also inhibited LTP ($n=6$, $p > 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **e**, Summary of the effects of the antibody WO1 and natural Aβ oligomers at 3 h post- HFS. There was a statistically significant difference compared with the vehicle group (# , $p < 0.05$) and the WO1 alone group (* , $p < 0.05$).

3.3.2 WO1 partly abrogates the inhibition of LTP by synthetic Aβ

Since we found that the (proto)-fibril antibody WO1 failed to prevent the inhibition of LTP by cell-derived Aβ for comparison we studied the effect of WO1 on soluble synthetic $A\beta_{1-42}$. In the present study animals given vehicle injection 15 min prior to HFS (Figure 3.2.4[a]) displayed LTP for at least 3 h post-HFS (138 ± 4%, n = 5; $p < 0.05$ compared with baseline 103 ± 1%). We determined the effects of WO1 (0.6 mg/ml) on the inhibition of LTP by soluble synthetic $A\beta_{1-42}$ *in vivo*. We pre-incubated the WO1 and $A\beta_{1-42}$ together at room temperature (23°C) 60 min before the i.c.v co-injection. Injection of soluble synthetic $A\beta_{1-42}$ (40 pmol), 15 min prior to HFS delivery (Figure 3.3.2[b]) completely inhibited LTP (103 ± 3, n = 5; $p > 0.05$ compared with baseline 97 ± 1%; $p < 0.05$ compared with vehicle injected controls).

Figure 3.3.2(c) shows the effects of acute 5μL i.c.v co- injection of antibody WO1 (100 pmol) + soluble $A\beta_{1-42}$ (40 pmol), 15 min prior to HFS delivery. WO1 antibody did not completely prevent the inhibition of LTP by the $A\beta_{1-42}$ (116 ± 3, n = 6; $p < 0.05$ compared with baseline 98 ± 3%; $p < 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the $A\beta_{1-42}$ + WFI group at 3 h ($p < 0.05$).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Aβ1-42 + WO1 (6)
EPSP (%)
Time (min)

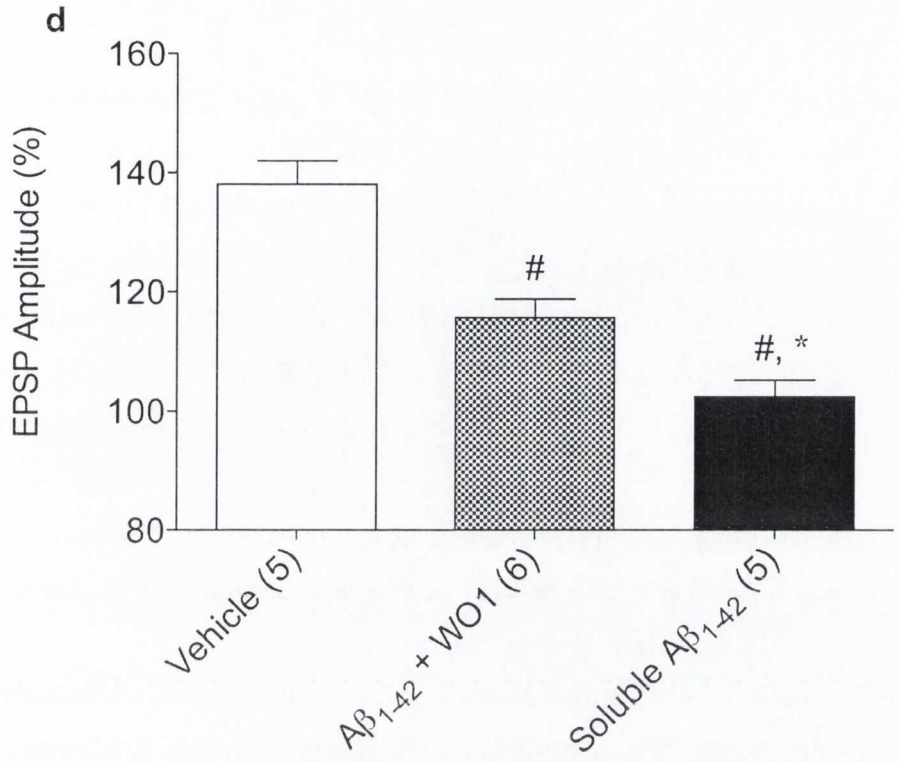
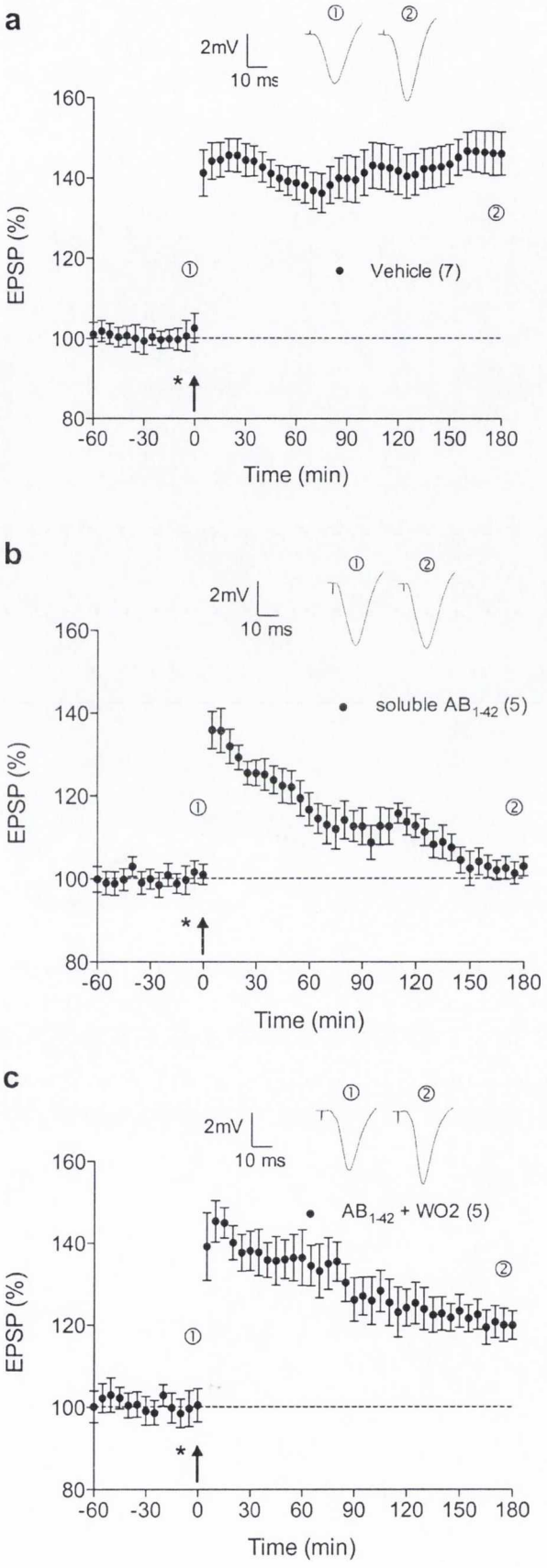


Figure 3.3.2 - The (proto)fibril antibody WO1 partially abrogated the inhibition of hippocampal LTP by soluble synthetic $A\beta_{1-42}$ *in vivo*. **a**, Vehicle controls exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Synthetic Aβ blocked hippocampal LTP (n=5, $p > 0.05$). **c**, $A\beta_{1-42}$ pre-incubated with WO1 partly inhibited LTP (n=6, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d**, Summary of the effects of soluble $A\beta_{1-42}$ preinjected with WO1 (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with synthetic Aβ + WO1 group (* , $p < 0.05$) and the vehicle group (# , $p < 0.05$).

3.3.3 WO2 partly abrogates the inhibition of LTP by synthetic Aβ

Since we found that the (proto)fibril antibody WO1 partially prevented the inhibition of LTP by $A\beta_{1-42}$ for comparison we studied the effect of a second (proto)fibril antibody, WO2, on soluble synthetic $A\beta_{1-42}$. In the present study animals given vehicle injection 15 min prior to HFS (Figure 3.3.3[a]) showed LTP for at least 3 h post-HFS (146 ± 6%, n = 7; $p < 0.05$ compared with baseline 101 ± 2%). We determined the effects of WO2 (1.6 mg/ml) on the inhibition of LTP by soluble synthetic $A\beta_{1-42}$ *in vivo*. We pre-incubated the WO2 and $A\beta_{1-42}$ together at room temperature (23°C) 60 min before the i.c.v co-injection. 5μL injection of soluble synthetic $A\beta_{1-42}$ (40 pmol), 15 min prior to HFS delivery (Figure 3.3.3[b]) completely inhibited LTP (102 ± 1, n = 5; $p > 0.05$ compared with baseline (101 ± 2%); $p < 0.05$ compared with vehicle injected controls).

Figure 3.3.3(c) shows the effects of acute 5μL i.c.v co- injection of antibody WO2 (240 pmol) + soluble $A\beta_{1-42}$ (40 pmol), 15 min prior to HFS delivery. WO2 antibody partially prevented the inhibition of LTP by the $A\beta_{1-42}$ (120 ± 2, n = 5; $p < 0.05$ compared with baseline (100 ± 3%); $p < 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the $A\beta_{1-42}$ + WFI group at 3 h ($p < 0.05$).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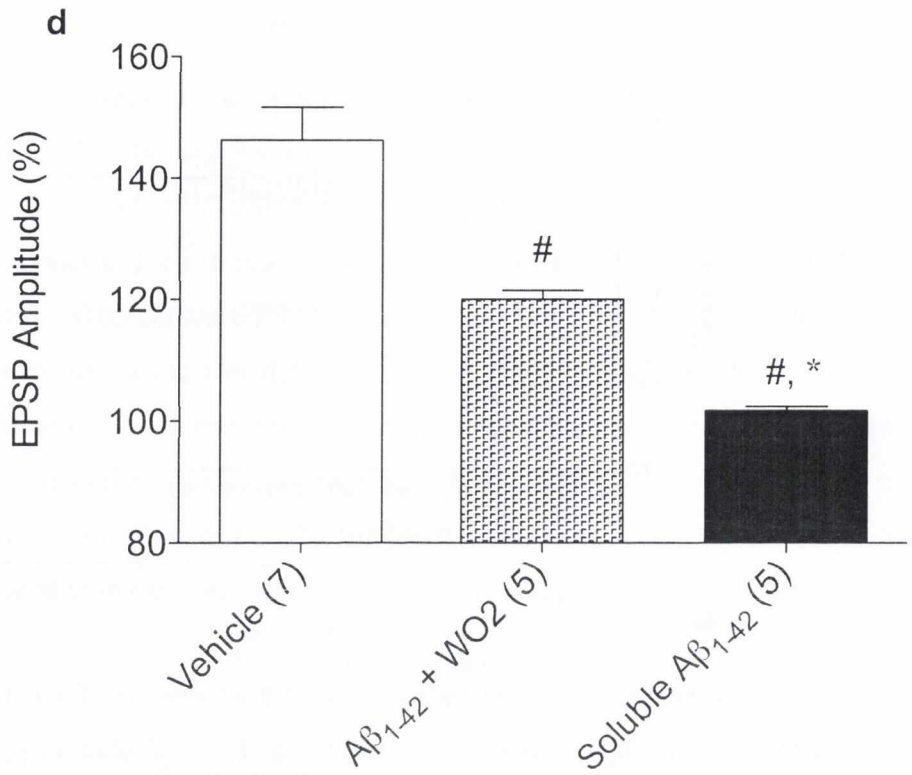


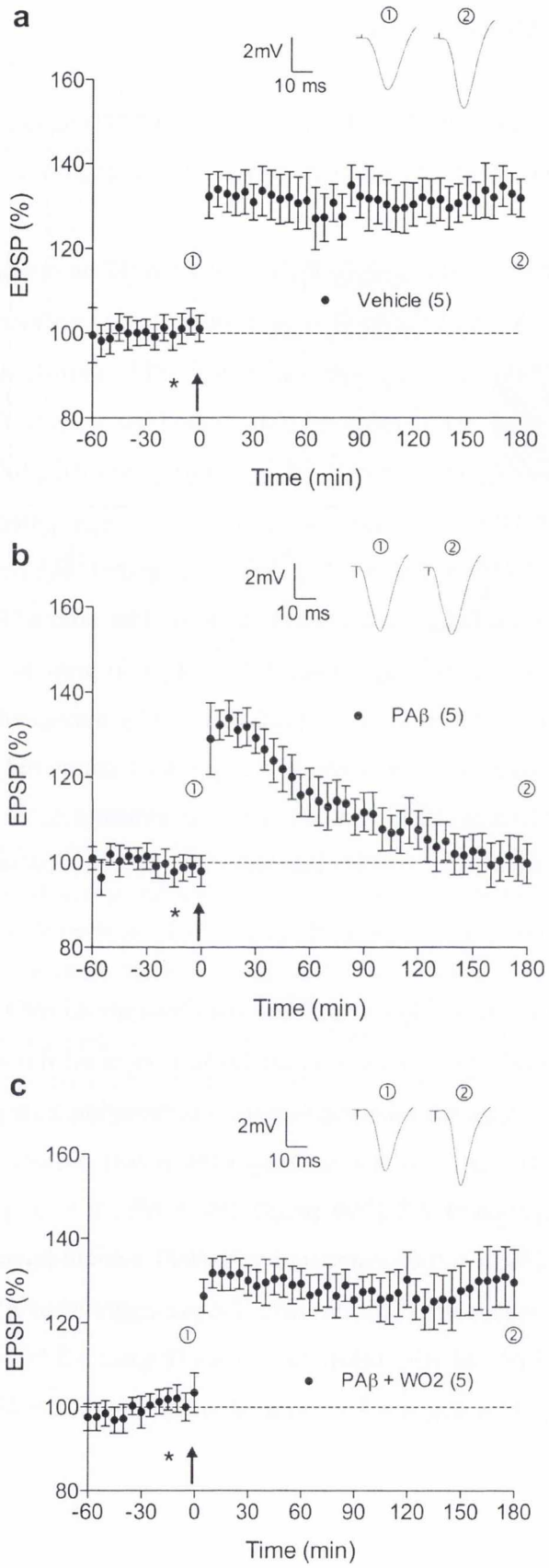
Figure 3.3.3 - The (proto)fibril antibody WO2 partially abrogated the inhibition of hippocampal LTP by soluble synthetic $A\beta_{1-42}$ *in vivo*. **a**, Vehicle controls exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, $A\beta_{1-42}$ pre-incubated with WO2 partially inhibited LTP (n=6, $p < 0.05$). **c**, Synthetic $A\beta_{1-42}$ blocked hippocampal LTP (n=5, $p > 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d**, Summary of the effects of preinjected soluble $A\beta_{1-42}$ and WO2 (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with synthetic Aβ + WO2 (* , $p < 0.05$) and vehicle (# , $p < 0.05$).

3.3.4 The antibody WO2 prevents the inhibition of LTP by pre-aggregated $A\beta_{1-42}$

As previously mentioned, O'Nuallain and Wetzel (2002) reported that WO2 binds to the amyloid fibril state of the Alzheimer's peptide $A\beta_{1-40}$ and not to its monomeric state.

In the present study we determined the effects of WO2 on pre-aggregated synthetic $A\beta_{1-42}$ *in vivo*. Figure 3.3.5(a) shows the results of an acute i.c.v injection of 10μL of vehicle (WFI) control, 15 min prior to HFS delivery. WFI controls demonstrate LTP for 3 h (132 ± 4%, n = 5, $p < 0.05$ compared to baseline (102 ± 2%)). Figure 3.3.5(b) shows the results of acute i.c.v injection of 10μL pre-aggregated Aβ (200 pmol), 15 min prior to the delivery of HFS. The administration of pre-aggregated $A\beta_{1-42}$ fully inhibited hippocampal LTP (100 ± 5%, n = 5; $p > 0.05$ compared with baseline 99 ± 1%; $p < 0.05$ compared with vehicle injected controls at 3 h). The effects of acute i.c.v injection of 10μL of pre-incubated pre-aggregated $A\beta_{1-42}$, 15 min prior to HFS delivery, were then investigated (Figure 3.3.5[c]). WO2 (480 pmol) fully abrogated the inhibitory effects of pre-aggregated Aβ at 3 h (130 ± 8%, n = 5; $p < 0.05$ compared with baseline 102 ± 1%; $p > 0.05$ compared with the vehicle injected controls at 3 h). These animals were statistically different from animals that were injected with pre-aggregated Aβ alone ($p < 0.05$).

We also tested a control IgMκ antibody- the isotype to WO2 (as well as WO1). To determine if co-injection of the control IgMκ prevented the inhibition of LTP by the pre-aggregated $A\beta_{1-42}$, we administered this combination i.c.v prior to HFS. The results in figure 3.3.5(d) show that the antibody (480 pmol) did not prevent the inhibition of LTP by pre-aggregated Aβ (200 pmol) (98 ± 6%, n = 5; $p > 0.05$ compared with baseline 103 ± 1%; $p < 0.05$ compared with WFI vehicle injected controls at 3 h). This group was not statistically different from the pre-aggregated Aβ alone group at 3 h ($p > 0.05$). Animals injected with IgMκ on its own (Figure 3.3.5[e]) showed LTP that lasted for at least 3 h (132 ± 4%, n = 3; compared to baseline 104 ± 4%).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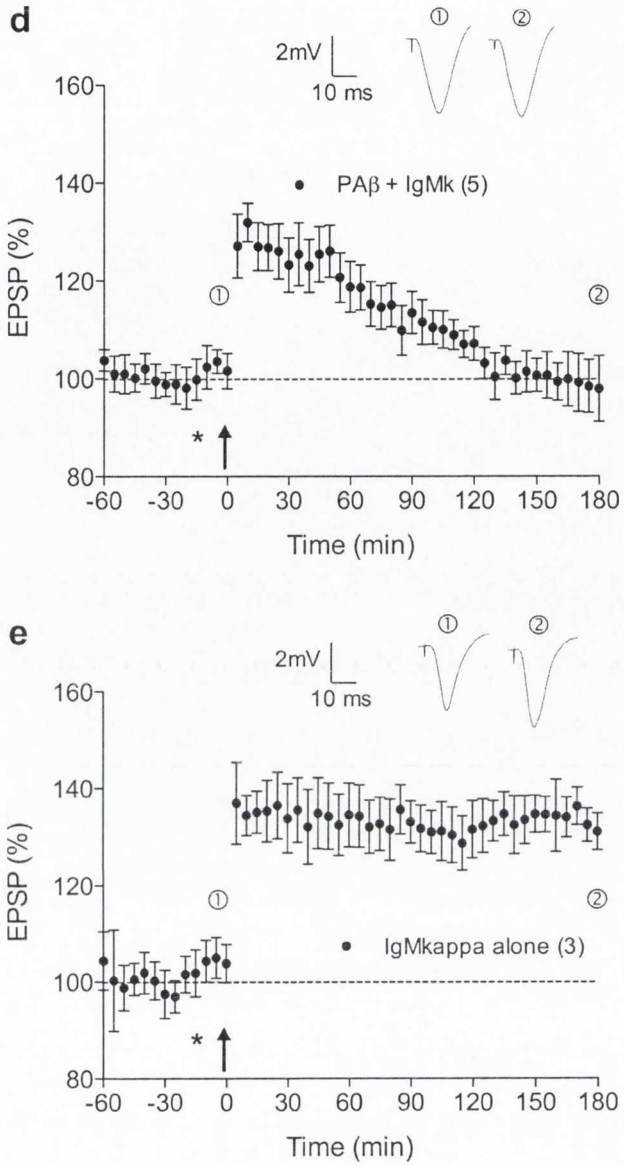
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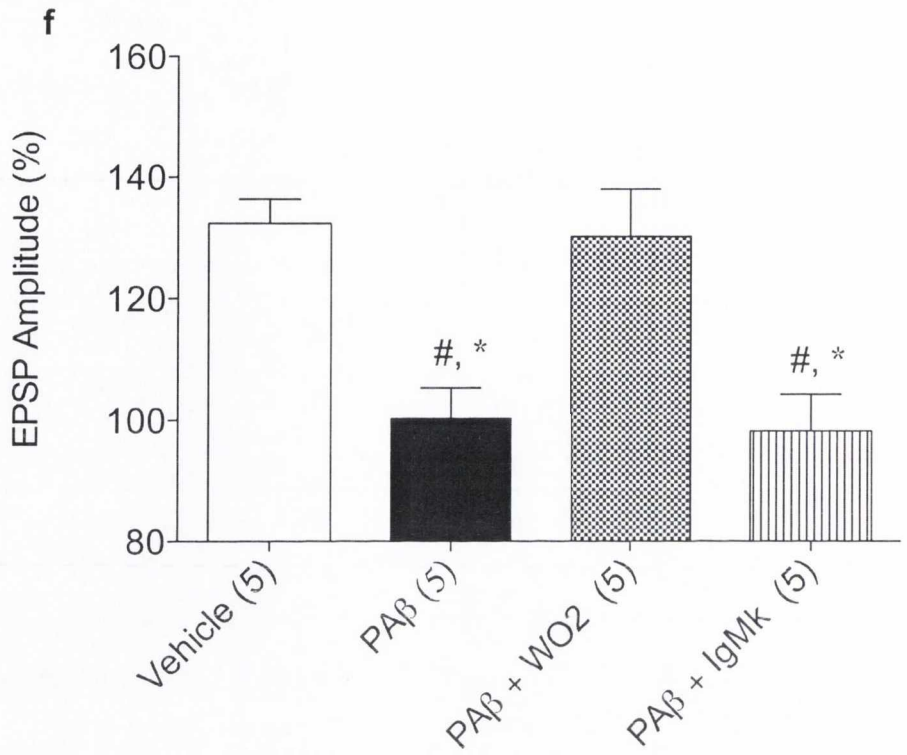


Figure 3.3.4 - The antibody WO2 reversed the inhibition of hippocampal LTP by preaggregated Aβ *in vivo*. **a**, Vehicle controls exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Preaggregated Aβ (PAβ) fully blocked hippocampal LTP at 3 h (n=5, $p > 0.05$). **c**, Pre-incubation WO2 abrogated the block of LTP by PAβ (n=6, $p < 0.05$). **d**, PAβ pre-incubated with IgMκ- a control antibody for WO2- fully blocked LTP (n=6, $p < 0.05$). **e,** Animals injected with the IgMκ control antibody injected on its own exhibited LTP at 3 h (n=5, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **f**, Summary of the effects of WO2 preinjected with preaggregated Aβ. The inhibition of hippocampal LTP by PAβ was abrogated by the (proto)fibril antibody WO2 *in vivo* (post hoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with vehicle (# , $p < 0.05$) and PAβ + WO2 (* , $p < 0.05$).

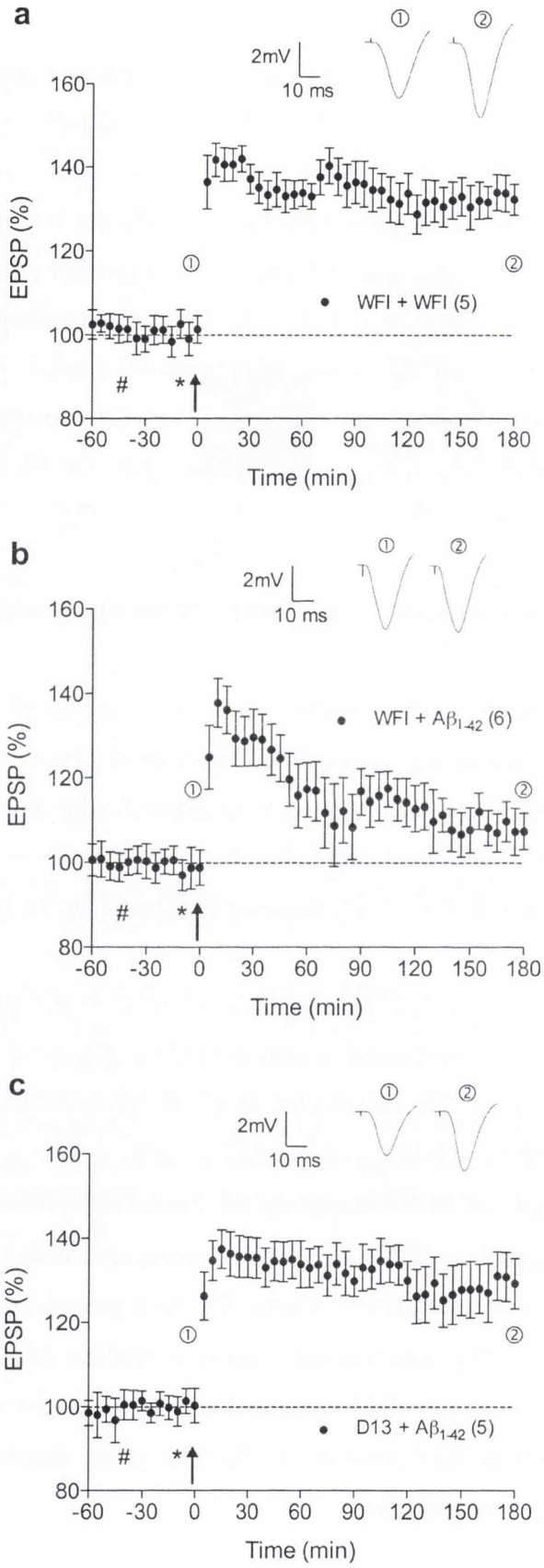
3.4 - Effects of a PrP_{95-105} Fab D13 on inhibition of LTP by Aβ and $PrP_{106-126}$

Recently, Lauren et al. (2009) reported that PrPc acts as a receptor to mediate $A\beta_{1-42}$-oligomer inhibition of LTP in the Schaeffer collateral pathway of the hippocampus *in vitro*. In hippocampal slices from normal mice, $A\beta_{1-42}$ oligomers reduced LTP significantly, but in slices from PrPc-null animals the oligomers had no significant effect. They also employed anti-PrP antibodies to localize $A\beta_{1-42}$ binding within PrPc. The antibody 6D11 blocked the binding of $A\beta_{1-42}$ assemblies to PrPc. The 6D11 blockade was epitope-specific since the 7D9 antibody binds avidly to a different epitope but failed to block $A\beta_{1-42}$ binding. The epitope for 6D11 corresponds to amino acid 93–109 of mouse PrPc, matching their conclusion that the 95–105 region is a primary determinant for binding.

3.4.1 Prevention of inhibition of LTP by $A\beta_{1-42}$ with a preinjection of D13

Monoclonal antibody binding studies have been used to elucidate the structure and function of the various regions of PrPc (Peretz et al., 1997; Williamson et al., 1998). Peretz et al. (2001) showed that monoclonal antibodies binding cell-surface PrPc inhibit PrPSc formation in a dose-dependent manner. Two antibody fragments stood out for their efficacy- Fabs D18 and D13, the latter binding to the 95-105 region of PrPc.

Animals given an acute 5μL i.c.v injection of WFI 30 min after 5μL of WFI (Figure 3.4.1[a]) showed LTP that lasted at least 3 h (133 ± 2%, n = 5; $p < 0.05$ compared with baseline 100 ± 1%). We determined if a 5μL pre-injection of D13 prevented the inhibition of LTP by soluble synthetic $A\beta_{1-42}$. Acute i.c.v injection of $A\beta_{1-42}$ (40 ρmol) 30 min after 5μL of WFI completely inhibited LTP (108 ± 3%, n = 6; $p > 0.05$ compared with baseline 100 ± 2%; $p < 0.05$ compared with vehicle injected controls). Figure 3.4.1(c) shows the effects of acute 5μL i.c.v pre-injection of D13 (165 pmol) 30 min before $A\beta_{1-42}$ (40 ρmol). The D13 Fab prevented the $A\beta_{1-42}$ –mediated inhibition of LTP (130 ± 5%, n = 5; $p < 0.05$ compared with baseline 101 ± 1%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the WFI + $A\beta_{1-42}$ group at 3 h ($p < 0.05$).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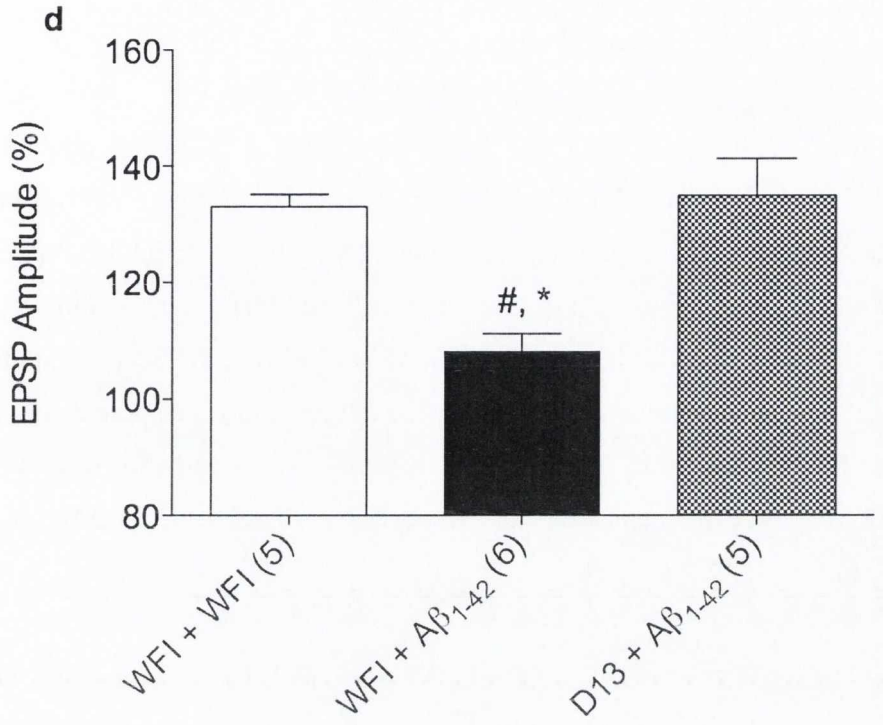


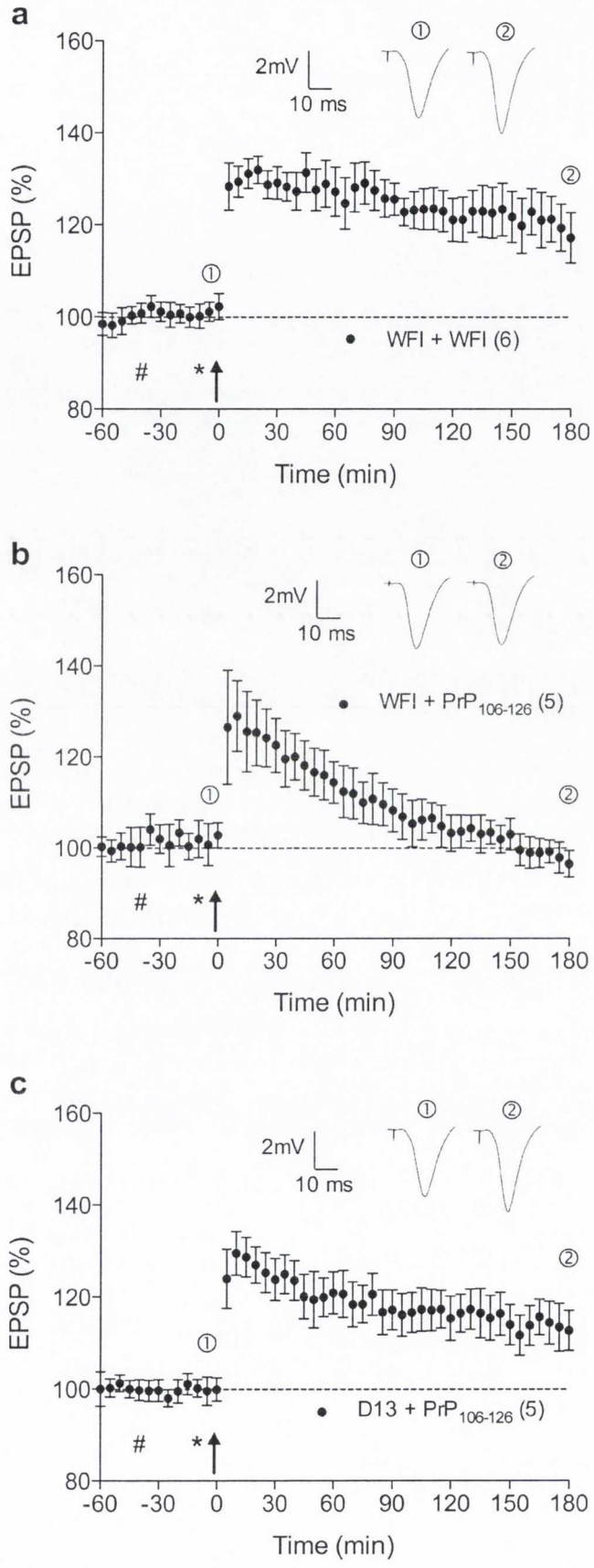
Figure 3.4.1 - The PrP95-105 Fab D13 prevented the inhibitory effect of soluble synthetic $A\beta_{1-42}$ on LTP *in vivo*. **a**, Vehicle controls (WFI + WFI) exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Synthetic $A\beta_{1-42}$ blocked hippocampal LTP (n=6, $p > 0.05$). **c**, A preinjection of D13, 30 min prior to $A\beta_{1-42}$ administration, fully abrogated the inhibition of LTP (n=5, $p < 0.05$). All animals received two injections at (#) 45 and (*) 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d**, Summary of the effects of pretreatment of D13 on $A\beta_{1-42}$ activity (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with the D13 + $A\beta_{1-42}$ group (* , $p < 0.05$) and the vehicle (# , $p < 0.05$).

3.4.2 A preinjection of D13 prevents the inhibition of LTP by prion protein peptide $PrP_{106-126}$

Peretz et al. (2001) examined the ability of several recombinant antibody antigen-binding fragments to inhibit prion propagation in cultured mouse neuroblastoma cells infected with PrPSc. They reported that antibodies binding PrPc inhibit PrPSc formation in a dose-dependent manner. In cells treated with the most potent antibodies, Fabs D18 and D13, prion replication was abolished and pre-existing PrPSc was rapidly cleared, suggesting that this antibody may cure established infection. The potent activity of Fabs D18 and D13 is thought to be associated with their ability to better recognize the total population of PrPc molecules on the cell surface, and with the location of the PrPsc-binding epitope on PrPc.

Here we determined if pre-injection of D13 prevented the inhibition of LTP by prion protein peptide $PrP_{106-126}$. Animals given a vehicle double-injection (10μL WFI + 5μL WFI) protocol (Figure 3.4.1[a]) showed LTP that lasted at least 3 h (116 ± 5%, n = 5; $p < 0.05$ compared with baseline 101 ± 1%). An acute 5μL i.c.v injection of $PrP_{106-126}$ (50 pmol) 30 min after a 10μL WFI pre-injection (Figure 3.4.1[b]) completely inhibited LTP (97 ± 2%, n = 6; $p > 0.05$ compared with baseline 102 ± 1%; $p < 0.05$ compared with vehicle injected controls).

Figure 3.4.1(c) shows the effects of 10μL pre-injection of D13 (330 pmol) 30 min before a 5μL i.c.v injection of $PrP_{106-126}$ (50 pmol). The D13 antibody prevented the $PrP_{106-126}$ –mediated inhibition of LTP (113 ± 5%, n = 5; $p < 0.05$ compared with baseline 100 ± 2%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h. This group was statistically different from the WFI + $PrP_{106-126}$ group at 3 h ($p < 0.05$).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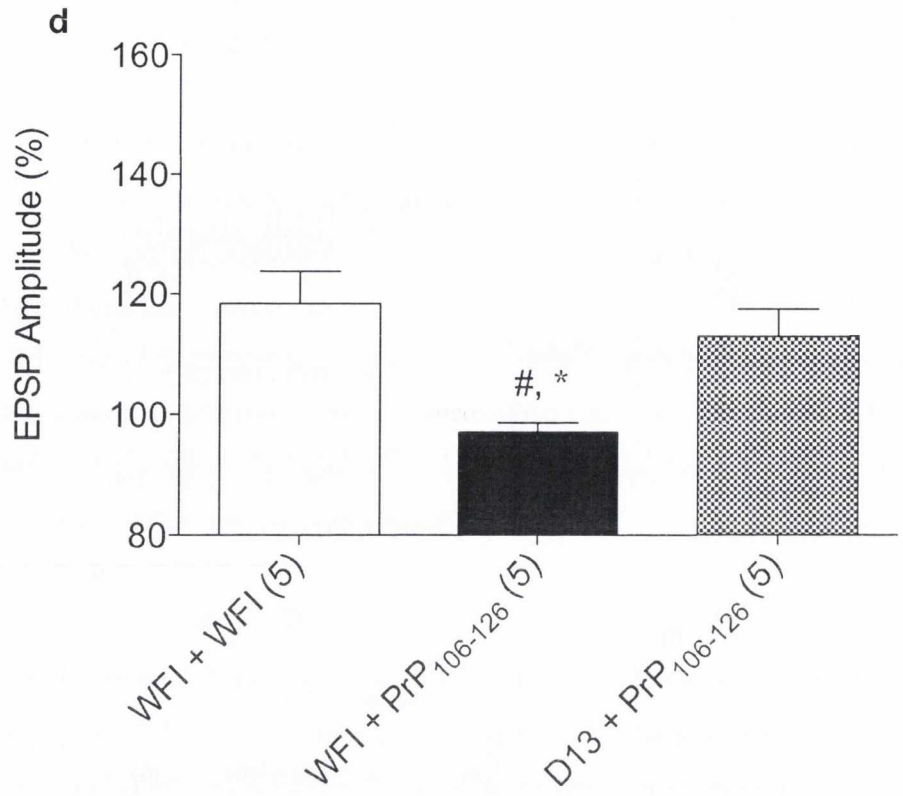


Figure 3.4.2 - The PrP95-105 Fab D13 prevents the inhibitory effect of prion protein peptide $PrP_{106-126}$ on LTP *in vivo*. **a**, Vehicle controls (WFI + WFI) exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Synthetic $PrP_{106-126}$ completely blocked hippocampal LTP (n=6, $p > 0.05$). **c**, A preinjection of D13, 30 min prior to $PrP_{106-126}$ administration, fully prevented the inhibition of LTP (n=5, $p < 0.05$). All animals received two injections at (#) 45 and (*) 15 min prior to HFS (↑). Insets show representative traces of field EPSPs recorded at times 0 (①) and 180 min (②). **d**, Summary of the effects of a preinjection of D13 on $PrP_{106-126}$ activity (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with D13 + $PrP_{106-126}$ (* , $p < 0.05$) and WFI + WFI (# , $p < 0.05$).

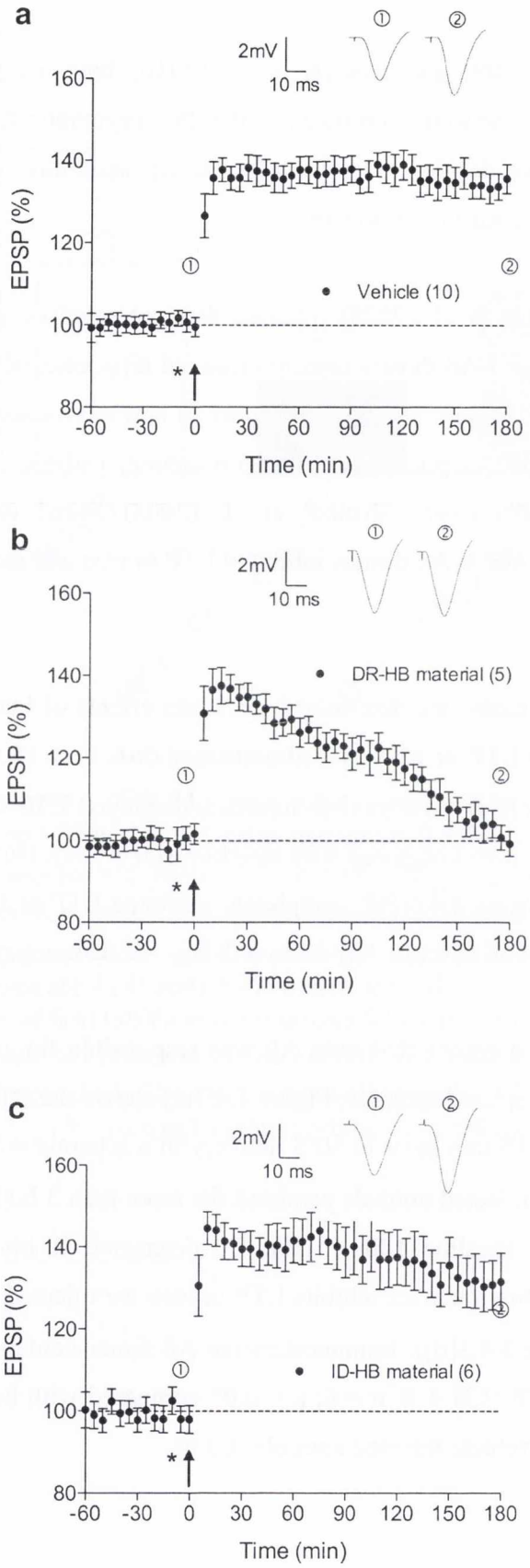
3.4.3 Aβ dimer-containing AD brain extract inhibits LTP *in vivo*

Lauren et al. (2009) and Kessels et al. (2010) have recently reported seemingly conflicting results as to the significance of PrPc in synthetic Aβ –mediated inhibition of *in vitro* LTP. We decided to test a different Aβ assembly- namely human AD brain derived dimers in our *in vivo* model.

Recently, Klyubin et al. (2008) reported that untreated *ex vivo* human cerebrospinal fluid that contained Aβ dimers rapidly inhibited hippocampal long-term potentiation *in vivo*, and that an acute systemic infusion of an anti-Aβ monoclonal antibody prevented this disruption of synaptic plasticity. Aβ monomer isolated from human CSF did not affect LTP. Futhermore, Shankar et al. (2008) found that human brain extract containing SDS-stable Aβ dimers inhibited LTP *in vivo* and disrupted passive avoidance learning.

First, in a pilot study, we determined the acute effects of human Aβ dimer-containing brain extract on LTP *in vivo*. We administered 5μL i.c.v 15 min prior to HFS (Figure 3.4.3[b]). Animals given a vehicle injection displayed LTP that lasted for 3 h (135 ± 3%, n = 10; $p < 0.05$ compared with baseline 100 ± 1%). Human Aβ dimer-containing brain extract (Figure 4.4.3 [a]) completely inhibited LTP at 3 h (100 ± 2%, n = 5; $p < 0.05$ compared with baseline 101 ± 1% at 3 h; $p < 0.05$ compared to vehicle).

Next, in order to ensure that only Aβ was responsible for inhibition of LTP we also tested immunodepleted samples. Figure 3.4.3(c) shows the effect of acute i.c.v injection of 5μL of WFI 15 min prior to HFS delivery in a separate control group. HFS-induced LTP in vehicle-injected animals persisted for more than 3 h (135 ± 3%, n = 5; $p < 0.05$ compared with baseline 100 ± 1%). To determine if immunodepleted Aβ dimer-containing AD brain extract inhibits LTP *in vivo* we injected 5μL 15 min prior to HFS delivery (Figure 3.4.3[d]). Immunodepleted Aβ dimer-containing AD brain extract had no effect on LTP (131 ± 6, n = 6; $p < 0.05$ compared with baseline 98 ± 2%; $p > 0.05$ compared with vehicle injected controls at 3 h).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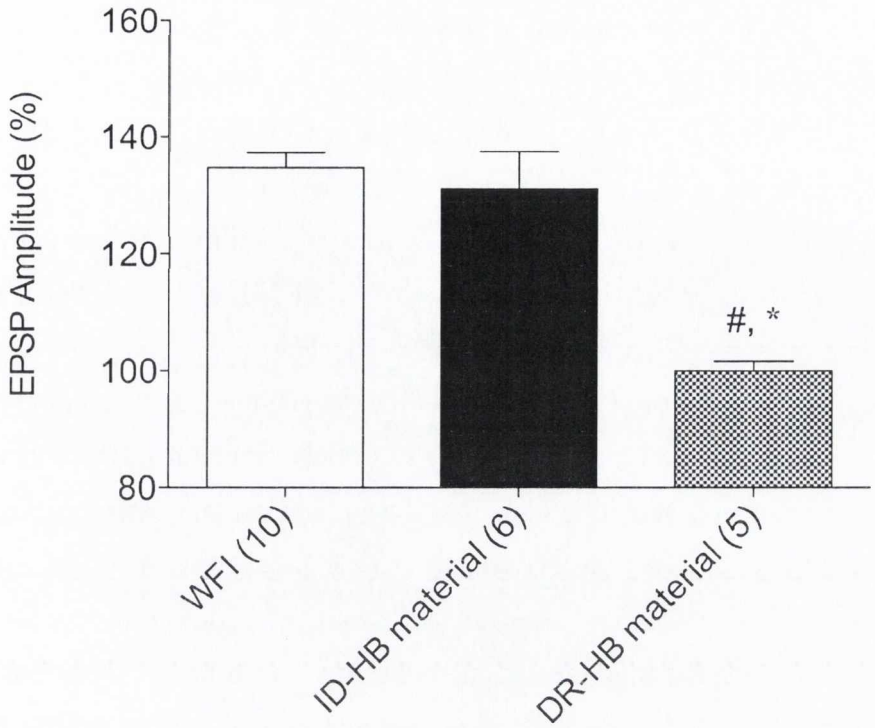
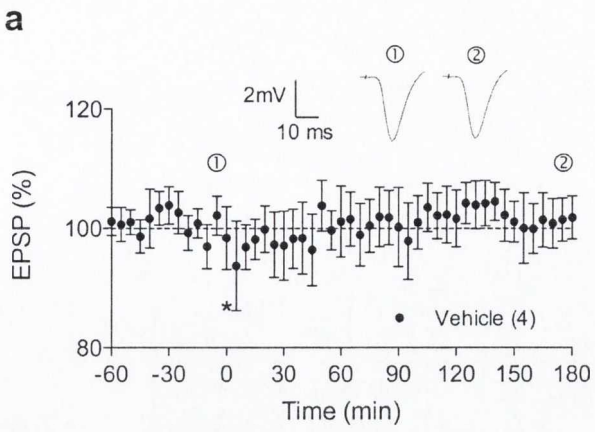


Figure 3.4.3 - Aβ dimer-containing AD human brain extraxt blocked hippocampal LTP *in vivo* at 3 h post- HFS. **a**, Vehicle control animals exhibited robust hippocampal LTP for 3 h ($n = 10$, $p < 0.05$, compared with baseline). **b**, Aβ dimer-containing human brain extract (DR-HB) completely blocked hippocampal LTP ($n = 5$, $p > 0.05$). **c**, Immunodepleted human brain (ID-HB) injected animals exhibited LTP for 3 h ($n = 5$, $p < 0.05$). All animals received an injection (*) at 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). Summary of the effects of dimer-rich human brain material and its immunodepleted form (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with WFI (* , $p < 0.05$) and immunodepleted human brain material (# , $p < 0.05$).

3.4.4 Aβ dimer-containing AD brain extract had no effect on hippocampal baseline synaptic transmission

In the present study, we determined the effects of i.c.v injection of Aβ dimer-containing AD human brain extract preparations on baseline synaptic transmission. We administered solutions at time 0 min; e.g. when HFS would have been delivered. Figure 3.4.4(a) shows the results of acute i.c.v injection of 5μL of WFI. Vehicle injection had no effect on baseline (102 ± 2%, 3 h post-injection, n = 4; $p > 0.05$ compared with baseline 100 ± 2%). To determine if Aβ dimer-containing AD human brain extract had an effect on baseline at the dose used (≈250 fmol) to inhibit LTP we administered an acute i.c.v injection of 5μL (Fig 3.4.4[c]). The human brain extract had no significant effect on baseline at 3 h after injection (103 ± 2%, n = 4; $p > 0.05$ compared with baseline 102 ± 1%; $p > 0.05$ compared to vehicle). As a control we also tested the effect of Aβ-immunodepleted Aβ dimer-containing AD human brain extract had on baseline. I.c.v injection of 5μL (Fig 3.4.4[b]) of the immunodepleted human brain extract also had no significant effect on baseline at 3 h after injection (102 ± 2%, n = 4; $p > 0.05$ compared with baseline 100 ± 1%; $p > 0.05$ compared to vehicle). This group was not statistically different from the human brain extract group at 3 h ($p > 0.05$).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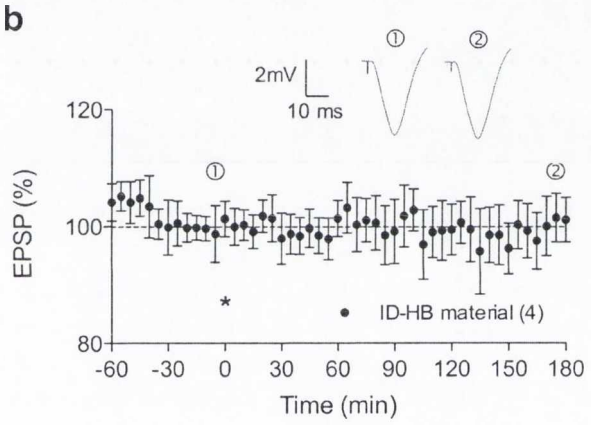
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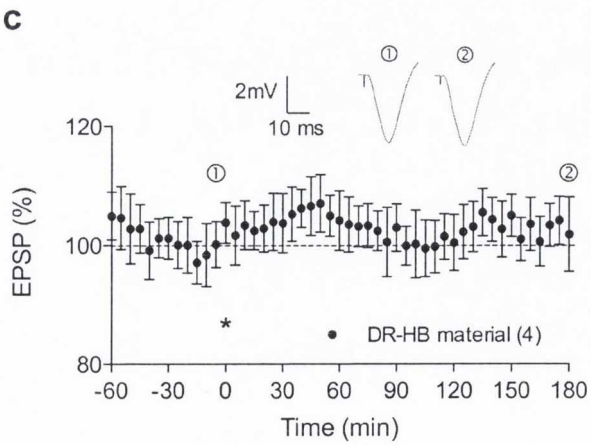
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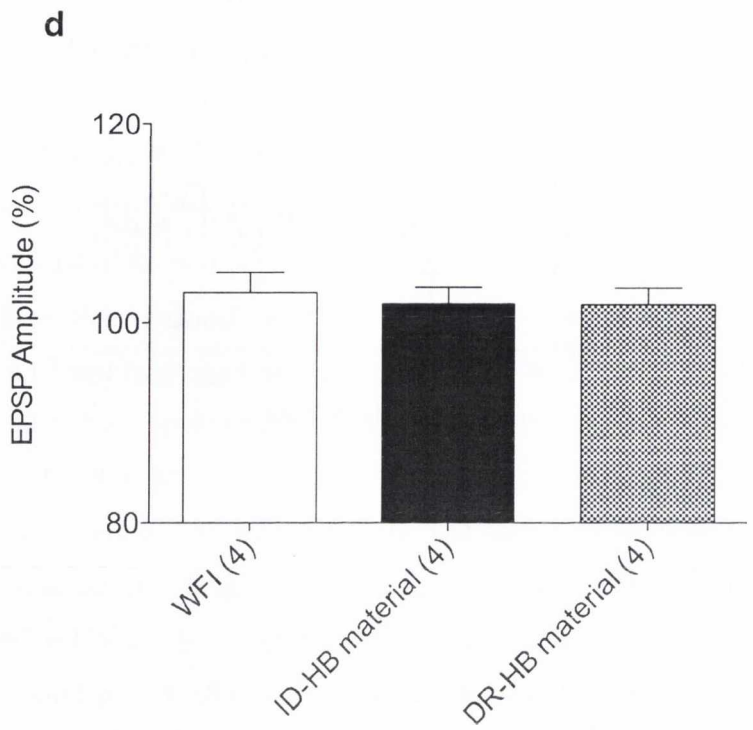
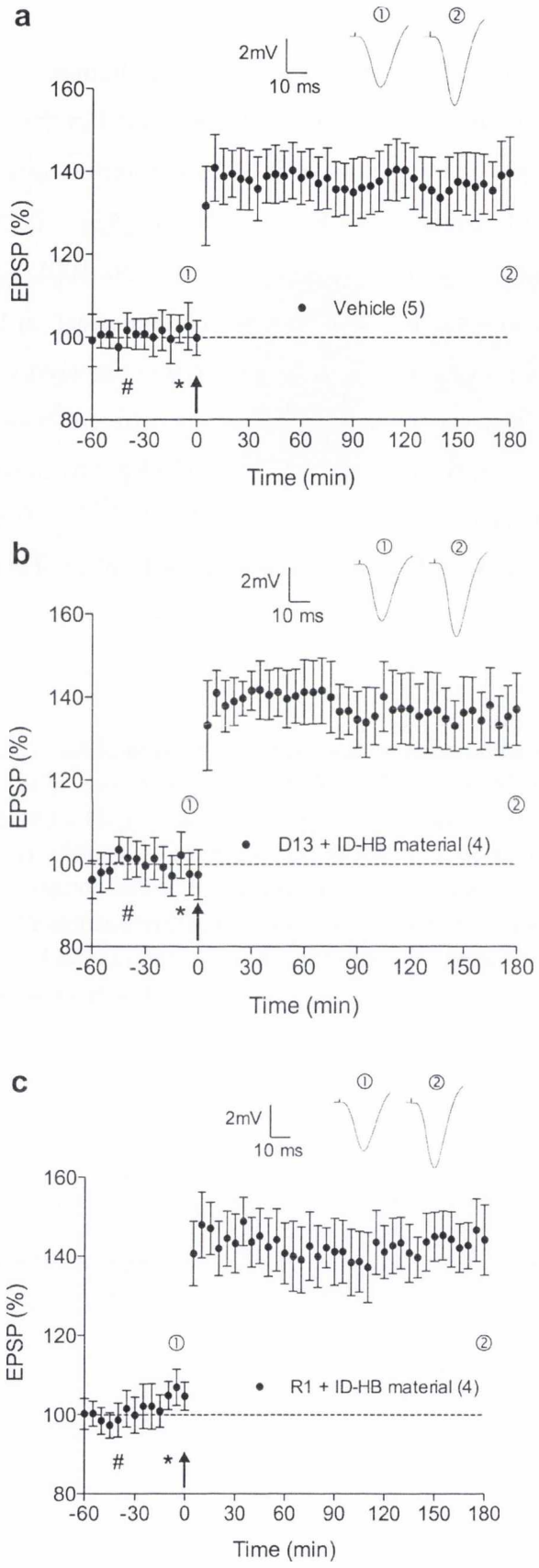


Figure 3.4.4 - Dimer-rich human brain material had no effect on hippocampal baseline synaptic transmission *in vivo*. **a**, Vehicle controls exhibited stable basline transmission for 3 h (n=4, compared with pre-injection). **b**, Immunodepleted dimer-enriched human brain material (ID-HB) had no effect on baseline at 3 h (n=4, $p>0.05$ compared with vehicle). **c**, Dimer-enriched human brain material (DR-HB) had no effect on baseline at 3 h (n=4, $p > 0.05$). All animals received an injection (*) at 0 min. Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d**, Summary of results at 3 h post-injection.

3.4.5 The Fabs D13 and R1 have no effect on hippocampal LTP

We investigated the effects of D13 and R1 (an alternate sequence on cellular prion protein (220-231) Fabs on their own at the dose used in the previous studies. Animals given an acute 5μL i.c.v injection of WFI 30 min after 10μL of WFI (Figure 3.4.5[a]) showed LTP that lasted at least 3 h (139 ± 8%, n = 5; $p < 0.05$ compared with baseline 101 ± 2%). Figure 3.4.5(b) shows the effects of acute 10μL i.c.v injection of D13 (330 pmol) 30 min before vehicle. The D13 Fab had no effect on LTP (136 ± 7, n = 5; $p < 0.05$ compared with baseline 98 ± 3%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was not statistically different from the WFI + WFI group at 3 h ($p > 0.05$). A 10μL pre-injection of R1 (330 pmol) 30 min prior to 5μL of WFI (Figure 3.4.5[c]) had no effect on LTP (145 ± 9%, n = 4; $p < 0.05$ compared with baseline 106 ± 2%; $p > 0.05$ compared with vehicle injected controls).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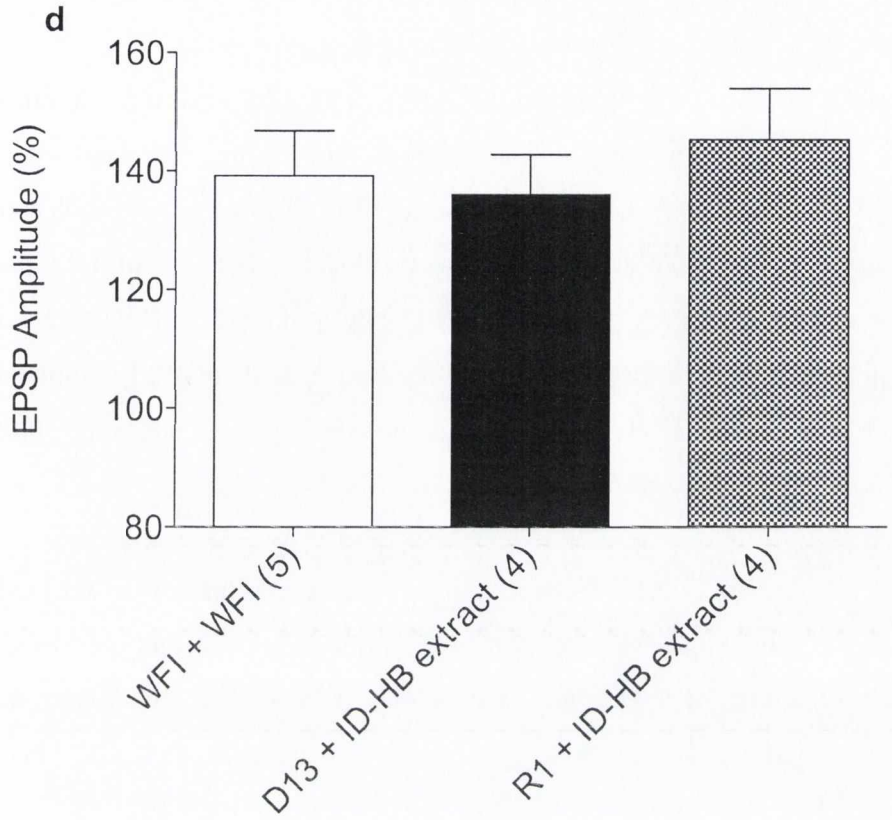


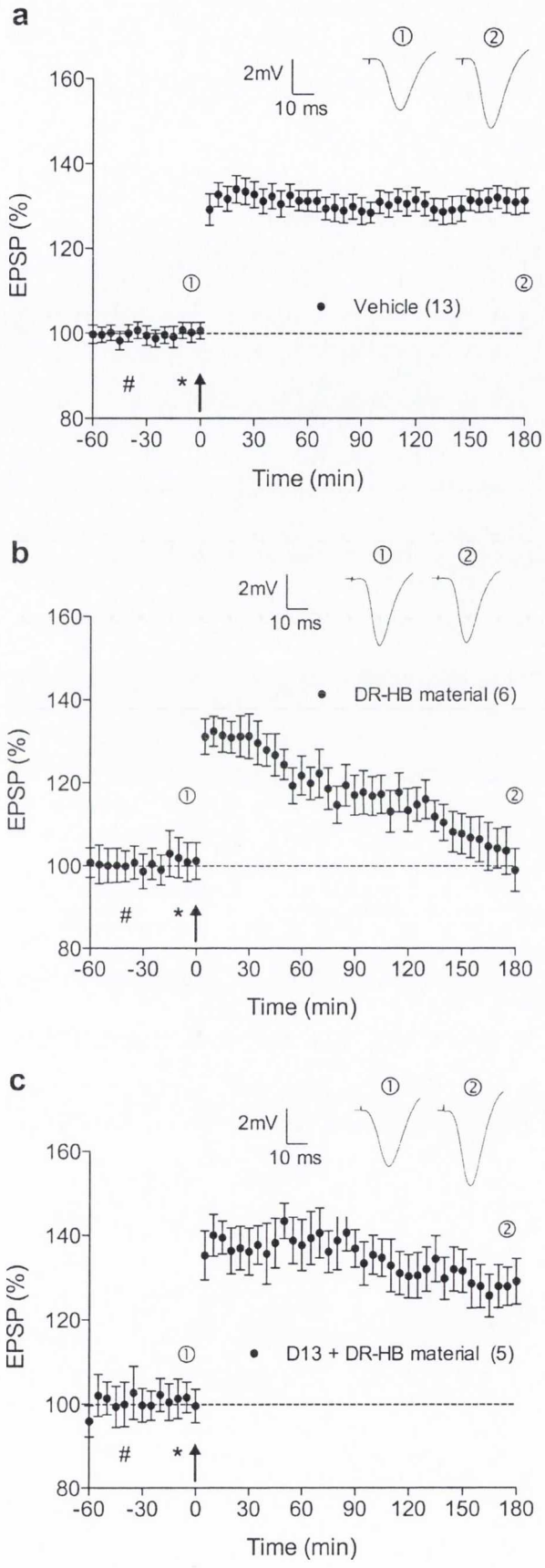
Figure 3.4.5 - The Fabs D13 and R1 had no effect on hippocampal LTP *in vivo*. **a**, Animals given vehicle (WFI + WFI) exhibited LTP for 3 h (n=5, $p < 0.05$, compared with baseline). **b**, Animals preinjected with D13, 30 min prior to immunodepleted human brain material (ID-HB) administration, showed LTP lasting for at least 3 h (n=4, $p < 0.05$). **c**, Animals that received preinjection of R1, 30 min prior to ID-HB administration, similarly showed LTP lasting for 3 h (n=4, $p < 0.05$). All animals received two injections at (#) 45 and (*) 15 min prior to HFS (↑). Insets show representative traces of field EPSPs receorded at times 0 (①) and 180 min (②). **d**, Summary effects of the injection of Fabs D13 and R1 at 3 h.

3.4.6 A preinjection of D13 abrogates the inhibition of LTP by Aβ dimer-containing AD brain extract

In these experiments we examined the ability of D13 to prevent the inhibition of LTP. Animals given an acute 5μL i.c.v injection of WFI 30 min after 10μL of WFI (Figure 3.4.6[a]) showed LTP that lasted at least 3 h (131 ± 2%, n = 13; $p < 0.05$ compared with baseline 100 ± 1%). We determined if a 10μL pre-injection of D13 prevented the inhibition of LTP by Aβ dimer-enriched human brain extract (Figure 3.4.6[b]). Acute i.c.v injection of dimers (≈250 fmol) 30 min after 10μL of WFI completely inhibited LTP (101 ± 4%, n = 6; $p > 0.05$ compared with baseline 101 ± 2%; $p < 0.05$ compared with vehicle injected controls).

Figure 3.4.6(c) shows the effects of acute 10μL i.c.v pre-injection of D13 (330 pmol) 30 min before 5μL human brain dimers (≈250 fmol). The D13 Fab prevented the dimer–mediated inhibition of LTP (128 ± 2, n = 5; $p < 0.05$ compared with baseline 100 ± 1%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was statistically different from the WFI + dimer group at 3 h ($p < 0.05$).

We also tested a control Fab (R1) to an amino sequence located at residues 220 to 231 of PrPc (Leclerc et al., 2003). Figure 3.4.6(d) shows the effects of acute 10μL i.c.v pre-injection of R1 (330 pmol) 30 min before 5μL human brain dimers (≈250 fmol). The R1 Fab failed to prevent the dimer–mediated inhibition of LTP (107 ± 6, n = 5; $p < 0.05$ compared with baseline 100 ± 1%; $p > 0.05$ compared with WFI vehicle injected controls at 3 h). This group was not statistically different from the WFI + dimer group at 3 h ($p > 0.05$).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*
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Time (min)
b
2mV
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DR-HB material (6)
EPSP (%)
Time (min)
c
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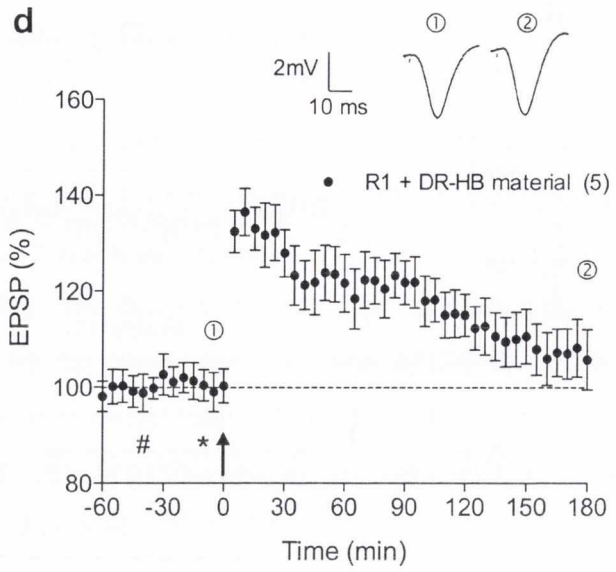
D13 + DR-HB material (5)
EPSP (%)
Time (min)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R1 + DR-HB material (5)
EPSP (%)
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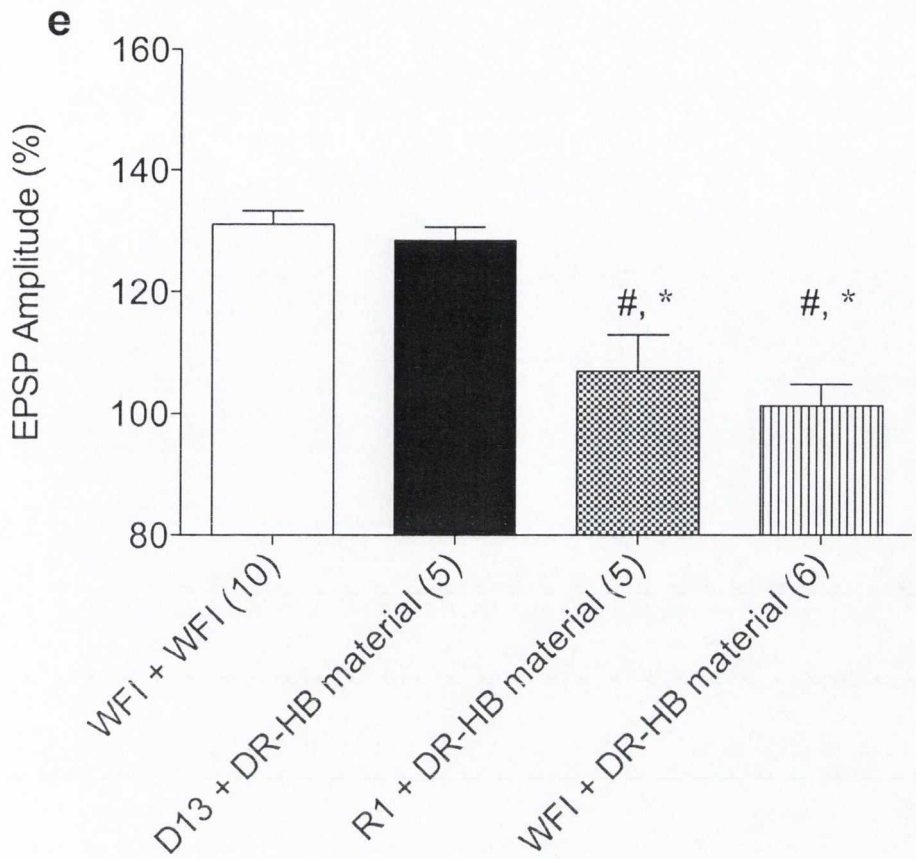

Figure 3.4.6 - The Fab D13, but not the control Fab R1, prevents the inhibitory effect of dimer-rich human brain material (DR-HB) *in vivo* at 3 h post- HFS. **a**,Vehicle controls (WFI + WFI) exhibited LTP for 3 h ($n = 13$, $p < 0.05$, compared with baseline). **b**, WFI + DR-HB fully blocked hippocampal LTP ($n = 6$, $p > 0.05$). **c**, A preinjection of D13, 30 min prior to DR-HB administration, fully prevented the inhibition of LTP ($n = 5$, $p < 0.05$,). **d**, A preinjection of a control Fab R1, 30 min prior to DR-HB administration, failed to prevent the inhibition of LTP ($n = 5$, $p > 0.05$,). All animals received two injections at (#) 45 and (*) 15 min prior to HFS (↑). Insets show representative traces of field EPSPs recorded at times 0 (①) and 180 min (②). **e**, Summary of the effect of pre-treatment of D13 on DR-HB activity (posthoc intergroup comparisons) at 3 h post- HFS. There was a statistically significant difference compared with WFI + WFI (# , $p < 0.05$) and D13 + DR-HB (* , $p < 0.05$) .

Chapter 4 Discussion

4.1 Natural and synthetic soluble Aβ oligomers

Since the establishment of the amyloid cascade hypothesis, significant research effort has been focused on the identification of specific Aβ assemblies in the brain that could be related to AD. In spite of this, it remains unclear exactly what happens to Aβ *in vivo* after it is cleaved from the APP by γ-secretase. It is known that the carboxy-terminal heterogeneity generated by γ-secretase may be an important contributing factor since *in vitro* preparations of the two major peptide fragments generated, $A\beta_{1-42}$ and $A\beta_{1-40}$, display a marked difference in neurotoxicity in a range of assays and this correlates with a clear difference in aggregation behavior (Jan et al., 2008). It is important to note that synthetic Aβ assemblies are highly dynamic. Synthetic Aβ fibrillogenesis is dependent on concentration and physiological parameters such as temperature (Harper and Lansbury, 1997). At body temperature and concentrations > 10-20 μM, synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ self-assembles to form a mix of low-n oligomers, protofibrils and fibrils (Walsh and Selkoe, 2004). Some forms of synthetic Aβ seem to behave like true fibril intermediates- they can form fibrils and dissociate into lower-n assemblies (Harper et al., 1999; Walsh et al., 1999).

In the present study we tested soluble synthetic $A\beta_{1-42}$, and confirmed what has previously been reported (e.g. Cullen et al., 1997; Lambert et al., 1998; Minogue et al., 2003), that synthetic soluble Aβ, at low concentrations, inhibits hippocampal LTP, with no statistically significant effect on baseline excitatory transmission (Figure 3.2.1). Klyubin et al., (2004) also examined the effects of synthetic Aβ derived from a human mutant-APP (G22 Arctic variant) on hippocampal potentiation *in vivo*. They found that a low dose (5 pmol) of soluble fraction of mutant $A\beta_{1-40}$ potently inhibited LTP. Hu et al., (2008) recently reported results of experiments using soluble synthetic $A\beta_{1-40}$ dimers from $A\beta_{1-40}$ S26C mutant-APP that rapidly inhibited LTP at relatively low doses of Aβ (10-42 pmol). However, the concentrations of synthetic soluble Aβ used in all of these experiments are not considered to be in the physiological range, reinforcing the need for studies of the effects of more naturally derived Aβ assemblies.

In the present study we compared the ability of oligomer-enriched fractions of 7PA2 CM from a previously described 1-step SEC alone protocol (Walsh et al. 2002) and a 2-step protocol SEC combined with IE. Because of the heterogeneity of non- Aβ proteins present in 7PA2 CM it is necessary to determine if they are important in the synaptotoxic effects of the Aβ oligomers. Consistent with what had been previously reported with the SEC alone, the SEC + IE fractions containing Aβ oligomers inhibited LTP in the rat hippocampus (Figure 3.1.2). The monomeric fraction failed to inhibit LTP, thus confirming that monomeric Aβ does not impair synaptic plasticity (Figure 3.1.2). Neither oligomer nor monomer fractions prepared using the two-step purification protocols had a statistically significant effect on baseline transmission at 3 h (Figure 3.1.1) consistent with previous experiments from this laboratory using the 1-step purification protocol (Klyubin et al., 2005).

From a research perspective, these cell-derived, low-n oligomers have several advantages over synthetic Aβ aggregates, most notably their natural production by these and other cells at low- to sub-nanomolar concentrations (total Aβ concentration of CM ≈ 2.6 ng/ml, of which ≈ 15% known to be $A\beta_{42}$). Importantly, the CHO-derived oligomers are entirely soluble: centrifuging the CM at >100,000 g for >1 h still leaves all of the monomers and oligomers in the supernatant. Furthermore, in striking contrast to the properties of various synthetic assemblies, Aβ peptides that are generated *in vivo* by humans and lower mammals or by cultured cells are diverse with regard to their N- and C-termini, occur naturally in extracellular fluids at ≤ 5 nmol concentrations (Gravina et al., 1995; Scheuner et al., 1996; Naslund et al., 2000; Walsh et al., 2002) and contain metastable dimers, trimers and higher oligomers while still at low nanomolar levels (Podlisney, 1998; Walsh et al., 2000). SDS-stable oligomers of varying sizes have been detected by Western blotting in APP transgenic mouse brain and human brain (Selkoe, 2008).

In the present study we tested a 4-step purification protocol (see Methods 2.4.4) that contained separated Aβ monomers and dimers, in our *in vivo* model. Results extend what has been previously reported (Klyubin et al., 2008; Shankar et al., 2008) that very low concentrations of natural Aβ dimers inhibit LTP in the rat hippocampus (Figure 3.1.3). Importantly in the present study, all other proteins of a similar size range to the dimers had been removed. Again, the monomer-containing fraction failed to inhibit

LTP, thus confirming that monomeric Aβ does not impair synaptic plasticity. Many laboratories have been hunting for a specific molecular assembly of defined size that is the main trigger of AD. In 2008, Dr Walsh's laboratory identified an enrichment of SDS-stable Aβ dimers in certain human CSF-samples (Klyubin et al., 2008). Injection of human CSF containing Aβ dimers, with no detectible higher-n Aβ oligomers, into animals completely abolished LTP. This adverse effect could be reversed by the systemic infusion of an Ab directed to the mid region of Aβ, 4G8. CSF samples that contained only Aβ monomer and no detectable dimer did not inhibit LTP. At this time it was also recognized that the isolation of large quantities of the SDS-stable dimer from human CSF was difficult, and a synthetic, disulfide stabilized Aβ dimer ($Aβ_{1-40}$Ser26Cys) was prepared (Xia et al., 2009) and used to further explore any detrimental effects of Aβ dimers on synaptic activity (Shankar et al., 2008). Studies using a combined approach of immunoblotting and western blotting techniques to study the Aβ population in J20 mice carrying Swedish and Indiana mutations in APP (Shankar et al, 2009) showed that before SDS-stable dimers can be detected, TBS and triton-insoluble Aβ aggregates are present, but also suggest that the assembly of Aβ assemblies throughout life is dynamic and heterogeneous. The authors concluded that based on transgenic APP mouse models alone, it would be difficult to attribute synaptotoxicity to one single Aβ assembly. Our *in vivo* results confirm and extend the recent literature implicating Aβ dimers as the smallest neurotoxic assemblies.

We validated our findings with a sample of purified cell-derived Aβ dimers, from a slightly different 4-step protocol (using different immunoaffinity- step #2; Methods 2.4.4), from the same 7PA2 cells. Again, in these experiments, small aliquots containing low- or subnanomolar concentrations of soluble dimers were found to inhibit LTP *in vivo* (Figure 3.1.4). Importantly, when co-injected with a well-characterized anti-Aβ antibody (6E10) the dimers failed to inhibit LTP. O'Nuallain et al., (2010) used synthetic disulfide cross-linked Aβ dimers to generate conformation-specific IgM monoclonal antibodies (mAbs)- providing a proof of principal of the usefulness of covalent dimers as immunogens. In our current study 6E10's ability to abolish the synaptic plasticity disrupting effects of dimeric Aβ *in vivo* suggests that the Ab neutralizes pathogenically relevant Aβ assemblies.

Through a series of systematic studies of soluble oligomers of human Aβ secreted by cultured cells, we have documented that low-n oligomers – but not monomers from the same source – can inhibit LTP without affecting basal synaptic transmission. We interpret these data to signify that small diffusible oligomers impair synaptic function on neurons in the hippocampus. Immunotherapy against Aβ is a promising treatment of AD, but the development of therapy has been hampered by an inability to define the optimal Aβ assembly to target. Evidence is accumulating that SDS-stable Aβ dimers may be the basic building blocks of AD-associated synaptotoxic assemblies. Therefore, targeting dimers may deliver the maximum therapeutic benefit and detection of dimers may prove useful for diagnosis of AD (Villemagne et al., 2010).

4.2 The anti-oligomer antibody A11

Charles Glabe and colleagues (Kayed et al., 2003) have developed a remarkably selective polyclonal antibody (named A11), produced in rabbits by immunization with a molecular mimic of oligomeric Aβ, which recognizes oligomers of different proteins. This pan-oligomer Ab is able to detect accumulations of these molecules even in tissue sections from the AD brain. Based on prior experimental evidence suggesting that soluble oligomeric Aβ exists as protein micelles (Soreghan et al., 1994), Dr. Glabe's laboratory generated an antigen that displayed many of the physical properties of synthetic Aβ oligomers, but was significantly more stable. Antibodies raised to this antigen specifically recognized Aβ oligomers, but not fibrils or monomer. Temporal analysis of *in vitro* aggregation of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ by EM and dot blot with A11 revealed that the appearance of ADDLs and prefibrillar assemblies were coincident with A11 immunoreactivity, and suggest that both ADDLs (trimer to dodecamer *in vitro* and *in vivo*) and prefibrillar assemblies share a common structural epitope (Glabe, 2008).

The present findings provide evidence that the pan-oligomer antibody A11 has the ability to prevent Aβ-induced impairment of LTP *in vivo*. This effect was seen following the co-incubation and co-injection of A11 and naturally derived soluble Aβ 60 min prior to injection. An i.c.v administration of A11 alone had no effect on hippocampal LTP (Figure 3.2.2). Here, we tested A11's effect on the disruptive action of synthetic soluble Aβ and natural Aβ oligomers. Natural Aβ oligomers failed to inhibit LTP when incubated with A11 and co-injected 15 minutes prior to HFS (Figure 3.2.3). Similarly, synthetic soluble Aβ failed to inhibit LTP when using the same experimental protocol (Fig 3.2.2). Kayed et al. (2003) have suggested that A11 does not recognize low-n oligomers well. Since our natural oligomers contain dimers and trimers (see Figure 2.4.5- immunoblots) it would appear that A11 does bind smaller oligomers. It is likely that A11 has a relatively low affinity for dimers and trimers, but can still bind low-n oligomers mediating synaptic plasticity. Deshpande et al. (2006) provide some evidence for this in their research showing that A11 binds ADDLs (being as short as a trimer) in human cortical neurons. Furthermore, Wang et al. (2007) give some evidence that the A11 Ab recognizes low-n amyloid assemblies, when they reported that A11 bound monomers or dimers in their experiments characterizing "functional" amyloids found in bacteria, fungi and humans.

We also tested the conformation-specificity of A11 in a non-amyloid beta model. Kayed et al., (2003) reported that the antibody A11 not only recognized amyloid beta, but also other amyloidogenic assemblies, including oligomeric aggregates of $PrP_{106-126}$. $PrP_{106-126}$ strongly inhibited in vivo LTP in our rat model. However, the synthetic soluble $PrP_{106-126}$ failed to inhibit LTP when using the 60 min co-incubation and co-injection protocol with A11 (Fig 3.2.4). These results are consistent with the idea that the oligomer-specific antibody A11 recognizes a unique common structural feature of the polypeptide backbone of amyloidogenic soluble oligomers that is independent of the amino acid side chains. As an additional control, we tested a polyclonal antibody (IgG isotype) to A11. In our present study, we demonstrated that synthetic soluble $PrP_{106-126}$ inhibited LTP when using the 60 min co-incubation and co-injection protocol with the control IgG antibody (Fig 3.2.4). Our present findings are consistent with the utility of A11 in recognizing different amyloidogenic proteins.

Although soluble oligomeric and prefibrillar assemblies of Aβ peptide cause synaptotoxicity and potentially contribute to AD, the role of mature Aβ-fibrils in mediating toxicity remains controversial. A widely held view in the field suggests that the fibrillization reaction proceeds 'forward' in a near-irreversible manner from the monomeric Aβ peptide through toxic soluble intermediates, which subsequently mature into biologically inert amyloid fibrils that are found in plaques. Here we tested different doses of pre-aggregated Aβ injected 15 min before delivery of HFS and found that both doses were able to fully block rat hippocampal LTP in vivo (Figure 3.2.5). These doses of pre-aggregated Aβ had no effect on baseline synaptic transmission (Figure 3.2.5). When co-incubated with A11 60 min prior to injection, pre-aggregated Aβ only partially inhibited LTP (Figure 3.2.6). One possible explanation for this finding is that the pre-aggregated preparation also contains soluble assemblies (e.g. oligomers), which may explain the partial protection afforded by A11. As A11 has been reported to be unable to bind fibrillar Aβ (Kayed el al., 2003), our results indicate that A11 bound toxic soluble assemblies in the preparation, resulting in the LTP inhibition that was observed. Our results suggest that assemblies of Aβ exist in dynamic equilibrium, where fibrillization reactions proceed 'forward', but may also occur in a reversible manner. Martins et al., (2008) have provided some evidence for this dynamic equilibrium. They observed that natural lipids destabilize and rapidly resolubilize mature Aβ amyloid

fibers. Interestingly, the equilibrium was not reversed toward monomeric Aβ but rather toward large soluble Aβ oligomer assemblies. They further characterized these 'backward' Aβ oligomer assemblies generated from mature Aβ fibers and compared them with previously identified 'forward' Aβ oligomer assemblies obtained from the aggregation of fresh Aβ monomers. Their results indicated that backward oligomer assemblies are biochemically and biophysically very similar to forward oligomer assemblies: they consist of a wide range of molecular masses, are toxic to primary neurons and cause memory impairment and tau phosphorylation in brain. In addition, they diffuse rapidly through the brain into areas relevant to AD. Our results are consistent with these findings, which imply that Aβ fibrils are potentially major sources of soluble toxic Aβ-aggregates that could readily be activated by exposure to biological lipids.

4.3 The (proto)-fibril antibodies WO1/WO2

It is rather remarkable that the polyclonal immune response to fibrillar and prefibrillar amyloid antigens is largely independent of protein sequence, type and conformation. Amyloid fibrils accumulate in many degenerative diseases as a result of the intermolecular hydrogen bonding of extended polypeptide strands that arise as a consequence of protein misfolding. It has been hypothesized that amyloids from different diseases may share a common pathway for fibril formation (Glabe, 2006).

WO1 and WO2 are interesting because they, like A11, have the unusual property of recognizing generic epitopes that are associated with specific aggregation states regardless of their primary amino acid sequence. The fact that these epitopes are widely distributed yet distinct and non-overlapping between fibrils and prefibrillar oligomers suggests that there is a fundamental structural difference in the organization of the polypeptide backbone between these two classes of amyloid structures that is shared by many different types of amyloids. Harper et al. (1997) and Walsh et al. (1997) reported that synthetic Aβ forms curvilinear, soluble assemblies, termed protofibrillar aggregates, which appeared to be intermediates on the pathway to amyloid fibril formation. Their transient and intermediate nature was confirmed by the finding that these aggregates grow slowly at first and then rapidly disappear in favor of the formation of mature amyloid fibrils (Harper et al., 1997). Morphological characterization of these protofibrillar structures by atomic force microscopy, transmission electron microscopy, quasi-elastic light scattering and SEC revealed that these curvilinear, soluble assemblies have an apparent mass of > 100 kDa, a diameter of 6 to 7 nm, and a length of ≤200 nm (Walsh et al., 1997).

In the present study we also assessed the role of Aβ conformations recognized by the (proto)-fibril antibodies WO1 and WO2 in mediating inhibition of LTP. Using our co-incubation 60 minute prior to injection protocol, we tested the WO1 antibody against cell-derived 7PA2 oligomers. These low-n oligomers inhibited LTP when co-injected with WO1 (Figure 3.3.1). Our finding in our *in vivo* model that the (proto)-fibrillar antibody WO1 does not neutralize low-n oligomers strongly indicates that these low-n oligomers do not aggregate to form protofibrils during the time-course of the experiment. Next we tested these antibodies against soluble synthetic Aβ using our co-

incubation 60 minute prior to injection protocol. This soluble Aβ preparation is likely composed of many different assemblies ranging from oligomers to larger soluble assemblies. Both WO1 (Figure 3.3.2) and WO2 (Figure 3.3.3) partially abrogated the inhibition of LTP by soluble synthetic Aβ. Although these antibodies were initially thought to be fibril-specific, Williams et al., (2005) demonstrated that WO1 was able to bind protofibrils in addition to fibrils. Therefore, our findings may indicate that there are protofibril assemblies in the soluble synthetic Aβ that are capable of inhibiting LTP. Although fibrils have often been operationally defined as insoluble material that sediments at 100,000 × g, the fact that protofibrils also react with fibril-specific antibodies indicates that protofibrils and possibly smaller assemblies of oligomers with the same type of structural organization as the insoluble fibril also exist (Kayed et al., 2007). Although fibrillar oligomers may sound like an oxymoron, the fact that fibril assembly is known to be a nucleation-dependent process indicates that the existence of these small "seed" aggregates, in which the peptide is organized in the same lattice structure of the fibrils, is to be expected. There is recent evidence that Aβ protofibrils can cause spatial learning impairment (Lord et al., 2009) and that covalently cross-linked Aβ dimers rapidly form stable synaptotoxic water-soluble protofibrils that inhibit LTP *in vitro* (O'Nuallain et al., 2010). Our results support the idea that targeting different soluble Aβ assemblies, including oligomers and protofibrils, could be a feasible and efficient means to mitigate cognitive dysfunctions in early Alzheimer's disease.

In separate experiments, we found that repeatedly washed pre-aggregated Aβ (i.e. containing fibrils and undetectable levels of soluble Aβ) had no effect on LTP, while various doses of freshly prepared pre-aggregated Aβ retained their LTP-inhibiting activity (Figure 3.2.5). In an attempt to further characterize the toxic assemblies of our freshly prepared pre-aggregated Aβ preparation, we co-incubated this with WO2. The WO2 antibody completely abrogated the inhibition of LTP by pre-aggregated Aβ (Figure 3.3.4). We also tested a control monoclonal IgMκ antibody- the isotype of WO2 (as well as WO1), which did not prevent the inhibition of LTP by pre-aggregated Aβ. As Williams et al., (2005) reported that WO1 was able to bind protofibrils in addition to fibrils, we conclude that the toxic assemblies in our pre-aggregated Aβ preparation are most likely soluble protofibrils. Whether these protofibrils are remnants of on-pathway fibrillization or products of backwards-dynamic equilibrium remains unanswered. What

is clear is that protofibrils display a rapid functional toxicity that mimics both soluble synthetic Aβ and naturally derived Aβ oligomers.

The lack of effect of the washed pre-aggregated Aβ may be explained by the poor penetration of brain by fibrils or the low synaptotoxicity of fibrils. Amyloid beta fibrils are found in the amyloid plaques as large insoluble macromolecular assemblies characterized by a 'cross-beta' fiber-like architecture. Mature Aβ fibrils are resistant to proteolytic cleavage (Booth et al., 1997; O'Nuallain et al., 2005) and are biologically inert; cytotoxicity appears primarily caused by soluble prefibrillar oligomers (Bucciantini et al., 2002). Taken together, these observations suggest an archetypal model for amyloid-associated pathologies in which disease is caused by transient toxic aggregates that eventually convert to inert amyloid deposits. These deposits would then be simple remnants of the aggregation process playing no significant role in the disease process (Lomas et al., 1992). On the other hand, amyloid fibrils are not strictly irreversible but rather in a slow dynamic equilibrium with soluble peptide (Carulla et al., 2005; O'Nuallain et al., 2005). For instance, amyloid fibrils recycle about half of their molecules over a period of weeks (Carulla et al., 2005). As noted previously, Martins et al., (2007) reported that Aβ fibrils are potentially major sources of soluble toxic Aβ-aggregates that could readily be activated by exposure to biological lipids. They focused on lipid rafts containing cholesterol, sphingolipids and gangliosides, which are associated with amyloid deposits (Kakio et al., 2002; Devanathan et al., 2006), promote Aβ aggregation and oligomer formation (Kakio et al., 2002; Yip et al., 2002; Zou et al., 2003; Gellermann et al., 2005; Kim et al., 2006), and stabilize toxic oligomers generated from monomeric Aβ (Johansson et al., 2007).

In vivo Meyer-Luehmann and colleagues (2008) showed that dense fibril-containing plaques in APP transgenic mouse models reach their maximum size in about a day and thereafter maintain a status quo. This rapid growth hints at a very fast and dynamic oligomerization/fibrillization process *in vivo*. Importantly, the quick growth of dense plaques suggests that dense plaques grow not only with Aβ monomer addition, but perhaps also by capturing oligomeric intermediates at the fiber ends, as shown in prion protein studies (Serio et al., 2000; Collins et al., 2004). In a follow-up to their study, Koffie and colleagues (Koffie et al., 2009) reported that the precipitated fibril-containing plaques seen by Meyer-Luehmann and colleagues (2008) act as a reservoir

of soluble oligomers. Our findings lend support to the idea that these pre-fibrillar assemblies are generated in dynamic equilibrium with the suddenly appearing plaques.

In the absence of more detailed structural information, conformation-dependent antibodies may provide a more rational means of classifying amyloid aggregates based on their underlying structural organization rather than on differences in size or sample preparation. This structure-based classification scheme may be more useful in comparing the toxic activity and pathological significance of different assemblies. The availability of novel conformation-dependent antibodies that specifically recognize these assembly states also affords us a unique opportunity to clarify their roles in Aβ fibril assembly and cellular toxicity. Besides aiding structural analysis, compounds capable of facilitating oligomer and protofibril formation might have therapeutic potential, if they act to sequester Aβ in a form and/or location that cannot engage the toxic pathway.

4.4 The cellular prion protein receptor

The present studies investigated the ability of the anti-PrPc antibody fragment D13 to affect the synaptic plasticity disrupting effects of various peptides. In the first set of experiments, a pre-injection of D13 antibody fragment prevented the inhibition of LTP by soluble synthetic $A\beta_{1-42}$ (Figure 3.4.1). This finding is consistent with Lauren et al. (2009) and earlier studies hinting at an association of Aβ with PrPc (Brown, 2000; Schwarze-Eicker et al., 2005). Lauren et al. (2009) reported that Aβ oligomers bind to PrPc and that the detrimental effect of Aβ on hippocampal LTP is not observed in PrPc knockout mice. They speculated that the prion protein, which is present on the surfaces of neurons, might activate a cell-damaging pathway leading to memory impairment when bound and activated by Aβ. Contradicting this proposal, Balducci et al. (2009) injected Aβ into mice lacking the prion protein, but found that the mice suffered memory deficits even in the absence of the prion protein. Calella et al. (2010) reached the same negative conclusion using mice that were genetically engineered to over-express Aβ. Calella and colleagues investigated the effects of expressing a soluble form of PrP (without its GPI-anchor) in their AD mouse model. In this case, whereas the levels of soluble and insoluble Aβ remained unchanged, learning was less affected; suggesting that secreted PrP might interfere with Aβ mediated toxic pathways by directly binding to the peptide, not unlike neutralizing the effect of Aβ antibodies. Furthermore, when Kessels et al. (2009) attempted to closely replicate the experiments of Lauren et al., they failed to replicate the main findings. In more recent work, Gimbel and colleagues (2010) reported that a lack of prion protein can, in fact, stave off memory loss due to Aβ accumulation in mice, using ones genetically different from those of Balducci et al. and Callela et al., and utilizing different behavioral tests. Differences aside, all of these groups agree that PrPc has a high affinity for Aβ, which is in line with our results.

In a defense of their work, Lauren et al. (2010) argued that the differences between their published work and negative findings of Kessels et al. (2010) might be due to different preparations of synthetic $A\beta_{1-42}$ oligomers being used. It is possible that the way the synthetic Aβ was administered may have changed the effective concentration of Aβ oligomers reaching the slice being recorded from. Because our initial PrPc experiments used soluble synthetic Aβ, we decided to test D13 with a human brain derived Aβ-dimer

containing preparation. TBS soluble extracts of AD brain containing Aβ-dimers inhibited LTP (Figure 3.4.3) *in vivo* similarly to previous reported studies in vitro (Shankar et al., 2008). Moreover, Aβ immuno-depleted preparations failed to inhibit LTP (Figure 3.4.3) and baseline excitatory transmission was not significantly affected by either of the preparations (Figure 3.4.4).

We investigated the ability of the anti-PrPc antibody fragment D13 to prevent the plasticity disrupting effect of human Aβ dimers by pre-injecting D13 30 min prior to the injection of the Aβ. D13 completely prevented the inhibition of LTP by Aβ dimer-containing extract (Figure 3.4.6). As a control for determining the specificity of the D13 Fab, we tested a second antibody fragment (R1) that targeted an alternate sequence on cellular prion protein (220-231). A pre-injection of R1 failed to prevent the inhibition of LTP by soluble dimers (Figure 3.4.6). We conclude that the effect of D13 was epitope-specific since the R1 antibody fragment binds avidly to a different epitope but failed to prevent the inhibition of LTP. Because the epitope for D13 corresponds to amino acids 96-105 of rat PrPc, our results are consistent with the conclusions of Lauren et al. (2009) that the 95–105 region is a primary determinant for binding to Aβ oligomers. In independent work, Chen et al. (2010) have confirmed the importance of the 95-110 segment for $A\beta_{1-42}$ binding, but have additionally implicated a second region of critical importance at the extreme N terminus of PrP (residues 23-27). These two binding sites may act in concert to provide a high affinity-binding site for $A\beta_{1-42}$ oligomers.

It is important to note that although Lauren et al. (2009) highlighted an Aβ-PrPc interaction they clearly showed that PrPc is not the only cell-surface molecule binding Aβ oligomers, as a high level of Aβ binding signals was still observed in Prnp−/− hippocampal neurons (50% compared to wild type). Furthermore, as pointed out by Callela et al. (2010), the *in vivo* generated synthetic Aβ oligomer pool is likely more complex than any *in vitro* generated Aβ oligomer mixture, and may therefore contain several 'strains' of toxic and less toxic conformers, somewhat resembling PrPsc (Aguzzi, 2008). Each of these conformations might act via different pathways. Therefore, and in conclusion, one cannot exclude that a remarkably high affinity of PrP to Aβ could be ascribed to a sub-pool of amyloid assemblies, but other Aβ assemblies may act independently of this interaction.

The present findings indicate a role for PrPc in Aβ-mediated synaptic dysfunction. Several investigators have shown that PrPc participates in cellular signaling (see review by Linden et al., 2008); and it is possible that some of these pathways may be altered, disturbed or over-activated by Aβ oligomers. Using a very different approach Parkin et al. (2007) suggested a link between prion protein and Aβ when they reported that the prion protein inhibits the β-secretase cleavage of APP thereby reducing Aβ levels in both cell and animal models. Renner et al. (2010) have recently reported that membrane-attached Aβ oligomers accumulate at synapses, with a concomitant clustering of metabotropic glutamate receptors (mGluR5). These authors proposed that Aβ-induced clustering of mGluR5 elevates intracellular calcium and causes synapse deterioration, and ultimately promotes synaptotoxicity. Of particular relevance to the present study, evidence was found using competition experiments with a cocktail of antibodies that mGluR5, PrPc, and NMDAR1 might be in proximity to each other to promote Aβ binding. In fact, Lauren et al. (2009) suggested that PrP may not be receptor for Aβ, but may help concentrate Aβ at the surface of neurons, thus increasing it's functional activity via other mechanisms.

Since D13 is known to bind a specific region on PrPc- residues 96-104 (Peretz et al., 2001) which forms part of the putative binding site of Aβ oligomers on PrPc (around 95-110; Lauren et al., 2009), our findings might support the Aβ-crosslinker-hypothesis (Blochl, 2008). This proposes that an Aβ-binding site within PrPc segment 91-123 would explain possible physiological and pathological effects of an Aβ-mediated interaction between the neurotrophin receptor p75 and PrPc, APP and possibly Aβ. This is somewhat similar to what Deshpande et al. (2006) suggested when they reported that Aβ accumulation at synapses is activity dependent and is required for Aβ to disrupt synaptic function. Some roles of PrPc may involve an interaction of PrPc with a surface receptor and via a binding site of PrPc that overlaps with segment 105-125 of PrPc (for a review Westergard et al., 2007). Indeed, Lauren et al. proposed that a putative PrPc-associated transmembrane co-receptor is likely to have a central role in Aβ-mediated toxicity. As several publications indicate that the neurotrophin receptor p75 is essential for Aβ-mediated toxicity (Capsoni and Cattaneo, 2006; Sotthibundhu et al., 2008), it is a possible candidate for such a co-receptor.

Curiously, the region in PrPc (amino acids 95-110) that has been reported to interact with Aβ oligomers (Lauren et al., 2009) also causes profound neurodegeneration when deleted from the prion protein (Baumann et al., 2007; Li et al., 2007). This also suggests a natural function for PrPc, which when perturbed can lead to neurodegeneration. Our results indicate that one way to perturb the natural function of prion protein is with soluble Aβ (Figure 3.4.1) or $PrP_{106-126}$ (Figure 3.2.4). Moreover, the antibody A11 prevented the inhibition of LTP by both $PrP_{106-126}$ and oligomeric Aβ. This may indicate that PrPc is, in some sense, a binding site for both of these toxic assemblies. Remarkably, D13 prevented LTP-mediated inhibition by $PrP_{106-126}$ (Figure 3.4.2). These results support the proposal that in addition to its involvement in synaptic plasticity disruption by Aβ, PrPc mediates $PrP_{106-126}$ toxic effects in neuronal cells (Henriques et al., 2008).

Our results have led us to hypothesize that PrPc may act as an oligomer-conformation-selective binding site. Looking at the studies available (Harper et al., 1997; Walsh et al., 1997; Kayed et al., 2003; Lanz and Sacher, 2006; Yu et al., 2005), a hypothesis can be formed that the key determinant for the neurotoxicity of Aβ not only involves the degree of oligomerization, but also the structural conformation of peptides in the assembly. This concept reconciles the apparently contradictory results that widely differing preparations of Aβ exert similar cytotoxic effects, and offers the therapeutic potential for targeting the key conformation with small molecules or monoclonal antibodies. Our results, showing that D13 blocks the binding of various oligomeric peptides, provide further evidence that PrPc is highly selective for oligomers.

To conclude, targeting a high-affinity PrPc binding site for toxic Aβ oligomers may avoid disrupting putative physiological roles of Aβ. Furthermore, evidence of a selective Aβ-binding site on PrPc opens up possibilities of exploring the role of PrPc in AD, and the existence of a putative binding site on PrPc mediating Aβ oligomer toxicity would be therapeutically appealing.

4.6 Conclusions

Citron et al., (1995) reported the appearance of soluble oligomers of Aβ in cell culture almost fifteen years ago, while Roher et al., identified SDS-stable oligomers in post-mortem human brains as early as 1996. Initially researchers considered these oligomers to be relatively inert intermediates in fibril formation, but most are now convinced that these oligomers are key mediators of AD. The search for Aβ assemblies that are capable of causing cognitive disorder as observed in AD patients led to several reports of animal-cell derived (Walsh et al., 2002), human derived (Klyubin et al., 2008) and synthetically prepared oligomers that have been found to induce disruptions of synaptic activity or impair cognitive function in animal models (Poling et al., 2008; Cleary et al., 2008).

In the present study we confirmed what has previously been reported, that synthetic soluble Aβ, at low concentrations inhibits hippocampal LTP, using doses that had no statistically significant effect on baseline excitatory transmission (Klyubin et al., 2005). The role of Aβ oligomers was investigated by purification of cell-derived Aβ. Consistent with what had been previously reported with SEC purification alone, 2-step (SEC + IE) purified fractions containing Aβ oligomers inhibited LTP. The monomeric fraction failed to inhibit LTP, thus confirming that monomeric Aβ does not impair synaptic plasticity. Neither oligomer nor monomer fractions prepared using the two-step purification protocols had an effect on baseline transmission consistent with previous experiments from this laboratory using the 1-step purification protocol. Futher purification of cell-derived Aβ using a 4-step protocol (1. remove the large amount of contaminating APPs from the CM, 2. use immunoaffinity to enrich Aβ species with either polyclonal or monoclonal Aβ antibodies, 3. use peptide ligand (a fragment of Aβ) as an additional affinity-binding reagent and, 4. separate the resultant Aβ assemblies by size on a superdex 75 SEC column), allowed us to test the possibility that other proteins may help mediate/facilitate Aβ-mediated disruption of plasticity. Our present studies using these highly purified cell-derived Aβ indicate that Aβ dimers are the smallest synaptotoxic assemblies, and that this activity can be ablated with antibodies.

Although abundant evidence suggests that amyloid accumulation plays a significant role in the pathogenesis of degenerative disease, the mechanism of amyloid formation and

toxicity remains elusive. Experimentally, oligomer-specific antibodies represent a new type of tool for illuminating the pathogenicity of disease-related proteins- it is now possible to have a fuller understanding of the comparative pathobiology of oligomerization. Our present studies show that the pan-oligomer antibody A11 has the ability to prevent Aβ-induced impairment of LTP *in vivo*. Complete prevention was seen with soluble synthetic and naturally derived Aβ assemblies, while partial prevention was observed in the case of inhibition of LTP by synthetic pre-aggregated Aβ. If we accept that A11 is selective for oligomers, this provides further evidence that it is these assemblies of Aβ in synthetic Aβ preparations that are the neurotoxic assemblies. A11-mediated prevention of the inhibition of LTP by $PrP_{106-126}$ in the present studies indicates that that the antibody recognizes a unique common structural feature of the polypeptide backbone of amyloidogenic soluble oligomers that is independent of the primary sequence of the amino acid side chains.

Our findings support previous work from this laboratory that a mechanistic basis exists for the proposal that Aβ-directed immunotherapy could target a rapidly reversible component of the cognitive impairment seen in AD (Klyubin et al., 2005). The primary focus of current clinical immunotherapy research is antibody-mediated clearance of AD plaques; our mechanism of soluble Aβ neutralization could work in parallel with this. In the future, an anti-oligo ELISA may prove a useful diagnostic tool for the screening of samples from suspected AD patients. In this regard, the detection of oligomers in brain homogenates from patients with AD and mild Braak changes, but not in non-demented controls, is encouraging (Glabe, 2008; Shankar et al., 2008), and provides evidence for the *in vivo* relevance of prefibrillar assemblies. Clearly, the availability of antibodies like A11 will facilitate a fuller understanding of the role of soluble oligomers in AD.

In both human AD and APP transgenic models of AD, Aß is in dynamic equilibrium between soluble oligomeric and fibrillar forms, implying that various biophysical parameters determine the relative abundance of different aggregation states. Investigating the role of soluble amyloid oligomers in pathogenesis presents a problem for distinguishing these aggregates from the mature fibrils. In the present study we assessed the role of Aβ conformations recognized by the (proto)fibril antibodies WO1 and WO2 in mediating inhibition of LTP. Our results indicate that the (proto)fibril antibody WO1 does not bind cell-derived low-n oligomers. In contrast, both WO1 and

WO2 partially abrogated the inhibition of LTP by soluble synthetic Aβ. In a separate study, we found that repeatedly washed Aβ fibrils (e.g. containing no detectable soluble Aβ) had no effect on LTP, while various doses of freshly prepared pre-aggregated Aβ retained their LTP-inhibiting activity, with no concomitant effect on baseline synaptic transmission. We also observed that the WO2 antibody completely abrogated the inhibition of LTP by pre-aggregated Aβ. As Williams et al., (2005) reported that WO1 was able to bind protofibrils in addition to fibrils, we conclude that the toxic assemblies in our pre-aggregated Aβ preparation is most likely protofibrils. The dynamic nature of this (proto)-fibrillization makes the definition of such toxic assemblies elusive and probably also explains why so many various direct and indirect interactions of Aβ peptides with membrane bound and intracellular proteins have been described (Ashe and Zahs, 2010).

Because the structure of Aβ depends on its source and the presence of cofactors, it is of great interest to determine whether human-derived oligomeric Aβ assemblies impair brain function. We report here that post mortem human AD brain material that contains Aβ dimers rapidly inhibits hippocampal LTP *in vivo*.

Selectively reducing the levels of potentially synaptotoxic Aβ oligomers is an appealing therapeutic approach to AD. Directly reducing the levels of toxic Aβ-assemblies is clearly one approach. However, if there are other proteins that are influencing the synaptotoxic effects of Aβ, than these could also become therapeutic targets. The role for PrPc in Aβ oligomer-induced synaptic impairment is a topic of great interest and some controversy. Researchers have recently begun to explore the contribution of PrPc to LTP inhibition and soluble Aβ levels in AD mouse models, with results demonstrating that the role of PrPc in Aβ related toxicity is far from clear- suggesting complex interpretations of the data available thus far.

The role of the anti-PrPc antibody fragment D13 to affect the synaptic plasticity disrupting effects of various Aβ assemblies was studied. Pre-injection of D13 antibody fragment prior to soluble synthetic $A\beta_{1-42}$ prevented the inhibition of LTP. Furthermore, D13 prevented the plasticity disrupting effect of human brain Aβ dimers. These findings are consistent with Lauren et al. (2009) and earlier studies hinting at an association of Aβ with PrPc (Brown 2000; Schwarze-Eicker et al., 2005). Remarkably we found that

D13 also prevented LTP-mediated inhibition by $PrP_{106-126}$, supporting the proposal that in addition to its involvement in synaptic plasticity disruption by Aβ, PrPc mediates $PrP_{106-126}$ toxic effects in neuronal cells (Henriques et al., 2008).

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