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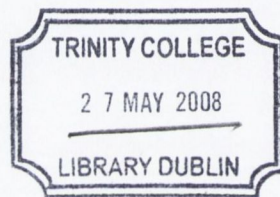
**Analysis of the effects of amyloid-beta in  
rat brain: effect of treatment with a novel  
anti-inflammatory agent VP025**

By

Anne-Marie Miller

Thesis submitted for the degree of Doctor of Philosophy at  
the University of Dublin, Trinity College.

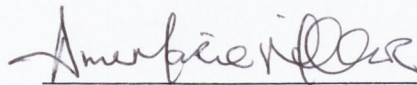
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## I. Declaration

This thesis is submitted by the undersigned for the degree of Doctor in Philosophy at the University of Dublin. I declare that this thesis is entirely my own work with the following exceptions; certain results were produced in collaboration with Dr. Darren Martin, Michelle Walsh and Alessia Piazza, and with the cooperation of Dr. Thelma Cowley and Dr. Anthony Lyons. All contributions are acknowledged within the thesis. This work has not been previously submitted for a degree to this or any other university. I give my permission to the library to lend or copy this thesis upon request.



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Anne-Marie Miller

## II. Summary

The data presented indicate that long-term potentiation was significantly impaired following acute intracerebroventricular injection of amyloid- $\beta_{1-40}$  in rat. Similarly chronic intracerebroventricular infusion of amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$  in rat for 8, 20 or 28 days led to a deficit in long-term potentiation which was more pronounced with longer exposure to amyloid- $\beta$ . Pre-treatment of rats with VP025 attenuated the amyloid- $\beta$ -induced deficit in long-term potentiation. Significantly VP025 also attenuated the effects of amyloid- $\beta$  on long-term potentiation, even when treatment was given following 14 days of A $\beta$  administration. Acute and chronic infusion of amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$  also resulted in increased expression of markers of microglial activation, cluster of differentiation 86 and intracellular adhesion molecule-1, in rat hippocampus. Evidence indicates that these amyloid- $\beta$ -induced changes were also attenuated by pre-treatment with VP025, suggesting a coupling between microglial activation and impairment in long-term potentiation.

Further analysis of the effect of amyloid- $\beta$  revealed that it triggered activation of caspases-8 and -3 in cortical tissue following 8, 20 and 28 days amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$  intracerebroventricular infusion and sphingomyelinase activation after 8 and 28 days. The evidence indicates that the neuroprotective effect of VP025 extends to the cortex, since treatment of rats with VP025 attenuated the amyloid- $\beta$ -induced changes in these 3 enzymes. In addition a significant increase in activation of secretory phospholipase A<sub>2</sub> was observed in cortical tissue prepared from rats, which received amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$  intracerebroventricularly for 8 days. An important role for sphingomyelinase in stimulating caspase-3 activity was demonstrated by analysis of changes in cultures enriched for cortical neurons; the data indicate that amyloid- $\beta$ -induced increases in caspase-3 in cortical neurons. In addition, it decreased neuronal viability, an effect that was mimicked by ceramide, and attenuated by the sphingomyelinase inhibitor GW4869.

The findings demonstrate that VP025 ameliorates the effects of chronic infusion of amyloid- $\beta$ , confirm its anti-inflammatory properties and highlight its ability to act as a neuroprotective agent in hippocampus and cortex.

### III. Acknowledgements

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## VI. List of Abbreviations

The following abbreviations are used:

AA	Arachidonic acid
A $\beta$	Amyloid $\beta$
AChE	Acetylcholine esterase
AD	Alzheimer's disease
ADDL	A $\beta$ derived diffusible ligands
ALS	Amyotrophic lateral sclerosis
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of variance
Apaf-1	Apoptosis protease activity factor
APC	Antigen presenting cell
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Asp	Aspartate
ATP	Adenosine tri-phosphate
BACE	$\beta$ -APP cleaving enzymes
Bax	Bcl-2 family of proteins, Bax has proapoptotic functions
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
bp	Base pair
CA	<i>Cornu ammonis</i>
CA <sup>2+</sup>	Calcium ions
CD86 / 68	Cluster of differentiation 86 / 68
CED	Cell death abnormal
CGC	Cerebral granule cells
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system

COX	Cyclooxygenase
CPP	3,3-(2 Carboxypiperazine-4-yl)-Propyl-1-Phosphonate
(a)CSF	(artificial)Cerebrospinal fluid
Cu <sup>2+</sup>	Copper ions
cDNA	Copy deoxyribonucleic acid
°C	Degrees Celcius
DDTC	Diethy-dithiocarbamate
DEPC	Diethyl pyrocarbonate
dH <sub>2</sub> O	Deionised water
DHA	Docosahexanoic acid
DISC	Death inducing signal complex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTNB	Dithio-bis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
EtBr	Ethidium Bromide
EtOH	Ethanol
fAβ	Fibrillar amyloid-beta
fAD	Familial Alzheimer disease
FADD	Fas-associated death domain
Fas	Cell death receptor ligand
FBS	Fetal Bovine Serum
5' / 3'	Five prime / three prime
g	G Force

GFAP	Glial fibrillary acidic protein
GSH	Glutathione
h	Hour
H <sub>2</sub>	Hydrogen
HCN	Human cortical neurons
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HEPC	2-hexadecanoylthio-1-ethylphosphorylcholine
HFS	High frequency stimulation
HMGR	Hydroxymethylglutary-CoA reductase
HSPG	Heparan sulfate proteoglycans
HRP	Horse radish peroxidase
Hz	Hertz
ICAM-1	Intercellular adhesion molecule-1
ICAD	Inhibitor of caspase-activated DNase
icv	Intracerebroventricular
IFN- $\gamma$	Interferon- $\gamma$
Ig	Immunoglobulin
IL-	Interleukin-
IL-1ra	Interleukin-1 receptor antagonist
im	Intramuscular
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneally
JAK STAT	Janus kinase signal transducers and activators of transcription
JNK	c-Jun amino-terminal kinase
K <sup>+</sup>	Potassium ions
LDS	Lithium dodecyl sulfate
LFA	Leukocyte function-associated antigens
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTP	Long-Term Potentiation
mA	milliamps

MAC-1	CD11b/CD18
MAP kinases	Mitogen activated protein kinases
MCP-1	Monocyte chemoattractant Protein-1
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeters
MHC II	Major Histocompatibility Complex class II
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
N <sub>2</sub>	Nitrogen
NAC	N-acetylcysteine
Na <sup>2+</sup>	Sodium ions
NBM	Neurobasal medium
nM	Nanometers / nanomolar
NFκB	Nuclear Factor κB
NK	Natural killer
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NTP	Nucleotide triphosphate
O <sub>2</sub>	Oxygen
PARP	Poly ADP ribose polymerase
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PC12	Porcine
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
pg	Picograms
PtG	Phosphatidylglycerol
PG(E <sub>2</sub> )	Prostaglandins (E <sub>2</sub> )

pH	Potential of hydrogen
pNA	p-nitroanilide
PS	Phosphatidylserine
PSen1	Presenilin-1
PUFA	Polyunsaturated fatty acid
QPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end-products
RCSI	Royal College of Surgeons Ireland
(r)(m)RNA	(r) ribosomal (m) Messenger ribonucleic acid
RT	Room temperature
RT-	Reverse Transcriptase
s	Second
SDS	Sodium dodecyl sulphate
SNpc	Substantia nigra pars compacta
SMase	Sphingomyelinase
SO	Superoxide
SOD	Superoxide dismutase
sPLA <sub>2</sub>	secretory Phospholipase A <sub>2</sub>
Tanneal	Temperature at which primers optimally anneal to a cDNA template
<i>Taq</i> Polymerase	<i>Thermus aquaticus</i> DNA polymerase
ThT	Thioflavin T
TGF- $\beta$	Transforming growth factor $\beta$
TMB	Tetramethylbenzidine
TNF $\alpha$	Tumor necrosis factor $\alpha$
TNFR	Tumor necrosis factor receptor
TBE	Tris borate EDTA
TBS(-T)	Tris Buffered Saline(-Tween)
TRAIL	Tumor necrosis factor-related Apoptosis-inducing ligand
TUNNEL	Transferase-mediated dUTP nick end labeling
UV	Ultra violet
v	Volts



$\mu\text{g}$	Microgram
$\mu\text{l}$	Microlitre
$\mu\text{M}$	Micromolar

# **Chapter 1**

## **Introduction**



## 1.1 Memory – An introduction

The nervous system and the brain are the physical foundation of the human learning process. Our brain actually contains three brains: the lower or reptilian brain that controls basic sensory motor functions; the mammalian or limbic brain that controls emotions, memory, and biorhythms; and the neocortex or thinking brain that controls cognition, reasoning, language, and higher intelligence.

Memory can be characterized as a process of information retention in which our experiences are archived and then recovered when we recall them; they derive from enhanced patterns of neuronal interconnections, which are subject to continual change. There are a number of ways to classify memory; two dichotomies arise when attempting to describe it. The first dichotomy is between procedural and declarative memory and the second between short-term memory and long-term memory. Procedural memory in humans is related to the knowledge of rules of action that can become quite automatic with repetition; procedural memory represents motor learning and does not require verbal mediation. Non-associative learning and most classical conditionings produce procedural memory. Declarative memory on the other hand involves explicit information about facts such as names and dates.

Long-term memory can however encompass both procedural and declarative memory. Procedural memory is slow to acquire but is more resistant to change or loss where as declarative memory is fast changing, quick to acquire but quick to be lost. There is a third form of memory known as "remote memory", it too can be classified as long-term memory. It simply refers to memories that were acquired early. They represent foundation memories upon which more recent memories are built. Short-term memory on the other hand describes the brain's system for remembering information currently in use. It involves the retention of a sensory stimulus, which is held for a fraction of a second in the sensory memory. Unless an individual pays attention to the stimulus for a number of uninterrupted seconds in order to encode it the memory will be lost.

Both long- and short-term memories are composed of three processes: encoding, storage, and retrieval. These processes take place in various locations in the brain, often simultaneously. Not much is known about the physiology of long-term memory but the hippocampus has been implicated in its formation. Procedural memory relies on the

cerebellum and amygdala with declarative memory depending on the hippocampus and temporal lobes. Irrespective of whether one speaks of memory in terms of it being “short- or long-term” or “procedural or declarative” it is now widely accepted that the hippocampus is central to the formation and eventual storage of memory (Kandel *et al.*, 2000).

### **1.1.1 Anatomy of the hippocampal formation**

The hippocampus, located in the temporal lobe in humans, forms a specialised part of the limbic cortex. It can be described as a horseshoe or C-shaped body of neurons located within the temporal lobes, adjacent to the amygdala (Figure 1.1). Its formation can be broken down into six sub-regions: the dentate gyrus, hippocampus proper, subiculum, presubiculum, parasubiculum and the entorhinal cortex (Amaral and Witter 1989). In this text as in several others, the term hippocampus is used to refer to a structure that is composed of hippocampus proper and the dentate gyrus. It possesses a characteristic laminar organization of cells packed into distinct groups. In rats, the hippocampus proper comprises three parts: CA1, CA2 and CA3. In humans, there are four parts: CA1, CA2, CA3 and CA4. The letters CA come from the Latin words *cornu ammonis*; "Ammon's horn" in English.

### **1.1.2 Intrahippocampal connections**

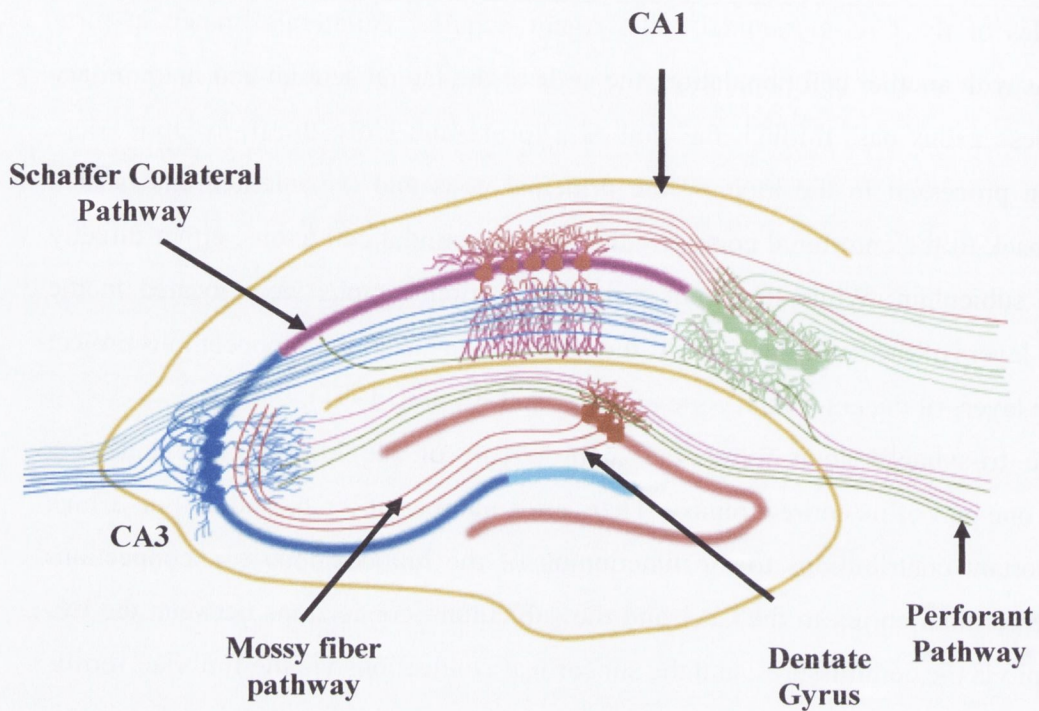
The intrahippocampal connections form a tri-synaptic loop, which is composed of the cells of dentate gyrus, CA3 and CA1 and their interconnections (Witter and Amaral 1991). The perforant pathway comprises the first synaptic connections of the tri-synaptic loop. It consists of connections that are formed between the entorhinal cortex and dentate gyrus. The cells in the superficial layers (mainly layer II) of the entorhinal cortex project their axons to the molecular layer of the dentate gyrus and they provide the hippocampus with its main glutamatergic input. Nerve fibers known as the mossy fibers form the second set of synaptic connections, and connect the dentate gyrus with the CA3. These axons from the granular cells of the dentate gyrus innervate the dendrites of the CA3 pyramidal cells. Mossy fibers also form other connections with another cell population, namely the mossy cells of the dentate gyrus. These interneurons provide feedback

excitation back to the granule cells. Schaffer collateral axons form the third and last stage of the tri-synaptic loop. Here axons of the CA3 pyramidal cells form connections with the dendrites of the CA1 pyramidal cells. Again Schaffer collaterals branch to form connections with another cell population: the cells of the lateral septum and mammillary bodies. These axons pass through the fimbria / fornix and close the tri-synaptic loop. Information processed in the loop by the principal cells and the interneurons is then projected back to the entorhinal cortex by the CA1 pyramidal cell axons, either directly or via the subiculum. While the input cells to the hippocampus were located in the superficial layers of the entorhinal cortex, the output axons from the hippocampus project to the deep layers of the entorhinal cortex (Witter and Amaral, 1991).

The tri-synaptic loop forms the main circuit of the hippocampus but only comprises one part of its entire circuitry. There are a number of inputs and outputs which make important contributions to the functioning of the hippocampus e.g. connections from the entorhinal cortex to the CA1 and the subiculum, connections between the two hippocampi via the commissures, and the subcortical connections via the fimbria / fornix, mostly with the septum. Among the projections that pass via the fimbria / fornix are noradrenergic projections from the locus coeruleus, serotonergic projections also pass from the raphe nuclei, histaminergic projections from supramammillary nucleus and dopaminergic connections from ventral tegmental area and the substantia nigra (Freund *et al.*, 1990). These projections provide sparse excitation but massive inhibition of the pyramidal cells of the hippocampus ensuring that only synchronization will enable the firing of the cells (Freund and Gulyas, 1997).

### **1.1.3 Role of hippocampus in memory**

The classic example illustrating the role of the hippocampus in memory tells of a human patient H. M., whose hippocampi were extracted in an attempt to treat severe epileptic seizures. As a result of the extraction H.M. was rendered incapable of forming new memories. Some of the events immediately before the surgery were also lost from his memory, but events from the distant past remained preserved (Scoville and Milner, 1957). Clinical observations, such as this, along with later studies with experimental animals (Maciejak *et al.*, 2003; Abbott *et al.*, 2007) led to a conclusion that the formation of



**Figure 1.1 Hippocampal formation**

The three major pathways in hippocampus: the perforant fiber pathway from entorhinal cortex forms excitatory connections with the granule cells of the dentate gyrus. The granule cells give rise to axons that form the mossy fiber pathway, which connects with the pyramidal cells in area CA3. The pyramidal cells of area CA3 project to the pyramidal cells in CA1 by means of the Schaffer collateral pathway.

University of Bristol, Department of Anatomy, School of Medical Sciences, University Walk, Bristol, BS8 1TD. [Updated 14/07/2003; cited 29/02/2008]. Available from: <http://www.bristol.ac.uk/Depts/Synaptic/info/pathway/hippocampal.htm>. CA = *cornu ammonis*.

new memories requires an intact and functioning hippocampus. These new memory traces are, however, eventually stored in other parts of the brain that are not dependent on the hippocampus (Squire and Zola-Morgan 1991).

### **1.1.3.1 Declarative and spatial memory**

In addition to clinical findings animal studies have served to further elucidate the importance of the hippocampus in memory formation. Wishaw and colleagues (1986) discovered that when the hippocampus is removed or partially impaired that the ability of a rat to form memory traces is disrupted. However, some fundamental differences exist between humans and experimental animals in the effects of hippocampal lesions on their ability to create new memories. In humans, hippocampectomy interferes with memory formation in almost all kinds of tasks that require new learning. Particularly affected is declarative memory. Procedural memory and priming, which are implicit forms of long-term memory, remain intact (Scoville and Milner, 1957; Warrington and Weiskrantz, 1968). In rats, however, tests conducted by Aggleton *et al.* (1986) and Phillips and LeDoux (1994) have shown that some tasks are relatively unaffected by damage to the hippocampus, in particular recognition memory and fear conditioning. The form of memory most dramatically affected in animals was that of spatial memory. For example in a study by Morris and colleagues (1982) it was observed that in negotiating the water maze, a task that requires spatial learning, rats with hippocampal damage were dramatically compromised.

The observation that the hippocampus is involved in spatial learning was supported by studies demonstrating that hippocampal pyramidal cells operate as so-called "place cells" (O'Keefe and Dostrovsky, 1971; Muller and Stead, 1996). Electrophysiological recordings made in freely moving animals have shown that some CA1 and CA3 hippocampal pyramidal cells fire only in a restricted environment known as the "firing field" of the cell. This observation, together with the finding that hippocampal damage causes spatial learning deficits, led to the hypothesis that the hippocampus creates an abstract representation of the environment, called a cognitive map (O'Keefe and Conway, 1978). CA1 and CA3 cells are not the only cells of the hippocampal formation that are involved in spatial representation. The cells in the



superficial layers of the entorhinal cortex (the input-area of the hippocampus) show location-selective firing, although the firing fields are larger and noisier than in CA1 and CA3 (Quirk *et al.*, 1992). The dentate gyrus also possesses cells that fire selectively to location (McNaughton *et al.*, 1989). The selectivity of these cells is higher than in the entorhinal cortex. The output area of the hippocampus, the subiculum, also contains location selective cells, with low selectivity (Sharp and Green 1994). Spatial memory and its mechanisms are thought to be similar in humans and rodents and are still the most universally accepted critical function of the hippocampus. However, the notion that the hippocampus is solely involved in spatial encoding in rodents has been challenged by a number of studies (Wood *et al.*, 1993; Bunsey and Eichenbaum 1996; Dusek and Eichenbaum 1997) whose findings indicate that the hippocampus in animals also governs non-spatial declarative processing in a pattern similar to that seen in humans (Scoville and Milner 1957).

## **1.2 Long-term potentiation**

### **1.2.1 Properties and induction of long-term potentiation**

It has been observed that trains of high frequency stimulation applied to the major synaptic pathways increase the amplitude of the excitatory postsynaptic potentials (EPSPs) in target hippocampal neurons. This sustained increase in synaptic firing is termed long-term potentiation (LTP) and was first described by Bliss and Lomo in 1973 (Figure 1.2). They reported that the synaptic changes, which underpin LTP, resembled those observed during the formation of certain types of learning and memory. LTP is considered a particularly important phenomenon, especially in the mammalian hippocampus, in that it is thought that the biological mechanisms involved are similar to that invoked during the establishment of stable memories. This role is consistent with "Hebbian" descriptions of memory formation. LTP displays three fundamental properties: that of 'cooperativity, input specificity and associativity'. In one definition of cooperativity Bliss and Collingridge (1993) state that in order to induce LTP, high frequency stimulation of a critical number of fibres is required. Lynch *et al.*, 1977 described 'input specificity' as a phenomenon that occurs when LTP is initiated at one set

of synapses on a postsynaptic cell and adjacent cells which were not activated don't undergo LTP. And finally, 'associativity' describes the ability of a set of synapses to undergo LTP in response to subthreshold stimulation only if their activation is in tandem with an LTP inducing stimulus at another synapse on the same cell (Levy and Steward; 1979).

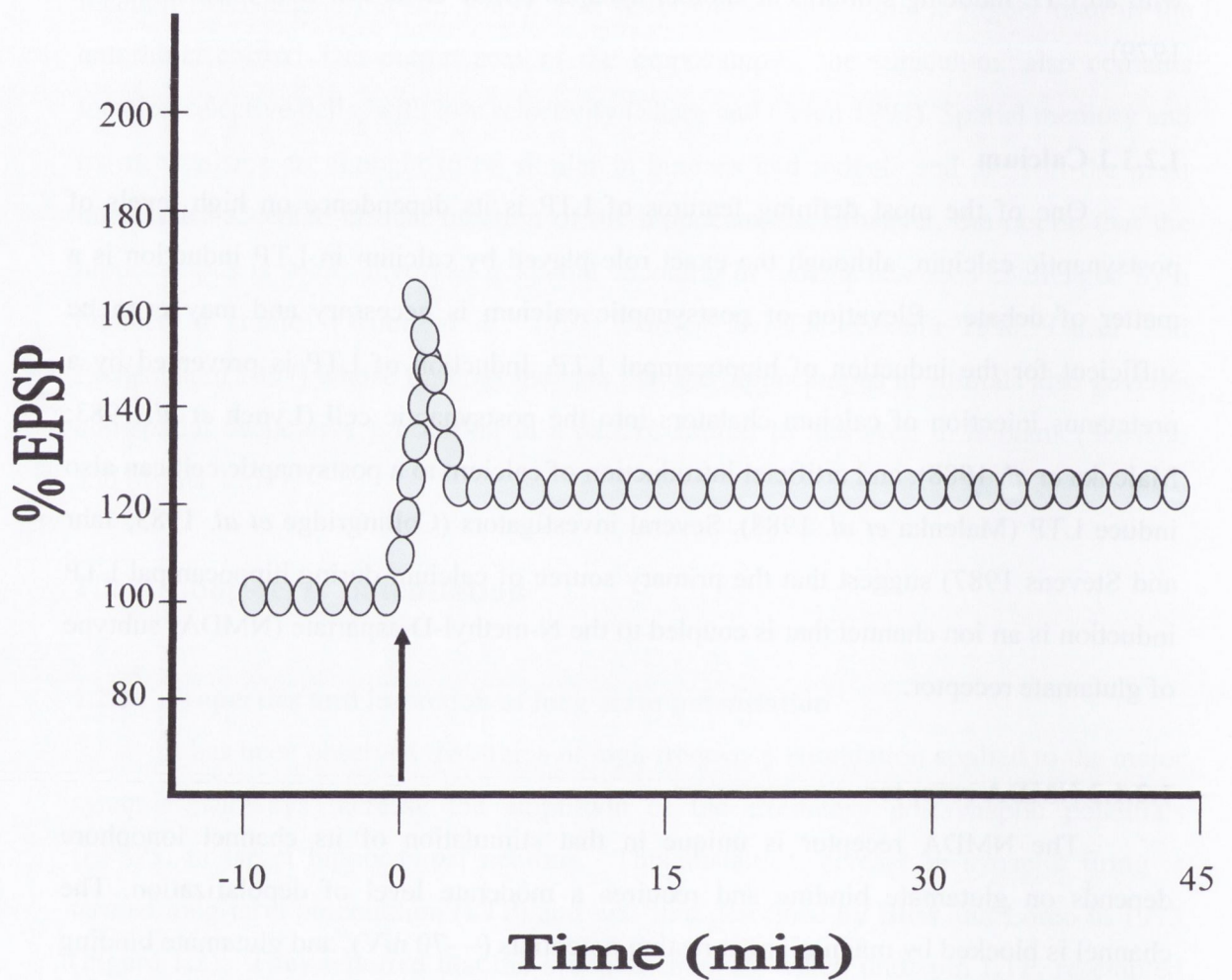
#### **1.2.1.1 Calcium**

One of the most defining features of LTP is its dependence on high levels of postsynaptic calcium, although the exact role played by calcium in LTP induction is a matter of debate. Elevation of postsynaptic calcium is necessary and may even be sufficient for the induction of hippocampal LTP. Induction of LTP is prevented by a pretetanus injection of calcium chelators into the postsynaptic cell (Lynch *et al.* 1983; Malenka *et al.* 1988), and artificial introduction of calcium to a postsynaptic cell can also induce LTP (Malenka *et al.* 1988). Several investigators (Collingridge *et al.* 1983; Jahr and Stevens 1987) suggest that the primary source of calcium during hippocampal LTP induction is an ion channel that is coupled to the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor.

#### **1.2.1.2 NMDA receptor**

The NMDA receptor is unique in that stimulation of its channel ionophore depends on glutamate binding and requires a moderate level of depolarization. The channel is blocked by magnesium at resting potentials ( $\sim -70$  mV), and glutamate binding alone is insufficient to open it. However, at depolarized membrane potentials ( $> -40$  mV), magnesium is expelled from the channel; it becomes sensitive to glutamate and highly selective for calcium ions causing it to open. Thus, the NMDA receptor complex is regulated by two factors: ligand and voltage.

A relatively long, high intensity presynaptic burst of activity (such as a high-frequency train of stimulation) can induce LTP by releasing glutamate onto the postsynaptic receptor. This then depolarizes the postsynaptic cell through stimulation of the non-NMDA type of glutamate receptors known as  $\alpha$ -amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptors. AMPA receptors in conjunction with kainate



**Figure 1.2 LTP**

A schematic of LTP recorded *in vivo*. The graph plots the slope of the rising phase of the evoked response (population EPSP), recorded from the cell body region in response to constant test stimuli, for 10mins before and 45mins following tetanus delivered at the time indicated by the arrow. EPSP = excitatory post synaptic potential

receptors give rise to the early phase of the EPSP. They both gate ion channels with low conductance and these channels allow for the passage of sodium and potassium but not calcium ions ( $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ ).

Shorter and more physiologically relevant levels of presynaptic activity can induce hippocampal LTP by stimulating the NMDA receptor with glutamate, while the postsynaptic cell is depolarized via an alternative means such as an input from a second afferent pathway. Other forms of LTP occur independently of the NMDA receptor, such as that induced in CA3 pyramidal cells following mossy fiber tetanization. LTP induced here is instead dependent on  $\text{Ca}^{2+}$  influx through voltage-gated channels. There is some debate as to whether the critical  $\text{Ca}^{2+}$  signal is pre- (Weisskopf *et al.* 1994; Castillo *et al.* 1994) or postsynaptic (Williams and Johnston 1989). A necessary role for calcium in LTP is, however, consistent with LTP's presumed role in learning. Calcium plays a critical role in many cellular modifications thought to underlie conditioned behavioral responses (Walters and Byrne, 1983; Abrams *et al.*, 1991; Matzel and Rogers, 1993).

### **1.2.1.3 Protein synthesis**

At least two phases of LTP can be distinguished, a) early LTP, that can be observed for up to 3 hours (h) and is protein synthesis-independent and b) late LTP, whose sustenance is more persistent and can be observed for a minimum of 24h. This second form of LTP is dependant on new protein and ribonucleic acid (RNA) synthesis. Experiments by Mullany and Lynch (1997) have shown that injection of protein synthesis inhibitors into rat hippocampus causes a shortening of the potentiation induced in the dentate gyrus following tetanic stimulation.

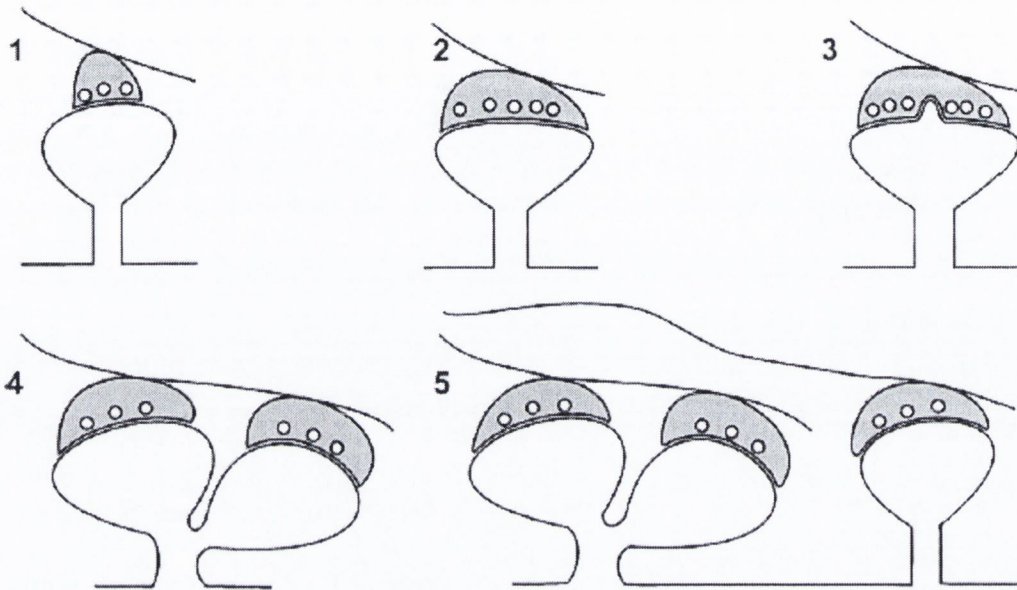
Yuste and Bonhoeffer (2001) propose 'a continuum of morphological events that can occur after the induction of LTP'. Changes observed range from slight enlargements of synaptic surfaces to the generation of completely new structures (Figure 1.3). The changes that occur at potentiated synapses consistent with early phase LTP are usually minor and functional in nature. These, it is suggested, include alterations in receptor composition (Malinow and Tsien, 1990) and changes in synaptic release properties (Nicoll and Malenka, 1999). It is thought that such changes occur within minutes (min) of potentiation; 30min following induction, the first detectable morphological changes

occur with the enlargement of spine heads (Desmond and Levy, 1990). As the late phase of LTP is dependant on a prolonged increase in synaptic strength, this enlargement in dendritic spine volume may provide the structural modification required for the changes in synaptic strength required for late phase LTP. Modifications in spine volume may also have an effect on calcium turnover thereby influencing conditions required for synaptic plasticity. Changes in the protein content in synapses following LTP may also be required as large synapses can bifurcate or perforate (Toni *et al.*, 1999), facilitating an enlargement in independent synaptic release sites as seen in some late phase LTP studies (Bolshakov *et al.*, 1997).

### **1.2.2 Inflammatory stress mediated signaling and LTP**

Short-term immunological stress in the central nervous system (CNS) is recognised as being often beneficial to an organism in that it facilitates appropriate activation of the immune response. Microglial cells comprise the network of endogenous immunocompetant cells that pervade the brain. Their primary function is to provide continuous surveillance of the parenchyma and protect the CNS during injury and disease. Microglia rapidly transform into an activated state in response to a variety of stimuli, including inflammation, immunological challenges and neurodegenerative changes. They respond to such stimuli by phagocytosing foreign material in an attempt to eliminate it and by producing inflammatory modulators such as cytokines or chemokines which further enhance the immune response. A prolonged CNS immune response can be damaging to the viability of neurons however, and can over extend the capabilities of their supporting network of cells (Nathan, 2002).

The part played by stressors in modulating hippocampal synaptic disruption has been investigated by studying their direct effects on synaptic mechanisms, especially LTP. Several studies indicate that LTP is impaired by a number of factors; including age (Martin *et al.*, 2002; Griffin *et al.*, 2006), oxidative stress (Kamsler and Segal, 2003; Rowan *et al.*, 2004), lipopolysaccharide (LPS) (Vereker *et al.*, 2000) the Alzheimer's disease (AD) associated protein amyloid- $\beta$  (A $\beta$ ) (Lyons *et al.*, 2007) and pro-inflammatory signaling molecules produced by activated microglia such as interleukin-1 $\beta$  (IL-1 $\beta$ ) (Bellinger *et al.*, 1993; Katsuki *et al.*, 1990; Cunningham *et al.*, 1996). The



**Figure 1.3 Possible sequences of events following LTP which require protein synthesis or rearrangement**

The post-LTP sequence of events may occur as follows: 1) within minutes; functional changes that are undetectable morphologically e.g. changes in receptor composition. 2) 30min post-induction; the first morphological changes occur with an enlargement of stimulated spine heads. 3 and 4) Synapses that were already large break apart and bifurcate and finally, 5) new spines form in the vicinity of activated spines. (Adapted from Yuste and Bonhoeffer (2001) *Annu. Rev. Neurosci*, **24**:1071–89).

inhibitory effects of these multiple factors in hippocampus have been linked with stimulation of the stress-activated kinase c-Jun-N-terminal kinase (JNK) (O'Donnell *et al.*, 2000; Vereker *et al.*, 2000; Nolan *et al.*, 2002; Minogue *et al.*, 2003; Wang *et al.*, 2004; Barry *et al.*, 2005; Griffin *et al.*, 2006 ). Among the documented consequences of enhanced activity of JNK are cell growth arrest and deterioration of cell function or even cell death (Chen *et al.*, 1996; Wu and Ng, 2007).

Barry and colleagues (2005) demonstrated that anti-inflammatory treatment (IL-4) and/or JNK inhibition (D-JNKI1) blocked the LPS-induced increases in IL-1receptorI (IL-1RI) expression and associated increases in phosphorylation of JNK. Both IL-4 and D-JNKI1 inhibited an LPS-induced increase in caspase-3 staining and abrogated the LPS-induced inhibition of LTP in perforant path-granule cell synapses. Nolan and colleagues also found that anti-inflammatory treatment (IL-10 or phosphatidylserine containing liposomes) reversed LPS-induced impairment in LTP and inhibited JNK activation in hippocampal homogenate. A $\beta$  also induces inhibition of LTP (Lyons *et al.*, 2006). The authors found the inhibition to be associated with increases in expression of markers of microglial activation such as major histocompatibility complex II (MHCII) as well as JNK phosphorylation and IL-1 $\beta$  concentration. Lyons and colleagues (2006) also found that these changes were again attenuated by anti-inflammatory treatment (IL-4). The authors suggest that their data show that glial cell activation and the consequent increase in IL-1 $\beta$  concentration mediate the inhibitory effect of A $\beta$  on LTP and indicate that anti-inflammatory treatment by down-regulating glial cell activation, antagonizes the effects of A $\beta$ .

### **1.3 Microglia**

Microglia are considered to be the resident immune cells of the CNS, they turn over slowly and are replenished by proliferation in the adult CNS (Vilhardt, 2005). The relationship between microglial activation and neurodegeneration remains unclear, although several cytokines and inflammatory mediators produced or up-regulated by

stimulated microglia can initiate or worsen the progression of neuropathology (Meda *et al.*, 2001). Several studies have reported that increased microglial activation is observed in neurodegenerative diseases such as AD (Benveniste *et al.*, 2001; O'Keefe *et al.*, 2002; Tan *et al.*, 2002). Microglia are derived from myeloid cells, and comprise approximately 12% of cells in the brain and constitute approximately 20% of the total glial population. Their densities vary from region to region in the brain but predominate in the grey matter with highest densities to be found in the hippocampus. Electron microscopy shows the surface of microglia to be covered with spines, a feature not seen in other macrophage cell types. Following brain injury the otherwise low infiltration of peripheral monocytes/macrophage increases, despite this, microglia remain the predominant immune cell type in the brain because of their ability to proliferate and their active migration towards sites of injury. Activated microglia may be classified into morphological subtypes (resting, enlarged and phagocytic-amoeboid) that represent successive stages of activation (Sheng *et al.*, 1995; Perry *et al.*, 2007) (Figure 1.4). In the non-compromised healthy brain they function as supportive glial cells with their activation being regulated by neurons through soluble mediators and cell-cell contact.

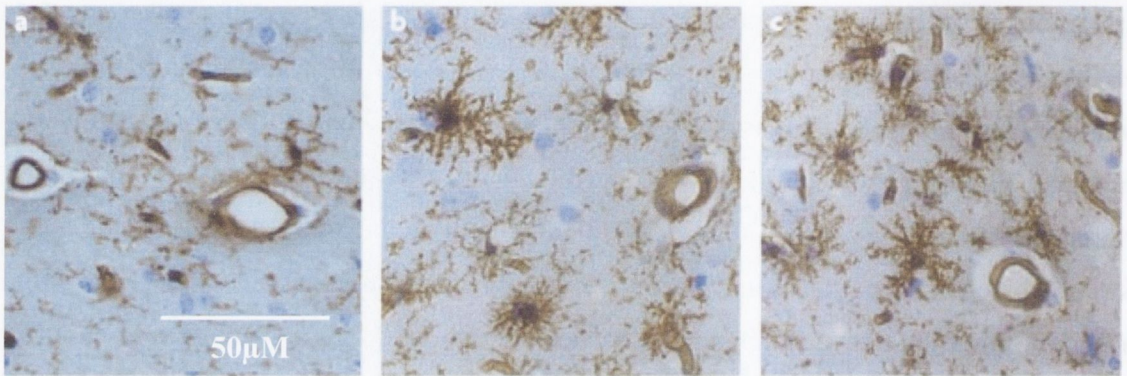
### **1.3.1 Microglia and brain development**

Microglia play a role in brain development. In the developing brain amoeboid microglia enforce the programmed elimination of neural cells by phagocytosing them. Microglia have been shown to directly determine cell fate. In experiments conducted by Marin-Teva and colleagues (2004), it was shown that microglia provoke the death of developing Purkinje cells by means of a superoxide-dependant mechanism. Microglia are also promoters of the migration, axonal growth and terminal differentiation of differing neuronal subsets. They enhance neuronal survival through the release of trophic and anti-inflammatory factors and also via direct cell-cell contact (Polazzi and Contestabile, 2002; Streit *et al.*, 2004).

### **1.3.2 Cluster of Differentiation 200**

The resting brain comprises microglia whose phagocytic activity is for the most part down-regulated. Cross-talk between neurons and microglia is believed to be a very





**Figure 1.4 Morphological changes displayed by activated microglia**

In the normal, mature brain microglia are in resting stage and appear morphologically ramified (a), in times of stress microglia display activated amoeboid morphology indicative of phagocytic activity (b and c). Pictures adapted from: Perry H.V, Cunningham C., Holmes C. (2007) Systemic infections and inflammation affect chronic neurodegeneration [Internet], Nature Publishing Group; ©2008 [cited 23 Jan 2008]. Available from [www.nature.com/.../v7/n2/full/nri2015.html](http://www.nature.com/.../v7/n2/full/nri2015.html)

important factor in maintaining microglia in a quiescent state. This cross-talk is thought to be governed by a number of cell surface proteins, cluster of differentiation 200 (CD200 for example). CD200 is a neuronal membrane protein that interacts with the myeloid cell receptor CD200R to dampen microglial activity. Hoek and colleagues (2000) have shown that mice deficient in CD200 display morphological and molecular evidence of increase microglial activation. Indeed, our own lab supports this hypothesis, with both *in vivo* and *in vitro* evidence for CD200 regulation of microglial activation (Lyons *et al.*, 2007).

### **1.3.3 Synaptic modulation**

Microglia have also been implicated in the control of synaptic modulation. Synapses are differentiated regions of adhesion between neurons and it has long been known that cell adhesion and apoptosis are functionally linked (Frisch and Francis, 1994). It has been suggested that microglia, activated by damaged neurons, may locally instruct the remaining non-lesioned neurons to form compensatory synapses. (Bessis *et al.*, 2007).

### **1.3.4 Cytokines**

A corollary of the neuroinflammation hypothesis is that suppression of neurotoxic products produced by persistent glial activation will result in neuroprotection. Amongst these products are cytokines; they are multifunctional proteins, which serve as humoral regulators in the body governing the functioning of individual cells as well as tissues. The numerous cytokines affecting the CNS have two possible origins. They could either be produced peripherally, crossing the blood brain barrier (BBB) to exert their effects, or they may be produced by glial cells and to lesser extents by neurons within the brain. Cytokine expression is tightly regulated and is usually only produced following cell activation in response to an induction signal. Within the CNS, both pro- and anti-inflammatory cytokines increase their expression following injury or the onset and progression of disease. The initial inflammatory response, characterized by intense glial activation, serves to remove pathogenic triggers and inhibit the neurodegenerative process.

#### 1.3.4.1 IL-1 $\beta$ plays a role in neurodegeneration

Among the number of cytokines most extensively studied is IL-1 $\beta$ . IL-1 $\beta$  is produced as a biologically inactive precursor, pro-IL-1 $\beta$ , which must be cleaved to release the active form. Plata-Salaman and collaborators (1988) were among the first to show that IL-1 $\beta$  affects activity of neurons in the brain and that these effects were biologically relevant. Intracerebral injection of IL-1 $\beta$  markedly exacerbates ischemic and excitotoxic brain damage following middle cerebral artery occlusion in mice (McColl *et al.*, 2007). Inhibition of endogenous IL-1 by means of the natural antagonist IL-1ra markedly diminishes several forms of neuronal damage following focal cerebral ischemia in rat (Loddick and Rothwell, 1996). The involvement of cytokines such as IL-1 $\beta$  in exacerbating neuronal damage has also been shown *in vitro*. Viviani and colleagues (2001) showed that glial cells exposed to a HIV-associated toxin (gp120) up-regulated their IL-1 $\beta$  production. IL-1 $\beta$  production was shown in this case to be directly and specifically involved in neuronal death; again, this was completely prevented by using neutralizing antibodies directed against IL-1 $\beta$ .

Koo and Duman (2008) showed that IL-1 $\beta$  is directly antineurogenic in the brain, with particular reference to the hippocampus. Administration of IL-1 $\beta$  suppressed hippocampal cell proliferation. Blockade of the IL-1 $\beta$  receptor, IL-1RI, in rat, by using either an inhibitor or IL-1RI null mice blocks the antineurogenic effect of IL-1 $\beta$  and blocks the anhedonic behavior caused by chronic stress in rats. Additional *in vivo* and *in vitro* studies demonstrated that hippocampal neural progenitor cells express IL-1RI and that activation of this receptor decreased cell proliferation via the nuclear factor- $\kappa$ B signaling pathway. These findings demonstrate that IL-1 $\beta$  is a critical mediator of the antineurogenic and depressive-like behavior caused by chronic stress and inflammation within the CNS.

Garvilán and colleagues (2007) showed a direct link between hippocampal neurodegeneration and IL-1 $\beta$ -mediated neuroinflammation in aged rats. They observed an increase in the mRNA expression of IL-1 $\beta$  and TNF-alpha, and the iNOs enzyme in aged hippocampus. In addition, numerous activated microglial cells were observed in aged rats. These cells were differentially distributed along the hippocampus, being more

frequently located in CA3 in particular. Concomitantly, the mRNA expression of somatostatin, a neuropeptide expressed by some GABAergic interneurons, and the number of somatostatin-immunopositive cells decreased in the hippocampus of aged rats. These represent the most vulnerable population of cells, in the hippocampus, to the aging process (Vela *et al.*, 2003; Cadiacio *et al.*, 2003). However, the number of hippocampal parvalbumin-containing GABAergic interneurons was preserved thus displaying selective age / inflammation-related degeneration of neurons in the CNS. Finally, intraperitoneal chronic LPS injection in young animals mimicked the age-related increase in hippocampal inflammation as well as the decrease of somatostatin mRNA expression. These results strongly support IL-1 $\beta$  driven neuroinflammation as a potential factor involved in age-related hippocampal neurodegeneration.

#### **1.4 Alzheimer's Disease**

Today, more than 35,000 people in Ireland have dementia, of which AD is the most common form (Alzheimer's Society of Ireland, 2007). AD is a disease characterized by a progressive loss of cognitive functions including memory, language, praxis, judgment, and orientation. Historically, AD diagnosis was made after exclusion of all other possible causes of dementia, including metabolic imbalances, such as vitamin deficiencies, or other neurological disorders with more definitive diagnostic criteria. Among the most commonly used criteria are those described in the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 2000) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (McKhann *et al.*, 1984).

Modern diagnostic criteria for AD require that a patient be given a diagnosis of AD only if they have a progressive loss of memory and at least one other cognitive function sufficient to interfere with social or occupational functioning. Two proven risk factors for AD are age and family history. It has been estimated that ~5% of the over 65s population is affected by AD. This number is thought to double with every 5 years beyond 65 and as many as 50% of over 85s are thought to be affected by AD to varying degrees (Forsyth and Ritzline, 1998). AD advances in stages, progressing from mild

forgetfulness and cognitive impairment to widespread loss of mental abilities. In advanced AD, people become dependent on others for every aspect of their care. The time course of the disease varies by individual, ranging from five to 20 years with the most common cause of death being disease-related infection.

AD can only definitively be diagnosed post mortem. At autopsy, the brain of a typical AD patient reveals a macroscopically visible cerebral atrophy involving brain regions implicated in learning and memory processes, including the temporal, parietal and frontal cortex as well as the hippocampus and amygdala. This brain volume reduction is due to a profound degeneration of neurons and synapses (Mattson, 2004). Other brain cells types affected in these areas include: microglia cells, which represent the brain innate immune system and astrocytes, whose role it is to maintain optimal brain homeostasis with respect to maintaining neuronal viability.

In addition to a marked reduction in brain volume in AD, post mortem examination of the brain shows two of the disease's most common histopathological hallmarks, A $\beta$  protein loaded plaques, which will be discussed further below, and neurofibrillary tangles, which consist of the filament protein tau. Tau, a highly asymmetric and heat-stable protein, is expressed mainly in the brain, where it regulates the orientation and stability of microtubules in neurons, astrocytes and oligodendrocytes. Tau is highly enriched in the axon where it is involved with anteretrograde transport.

Tau-bearing lesions appear initially in the entorhinal cortex and hippocampus, then progress to neocortex of the frontal and temporal poles, and finally involve much of the temporal, frontal, and parietal cortices. A significant consequence of Tau hyperphosphorylation in AD is a reduction in its ability to bind microtubules and promote microtubule assembly. In AD, normal soluble cytoskeletal elements, such as Tau and neurofilaments are transformed into insoluble paired helical filaments. This is linked to the post-translational change in Tau, primarily the hyperphosphorylation of Tau by a number of protein kinases. Hyperphosphorylated Tau may contribute to a destabilized microtubule network, impaired axonal transport, and ultimately in neurofibrillary tangle formation and neuronal death associated with the disease.

In addition to dysregulation in protein processing, AD is also associated with imbalances in neurotransmitter concentration in the brain. Acetylcholine is widely

distributed in the nervous system and has been implicated to play a critical role in cerebral cortical development, cortical activity as well as in modulating cognitive performances and learning and memory processes (Schliebs and Arendt, 2006). Over 30 years ago a number of research groups reported a severe deficiency in presynaptic cholinergic markers in the cortex of patients with early AD (Bowen *et al.*, 1976; Perry *et al.*, 1977). Correlation between clinical dementia scores with reductions in brain acetyltransferase, acetylcholine or acetylcholine receptor binding (Bierer *et al.*, 1995; Gsell *et al.*, 1993; Nordberg, 1992) suggested an association between cholinergic hypofunction and cognitive decline, which led to the development of the cholinergic hypothesis of memory dysfunction in AD (Bartus, 2000). The presence of acetylcholine is not only necessary for the above-mentioned processes, but can also ameliorate learning deficits and restore memory following the damage to the basalis magnocellularis, the brain area that provides the major cholinergic innervation of the neocortex (Winkler *et al.*, 1995). However, whilst there is no doubt that loss of cortical cholinergic innervation exists in the late stages of AD its role in the initial stages or in mild AD has been debated (Mesulam, 2004).

#### **1.4.1 Amyloid- $\beta$ and Alzheimer's disease**

As a chronic neurodegenerative disease AD causes progressive deterioration in cognitive function. One of the hallmarks of the disease is the accumulation of A $\beta$  peptide in the brain and its deposition as extracellular plaques. Selkoe (2002) describes AD as a syndrome, which in its earliest clinical phase produces a remarkably pure impairment of memory. The author suggests that AD begins with subtle A $\beta$ -induced alterations of hippocampal synaptic efficacy prior to frank neuronal degeneration. In support of this a number of research groups have shown that A $\beta$  impairs hippocampal synaptic plasticity, as evidenced by an A $\beta$ -induced attenuation of LTP in aged transgenic mice and in rat (Chapman *et al.*, 1999; Minogue *et al.*, 2003; Lyons *et al.*, 2007) and *in vitro* (Freir *et al.*, 2003; Townsend *et al.*, 2006).

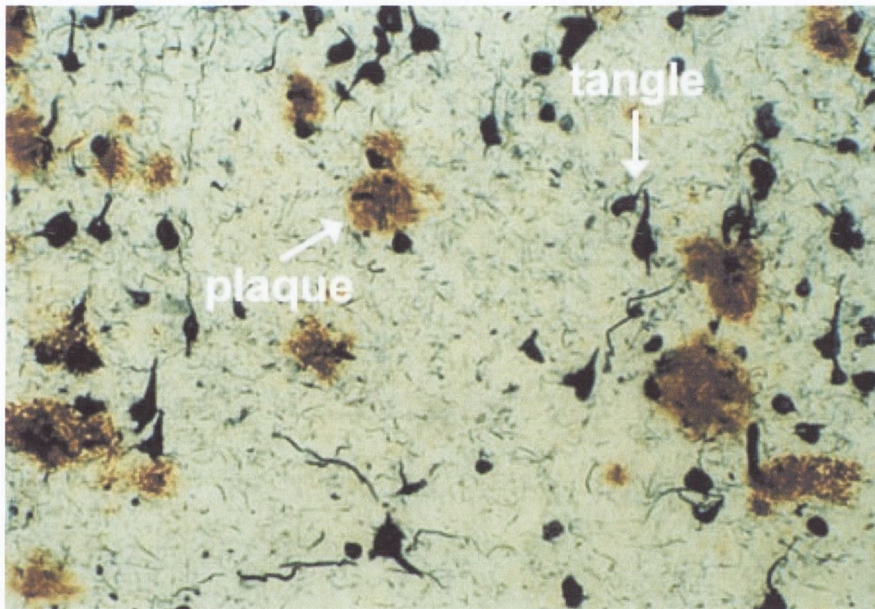
Ageing, a major risk factor for AD is associated with impairments in learning and memory and its effects are comparable to impairments induced by hippocampal damage. Electrophysiological studies indicate that the ageing process alters the hippocampal

function and deficits in LTP in aged rats have been reported several times (Almaguer *et al.*, 2002; Griffin *et al.*, 2006; Lynch *et al.*, 2007). AD is the most prevalent form of dementia in the elderly. After the age of 60, the risk of AD increases exponentially doubling every five years (Finch and Morgan, 2003). The current most common treatment for AD sufferers includes use of acetylcholinesterase (AChE) inhibitors to improve cognitive function (Doody *et al.*, 1999) and an NMDA antagonist, memantine. Both forms of treatment have been shown to rescue A $\beta$ -impaired LTP in rat hippocampus, in humans and in transgenic mice (Ye and Qiao, 1999; Reisberg *et al.*, 2003; Minkeviciene *et al.*, 2004; Geerts and Grossberg, 2006) Whilst these treatment regimes are not harmful to the individual they only serve to alleviate symptomatic manifestations of AD i.e. memory loss, with little or no disease modifying effect.

One central point of interest in AD research is the role that A $\beta$  peptides play in the pathogenesis of dementia. A $\beta$  is a metabolic product of the transmembrane protein amyloid precursor protein (APP) (Hardy and Selkoe, 2002). APP is cleaved by  $\alpha$  and  $\gamma$  secretases yielding A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub>. The latter species of A $\beta$  is highly hydrophobic and has been particularly implicated in causing AD. AD is diagnosed histopathologically by the presence, in sufficient numbers, of extracellular deposits of A $\beta$  fibrils and plaques, and intracellular accumulations of tau protein commonly called tangles (Figure 1.5). There is much debate as to which comes first the tangle or the plaque (Duyckaerts, 2004). In many cases tangles in the entorhinal cortex are the first morphological lesions to be detected with age but these findings cannot be generalized to the whole brain. In many subjects, who have been evaluated to be at the threshold of dementia, high densities of predominantly diffuse senile plaques are observed (Morris *et al.*, 1996).

#### **1.4.2 The role of inflammation in AD; a clinical perspective**

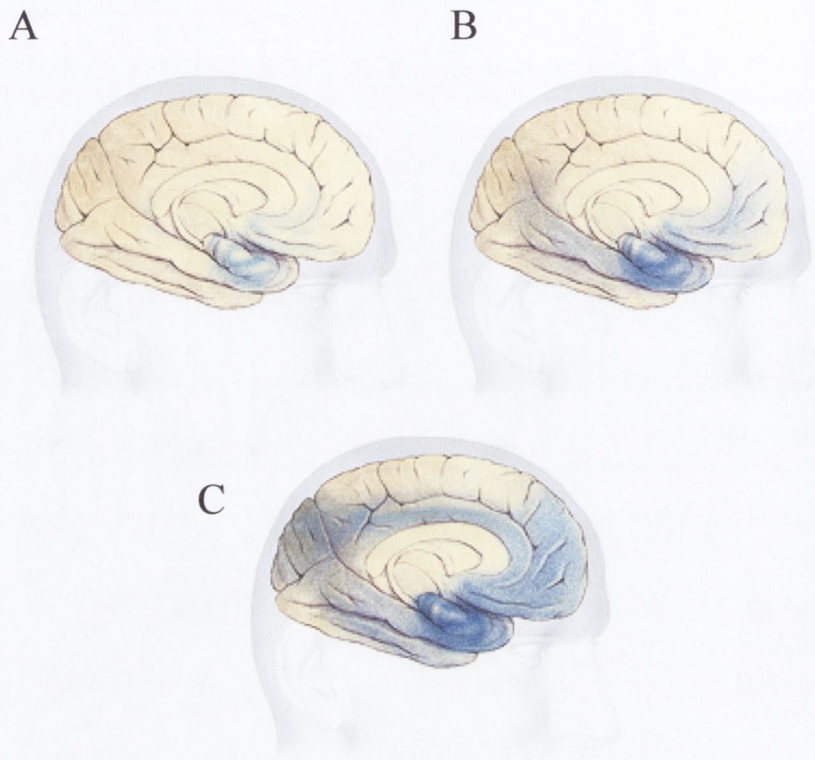
The regions of the brain most affected by AD are the temporal lobe and, in particular, the hippocampus, the seat of declarative memory functioning (Figure 1.6). A $\beta$  peptide accumulation in the hippocampus initiates local inflammation. The chronic inflammatory nature of the disease results in persistent microglial activation in the brain leading to synaptic dysfunction and neuronal loss thus giving rise to the clinical symptoms of the disease (Stephan and Phillips, 2005). A correlative study by Arends and



**Figure 1.5 Plaques and tangles in the AD brain.**

AD is pathologically characterized by the presence of extracellular amyloid plaques (composed of aggregated amyloid- $\beta$  peptide) and intracellular neurofibrillary tangles (consisting of aggregated tau proteins). University of Illinois at Chicago, 909 S Wolcott Rm 7060, Chicago Illinois 60612; [updated 03/16/2006; cited 23/01/2008]. Available from: <http://ladulab.anat.uic.edu/>





**Figure 1.6 Gradual spread of amyloid load over the progression of AD**

The disease is characterized by the gradual spread of plaques that disrupts the delicate organization of nerve cells in the brain. As brain cells stop communicating with one another, they atrophy causing memory and reasoning to fade. A. Plaques first appear in the entorhinal cortex, an essential memory-processing centre needed for making new memories and retrieving old ones. B. Over time they move higher, invading the hippocampus, the part of the brain that forms complex memories of events or objects. C. Finally the plaques reach the top of the brain, or neocortex, the "executive" that sorts through stimuli and orchestrates all behaviour. Alzheimer's Association Web Site [Internet]; © 2008 [cited 24 Jan 2008]. Available from: [www.alz.org/brain](http://www.alz.org/brain).

colleagues (2000) investigated the relative volume of tissue occupied by activated microglia and A $\beta$  deposits in neocortex of clinically well evaluated patients. The volume density of activated microglia (CD68<sup>+</sup>) correlated highly with the volume density of congophilic deposits but not with the volume density of A $\beta$ . This association between microglia and congophilic deposits peaked in those individuals diagnosed as moderately affected cases, whereas A $\beta$  steadily accumulated with progression of the disease.

Another pathological study, conducted by Vehmas and colleagues (2003), confirmed the association of microglia and congophilic amyloid, and also reinforced the idea that the prevalence of activated microglia were significantly increased in early stages of the disease. They also found that the clinical association between astrocytic activation and the clinical manifestation of dementia suggested that astrocyte activation occurs in later stages of the disease when dementia is evident.

### 1.4.3 Physiological functions of APP

APP is an integral membrane protein; it is ubiquitously expressed in many tissues and in mammalian brain is concentrated in the synapses of neurons. APP-like proteins have been described in evolutionarily distant organisms including *Caenorhabditis elegans* (*C.elegans*) and *Drosophila melanogaster*. In humans three isoforms exist, producing proteins of different length – 695, 751 and 770 amino acids long. APP695 is the most abundant in the brain. Currently there is little consensus as to the functioning of APP in the nervous system. It has been described as having sequence elements that are indicative of a housekeeping gene (Pollwein *et al.*, 1992). Such genes are functionally important in all cell types, irrespective of the specialized role of the cell. It has also been argued that APP's actions may depend on the cell type in which it is expressed.

Some experiments have shown it to have neurotrophic properties and to support synaptogenesis; this is thought to spring from its adhesive properties. E1 and E2 of the APP ectodomain interact with extracellular matrix proteins and heparin soluble proteoglycans thereby supporting cell substratum adhesion (reviewed by Small *et al.*, 1999). APP forms anti-parallel dimers shown to function in trans cellular adhesion (Wang *et al.*, 2004). APP is thought to support neurite outgrowth both as a whole transmembrane protein and in soluble form, giving it both auto- and paracrine functions

and its expression is up-regulated during neuronal maturation and differentiation and its expression is rapidly induced following traumatic brain injury in mammals (Hung *et al.*, 1992; Murakami *et al.*, 1998; Bibel *et al.*, 2004).

APP undergoes rapid anterograde transport and is targeted to synaptic sites, where levels of secreted APP coincide with synaptogenesis (Moya *et al.*, 1994). Indications that APP supports cell growth came from observations of fibroblasts treated with antisense APP constructs. These cells grew slower and the growth retardation was restored by treatment with secreted APP (Saitoh *et al.*, 1989). In addition, Zheng and Colleagues (1995) generated APP null mutation mice in an effort to understand the *in vivo* function of APP and its processing. APP<sup>-/-</sup> mice were viable and fertile but were 15-20% smaller than age-matched controls. Mutant mice displayed decreased locomotor activity and forelimb grip strength indicating compromised neuronal and/or muscular function. These mice also exhibited reactive gliosis, suggestive of neuronal damage in brain activity. Subsequent analysis of these APP<sup>-/-</sup> mice revealed that they performed poorly in Morris water maze tasks and were also defective in LTP and GABA-mediated postsynaptic responses (Dawson *et al.*, 1999; Phinney, 1999; Seabrook *et al.*, 1999).

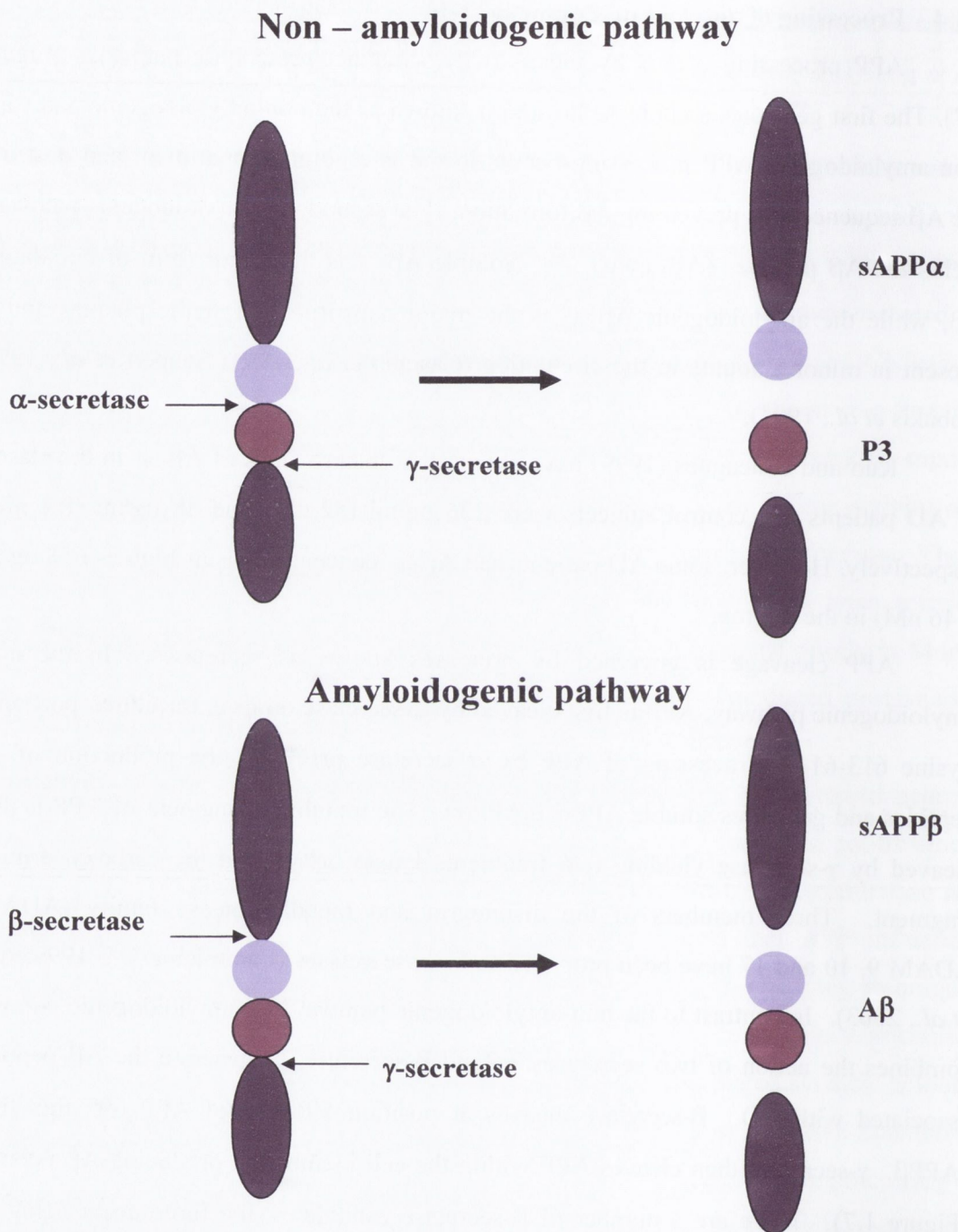
Mattson and colleagues (1993, 1994) have shown that rat hippocampal neurons when treated with APP, display decreased concentrations in Ca<sup>2+</sup> and that pre-treatment of the neurons with APP reduces glutamate-mediated increases in Ca<sup>2+</sup> concentration and associated toxicity. Furukawa and colleagues (1996) reported that APP influences synaptic plasticity in cultured hippocampal neurons. Using patch-clamping techniques the authors showed that APP was capable of activating high conductance potassium channels, leading ultimately to suppressed glutamate receptor-mediated synaptic activity. Seabrook and colleagues (1999) reported age-dependant deficits in cognitive function in APP null mice as well as impairments in LTP. It appears that inappropriate processing of APP, such as occurs in AD, may contribute to cognitive decline, firstly by increasing the production of neurotoxic A $\beta$  peptides and secondly by decreasing the relative concentrations of neuroprotective full length APP or the pool of soluble APP $\alpha$  fragments.

#### 1.4.4 Processing of amyloid precursor protein

APP processing occurs by means of two distinct proteolytic pathways (Figure 1.7). The first generates soluble APP $\alpha$  and is known as the non-amyloidogenic pathway. Non-amyloidogenic APP processing occurs in the late golgi compartment and destroys the A $\beta$  sequence thus preventing A $\beta$  formation. The second, the amyloidogenic pathway, yields the A $\beta$  peptides (A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>). Soluble A $\beta$ <sub>1-40</sub> is the major form of circulating A $\beta$ , while the amyloidogenic A $\beta$ <sub>1-42</sub> is the major constituent of senile plaques and is present in minor amounts in the circulation (Castano *et al.*, 1991; Seubert *et al.*, 1992; Robakis *et al.*, 1994),

Kuo and colleagues (1999) have shown that mean values of A $\beta$ <sub>1-42</sub> in the plasma of AD patients and control subjects were 236 ng/ml (52 nM) and 38 ng/ml (8.4 nM), respectively. However, some AD patients had A $\beta$ <sub>1-42</sub> concentrations as high as 658 ng/ml (146 nM) in the plasma.

APP cleavage is governed by proteases known as secretases. In the non-amyloidogenic pathway, APP is first cleaved by  $\alpha$ -secretase on its extracellular portion at Lysine 613-614. Processing of APP by  $\alpha$ -secretase precludes the production of A $\beta$  peptides and generates soluble APP $\alpha$  (sAPP $\alpha$ ). The remaining fragment of APP is then cleaved by  $\gamma$ -secretase yielding two fragments known as p3 and the carboxy-terminal fragment. Three members of the disintegrin and metalloprotease family (ADAM) ADAM 9, 10 and 17 have been proposed to be  $\alpha$ -secretases (Lammich *et al.*, 1999; Asai *et al.*, 2003). In contrast to the non-amyloidogenic pathway, the amyloidogenic pathway combines the action of two secretases,  $\gamma$ - and  $\beta$ -secretase, to generate the A $\beta$  peptides associated with AD.  $\beta$ -secretase cleaves at position 596-597 of APP 695 this frees sAPP $\beta$ .  $\gamma$ -secretase then cleaves APP within the cell membrane, producing A $\beta$  peptides (Figure 1.7). There are a number of  $\beta$ -secretase candidates, the forerunners being the aspartyl proteases  $\beta$ -APP cleaving enzymes 1 and 2 or BACE 1 and 2 (Farzan *et al.*, 2000). The presenilins and nicastrin are considered as possible candidates for  $\gamma$ -secretase (Wolfe *et al.*, 1999, Esler *et al.*, 2002). It is thought that  $\gamma$ -secretase activity requires the formation of a complex (Leem *et al.*, 2002).



**Figure 1.7 Schematic drawing of the processing of APP**

(Adapted from Minogue (2005) Analysis of the effects of Beta-Amyloid and age on cell signalling in rat hippocampus: effect of treatment with polyunsaturated fatty acids; PhD Thesis).

## 1.4.5 Amyloid- $\beta$ toxicity and the central nervous system

Papers first published by Fischer in 1907 and 1910, described plaque formation in the brains of patients who suffered from dementia. He stated that plaque formation resulted from the deposition of a foreign substance in the cortex that provokes a local inflammatory response, which spurs the regenerative response of the surrounding nerve fiber. It was only in the last 25 years of the 20<sup>th</sup> century that this ‘foreign substance’ was identified as the A $\beta$  peptide by Glenner and Wong (1984).

### 1.4.5.1 Pathogenic mechanisms in Alzheimer’s disease

There are multiple mechanisms that contribute to the pathogenesis and progression of AD. Causative mechanisms include genomics, A $\beta$  production, the inhibition of A $\beta$  removal from the brain and the transformation of A $\beta$  to toxic forms through aggregation. In addition, A $\beta$ -induced oxidative stress and inflammation also contribute to the severity of AD. Aggregation of A $\beta$  into fibrils follows multiple conformational changes including trimer, pentamer, or higher molecular weight complex formation, also known as A $\beta$ -derived diffusible ligands (ADDLs) (Lambert *et al.*, 1998) as well as A $\beta$  oligomers (A $\beta$ O) composed of 15–20 monomers (Kayed *et al.*, 2003), protofibrils (Nguyen and Hall, 2004), and dodecameric oligomers (Lesne’ *et al.*, 2006). These intermediate A $\beta$  species are designated collectively “soluble A $\beta$ ” (Glabe, 2004). Soluble A $\beta$  species are found in the cerebrospinal fluid (CSF) of AD patients (Kuo *et al.*, 1996; Georganopoulou *et al.*, 2005), can be neurotoxic at low concentrations, and induce inhibition of LTP and cognitive dysfunction in rodents (Lambert *et al.*, 1998; Hartley *et al.*, 1999; Dahlgren *et al.*, 2002; Walsh *et al.*, 2002; Lesne’ *et al.*, 2006). Most relevant, the brain levels of soluble A $\beta$  species are thought by some to correlate better than density of plaque deposition with severity of cognitive impairment (Lue *et al.*, 1999; Naslund *et al.*, 2000). Klyubin and colleagues (2005) show that intracerebroventricular (icv) injection of naturally secreted human A $\beta$  inhibits LTP in rat hippocampus *in vivo* and that a monoclonal antibody to A $\beta$  completely prevented the inhibition of LTP when injected following A $\beta$  administration. Size fractionation showed that A $\beta$ O, not monomers or fibrils, were responsible for inhibiting LTP in these animals. There is much debate as to

whether soluble assemblies of A $\beta$  or the later stage aggregates of A $\beta$  peptides contribute more to the pathology of AD but it is in no doubt that both contribute to aberrant nerve cell signaling and synaptic failure with the ultimate outcome being nerve cell death.

#### **1.4.5.2 Defective amyloid- $\beta$ clearance and plaque formation**

Hypoperfusion affects the formation and absorption of artificial CSF (aCSF), which in turn further exacerbates the slow down in A $\beta$  clearance and promotes A $\beta$  deposition thus initiating a vicious cycle in the human brain (Chakravarty, 2004). Chronic vascular hypoperfusion in the human brain has been shown to induce mitochondrial failure resulting in oxidative damage, this in turn drives caspase-3-mediated A $\beta$  peptide secretion and enhances amyloidogenic APP processing, creating neurotoxic processes which accelerate one another (reviewed by Aliev and colleagues, 2003). Lesne and colleagues (2003) have shown in transgenic mice that distinct from its role in microglial activation, A $\beta$  can also activate astrocytes, which adds to the oxidative imbalance in the CNS (Lesne *et al.*, 2003). They found that this oxidative imbalance up-regulates the expression of APP in astrocytes and that astrocytes are capable of expressing the putative  $\beta$ -secretase, BACE1. In addition to microglial activation, these 3 factors could further accelerate the larger cycle of cholinergic neuron destruction seen in AD.

#### **1.4.5.3 Amyloid- $\beta$ - microglial interaction in Alzheimer's Disease**

Although it is now acknowledged that the formation of fibrillar forms of A $\beta$  play a crucial role in the etiology of AD, the presence of diffuse deposits of A $\beta$  in the brains of non-demented elderly and in brain regions not normally associated with the disease such as the cerebellum suggests that the deposition of A $\beta$  by itself is not sufficient to produce AD associated clinical symptoms (Rozemuller *et al.*, 1990). The role of inflammation in the pathology of AD has garnered support from studies indicating the efficacy of anti-inflammatory drugs that have proven effective in retarding the development of the

disease (Rogers *et al.*, 1996; McGeer and McGeer, 2007). During the past 20 or more years a number of inflammatory proteins have been reported to be associated with A $\beta$  plaques; McGeer and colleagues (1989) showed the activation of the terminal membrane attack complex in senile plaques. Several groups have indicated, by means of monoclonal antibodies directed against cells of the monocyte-macrophage cell line, an immunohistochemical association between senile plaques and clusters of activated microglia (McGeer *et al.*, 1987; Rogers *et al.*, 1988), which would be indicative of a plaque-induced inflammatory response. In fact, A $\beta$  has been shown to both recruit and activate microglia (Davis *et al.*, 1992; Meda *et al.*, 1995; Sasaki *et al.*, 1997) (Figure 1.8).

Experiments conducted by Weldon and colleagues (1998) showed that a single injection of fibrillar A $\beta$  (fA $\beta$ ) into the rat striatum was stable *in vivo* for up to 30 days whereas soluble A $\beta$  was cleared within 1 day. Following injection of fA $\beta$  microglial activity increased in the form of phagocytosis and the surrounding astrocytes formed a protective wall between the fA $\beta$  containing microglia and the surrounding neuropil. The activated glia also displayed elevated levels of inducible nitric oxide synthase (iNOS) expression and caused a significant loss in neuronal number. These data, they believe, demonstrate that fA $\beta$  neurotoxicity is mediated, in large, by factors released from activated glia as opposed to direct interaction between A $\beta$  fibrils and neurons.

#### **1.4.6 Amyloid- $\beta$ induces microglial activation**

One of the main targets of AD immunologic research has been the characterisation of the pathological stimuli in senile plaques that lead to microglial activation. The first and the most widely investigated of these stimuli is A $\beta$ .

A $\beta$  peptides have been shown to be amyloidogenic under experimental conditions and have been shown to be lethal to neurons in the presence of microglia (Meda *et al.*, 1995). Studies examining the structure and make up of the A $\beta$  peptide have been performed to identify the specific regions required for neurotoxicity and glial activation. Several reports have identified the amino terminus of A $\beta$ <sub>1-42</sub> as being one of the main players in microglial mediated neurotoxicity. A $\beta$ <sub>1-42</sub> has several domains necessary for cellular binding and the execution of its biologic effects such as complement activation



(Van Muiswinkel *et al.*, 1999). Giulian and colleagues (1998) identified a cluster of amino acids HHQK in the A $\beta$  13-16 region of A $\beta$ <sub>1-42</sub>, which are thought to act as a plaque-anchoring site for microglia and to be necessary for their subsequent activation. In addition, the A $\beta$  25-35 portion of A $\beta$ <sub>1-42</sub> is thought to be responsible for the activation of its non-complement based mechanisms of inflammation such as cytokine signaling (Pike *et al.*, 1993).

A post-mortem gene array study involving A $\beta$ <sub>1-42</sub> (2.5 $\mu$ M) stimulation of human brain microglia (Walker *et al.*, 2001) showed that 104 genes were either up- or down-regulated by A $\beta$  treatment. Included amongst them were a) IL-1 $\beta$  precursor which was increased 3.2 fold, b) intracellular adhesion molecule-1 (ICAM-1), 2.6 fold and c) the pro-survival protein, extracellular signal-regulated kinase (ERK), was down-regulated 2.1 fold. Ralay Ranaivo and colleagues (2007) reported that by using selective inhibitors of glial activation they succeeded in suppressing up-regulation of IL-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the hippocampus of mice following icv infusion of A $\beta$ <sub>1-42</sub>. It was found that suppression of neuroinflammation and excessive glial activation by these inhibitors resulted in a restoration of hippocampal synaptic dysfunction as evidenced by maintenance of synaptophysin levels in synapses of A $\beta$ -treated mice on a level comparable to control animals.

Using cultures of rat brain microglia cells, Garção and colleagues (2006) investigated whether fA $\beta$  peptides were crucial for microglial activation *per se*, and whether substances released by A $\beta$ -activated microglia induced neuronal death. They found that A $\beta$  elicited changes in the shape and size of microglial cells causing them to become amoeboid in shape and a thickening of their branches, they also observed a clustering of the microglia relative to ramified dispersed controls. Following 6h of A $\beta$  (5 $\mu$ M) treatment, NO levels produced by the microglia increased by 50% in treated cells relative to untreated controls, with a concomitant increase in iNOS protein concentrations. This was accompanied by up to 170% increases in production of IL-6 in A $\beta$ -treated microglial cells. Using co-culturing techniques it was concluded that cell-cell contact was not necessary for microglial activation and that substances released by

microglia caused significant neuronal death, this neuronal demise was significantly reversed by the addition of anti-IL-6 antibody.

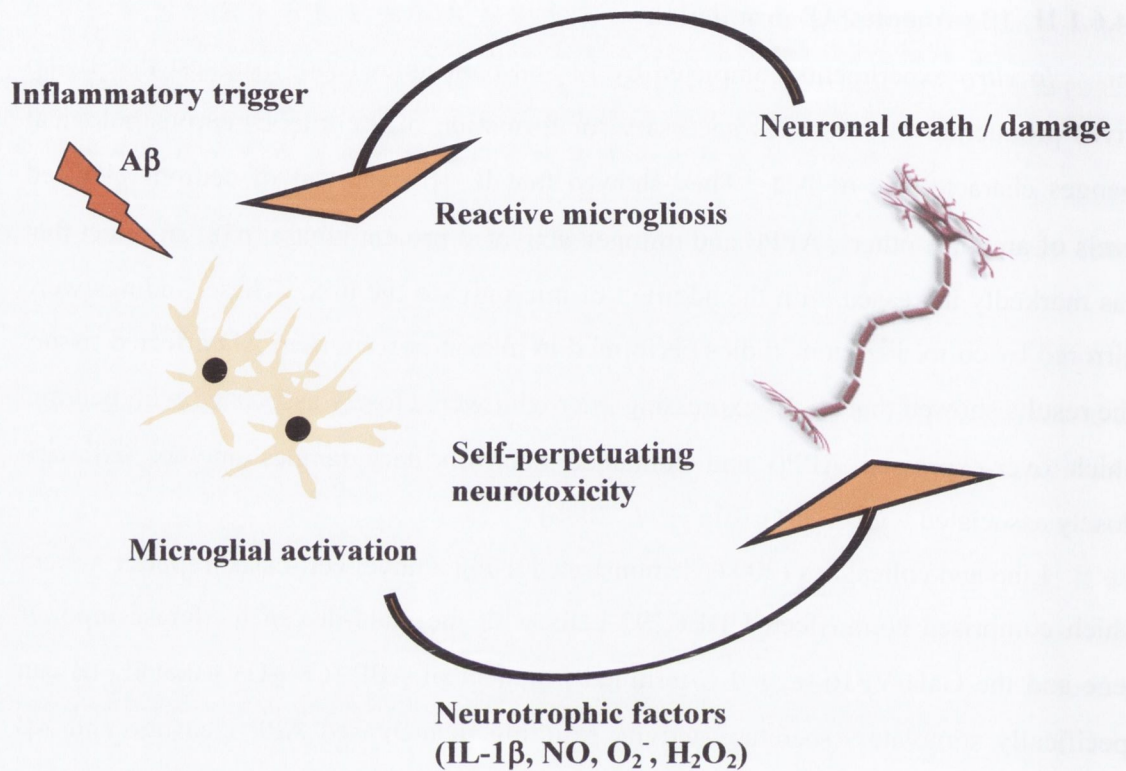
#### **1.4.6.1 IL-1 $\beta$ promotes A $\beta$ production**

*In vitro* experiments completed by Li and colleagues (2003) suggest that IL-1 $\beta$  drives production of substrates necessary for formation of the major neuropathological changes characteristic of AD. They showed that IL-1 $\beta$  treatment of neurons elevated levels of amongst others, APP $\beta$  and mitogen activated protein kinase, p38, an effect that was markedly increased with the addition of microglia to the mix. These findings were mirrored by co-localization studies performed in human post mortem AD affected tissue. The results showed that IL-1 $\beta$  expressing microglia were closely associated with neurons which over-expressed APP $\beta$  and contained neurofibrillary tangles another hallmark closely associated with AD (Griffin *et al.*, 2006).

Liao and colleagues (2004) demonstrated using a novel cell-based reporter system which comprised cotransfected HEK293 cells with the Gal4-driven luciferase reporter gene and the Gal4/VP16-tagged C-terminal fragment of APP (C99-GV) that IL-1 $\beta$  can specifically stimulate  $\gamma$ -secretase activity resulting in increased APP cleavage and A $\beta$  production. However, Koenigsnecht and Landreth (2004) reported that pro-inflammatory cytokines, such as IL-1 $\beta$ , attenuate microglial phagocytosis stimulated by fA $\beta$  and argue that this may, in part at least, contribute to the accumulation of fA $\beta$  containing plaques in the AD brain. The inhibition of phagocytic activity by A $\beta$  was relieved, in there hands at least, by co-incubation with anti-inflammatory cytokines, findings that support anti-inflammatory strategies as therapies for AD.

#### **1.4.7 Amyloid- $\beta$ -induced markers of microglial activation**

The molecular mechanisms by which A $\beta$  activates microglia are unknown at present. However they are likely to reflect modulation of cell membrane components and intracellular signal transduction pathways (Meda *et al.*, 2001). Activated microglia secrete a whole host of soluble factors, the majority of which are pro-inflammatory and neurotoxic, such as IL-1 $\beta$  and TNF $\alpha$  (Davies *et al.*, 1999; Li *et al.*, 2003). In addition to



**Figure 1.8 Microglial responses to activating stimuli**

In response to noxious stimuli microglia undergo activation. This activation may initially be beneficial to the host and microglial activation may be neuroprotective. If, however, they exceed a threshold level of host tolerance these mechanisms become neurotoxic and ultimately result in neurotoxicity and cell death. This in turn may further contribute to microglial activation. (Adapted from Bessis *et al.*, 2007, *Glia* **55**(3): 233-238). Nitric Oxide ( $NO$ ), Oxygen radical ( $O_2^-$ ), Hydrogen Peroxide ( $H_2O_2$ ).

their secretory role, activated microglia up-regulate cell surface molecules such as MHCII and the co-stimulatory ligand cluster of differentiation 86 (CD86) (Allison, 1994; June *et al.*, 1994).

#### **1.4.7.1 Major histocompatibility complex II; Cluster of differentiation 86**

MHCII and the co-stimulatory molecules CD80/CD86 are essential for the initiation of immunological responses (Unanue, 1984; Kalaria, 1993; Menendez Iglesias *et al.*, 1997). In specific, they are required to present antigenic peptides to CD4<sup>+</sup> T lymphocytes. To do this they interact with their lymphocyte partners, the T cell receptor and CD28 respectively (Freeman *et al.*, 1993; Neuman *et al.*, 1998) (Figure 1.9). The expression of antigen presenting peptides on both microglia and infiltrating macrophages is regulated through a complex network of cytokines in the inflamed brain (O'Keefe *et al.*, 2002). Thus it can be inferred that in an AD affected brain the aberrant modulation of microglial and macrophage activity may result in a self perpetuating cycle of increased production of pro-inflammatory cytokines which, when allied to an up-regulation of MHCII and/or CD86, ends ultimately in neuronal compromise and cognitive impairment.

#### **1.4.7.2 Intracellular adhesion molecule-1**

A prominent part of many neuroinflammatory reactions is the activation of complement. Complement is a sophisticated attack system designed to destroy invaders, stimulate inflammation and assist in the phagocytosis of waste materials. Integrins, which exist as heterodimers are comprised of  $\alpha$  and  $\beta$  subunits. These subunits combine to form activated complement systems. Integrins comprise a family of molecules that promote adhesion to other cells as well as extracellular matrix and other proteins.  $\beta$ 2 integrins are expressed at elevated levels by microglia in AD (Sabo *et al.*, 1995). Integrin induction provides further evidence for a potential link between activated complement systems suggested in AD and pro-inflammatory effects observed in this disease. The  $\beta$ 2 integrin/CD11a form a complex known as leukocyte function-associated antigen (LFA-1), which acts as a receptor for ICAM-1 (Figure 1.10). The expression of this complex is ubiquitous, seen on blood microvessels, endothelial cells, astrocytes and microglia and is shown to be increased in AD brains (Akiyama *et al.*, 1993; Frohman *et al.*, 1991). ICAM-

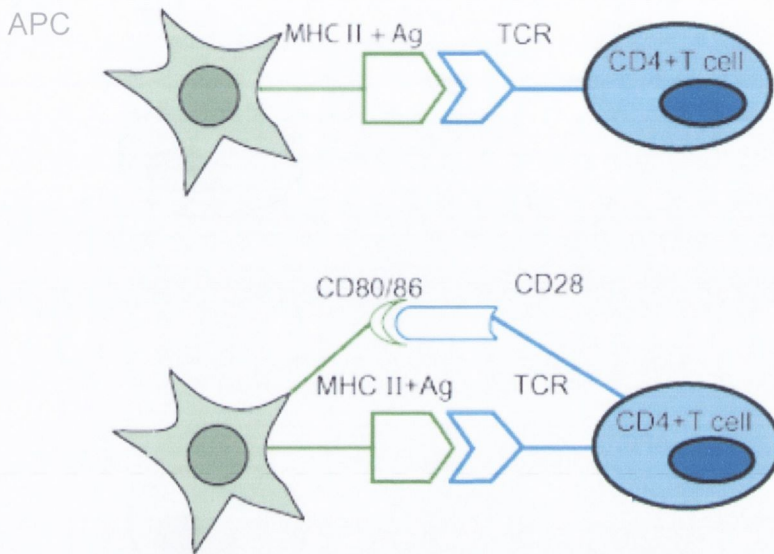
1, a member of the immunoglobulin superfamily, is up-regulated at sites of inflammation and promotes the adhesion of LFA-1 expressing cells to those sites; it may act in AD to adhere microglia to A $\beta$  fibrils in the context of senile plaques. Cytokines such as interferon-gamma (IFN- $\gamma$ ), IL-1, and TNF- $\alpha$  (Verbeek *et al.*, 1994) have been shown to up-regulate ICAM-1 expression in endothelial cells. Grace and colleagues (2003) have demonstrated that A $\beta$ -induced neuronal dystrophy is mediated by the aberrant activation of adhesion proteins. They report that APP is co-localized with integrins in neurons, and suggest that APP may bring A $\beta$  fibrils into physical contact with integrin receptors.

In addition to its role in the complement system, LFA-1/ICAM-1 interaction has been implicated in facilitating activated peripheral lymphocytes to migrate into brain tissue. ICAM-1 is localized immunohistochemically in senile plaques in the brain tissue of patients with AD (Frohman *et al.*, 1991; Akiyama *et al.*, 1993), which may correlate with an increase in the intracerebral localization of T lymphocytes in the AD brain (Itagaki *et al.*, 1988; Hartung *et al.*, 1995). Not only this, findings by Cottman and colleagues (1998), show that A $\beta$  activates signal transduction via the cross linking of adhesion molecules. Recent experiments (Grace *et al.*, 2003) indicate that, fA $\beta$  induced integrin receptor clustering, causes their translocation to the cytoskeleton causing the formation of aberrant focal adhesion-like structures, suggesting the activation of focal adhesion signaling cascades in promoting neuronal dystrophy.

#### **1.4.7.3 Alternative sources of A $\beta$ -related neurotoxicity**

Other data suggest that A $\beta$  may be toxic to neurons in several other ways. A $\beta$  has been shown to augment Ca<sup>2+</sup> signaling and induce oxidative stress in neurons (Mattson *et al.*, 1994, Kawahara and Kuroda, 2000) as well as perturb the fluidity of neuronal membranes thereby lowering the integrity of the cell as a whole (Muller *et al.*, 2001). Resende and colleagues (2007) showed that A $\beta$  peptides affected both cortical neuronal viability and morphology. A $\beta$  induced apoptotic cell death as displayed by caspase-3 activation and caused neuritic dystrophy. They also found, that in culture at least, that hippocampal neurons were more susceptible to A $\beta$  than cortical neurons but that both cell types were affected by similar mechanisms i.e. Ca<sup>2+</sup> homeostatic imbalance, and a fall

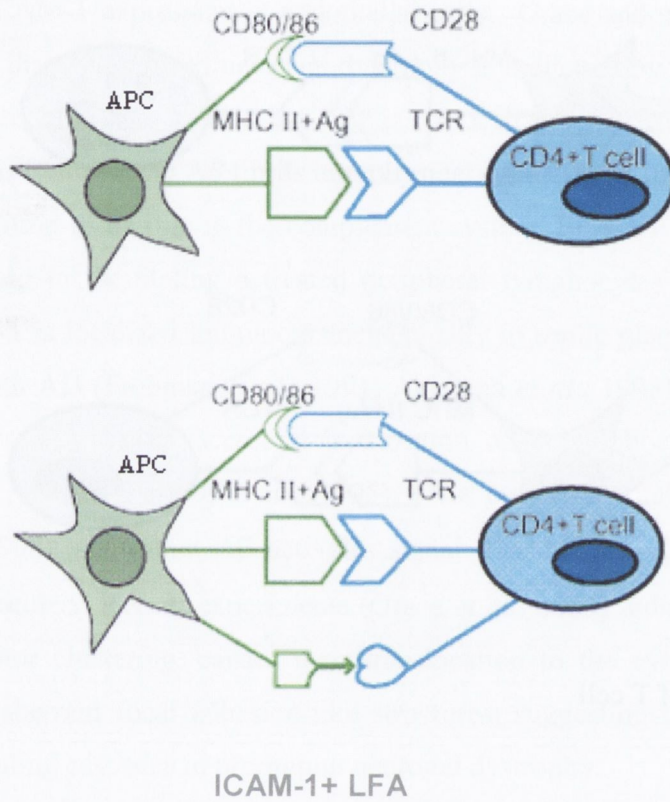
**Signal 1** No activation of T cell



**Signal 2** Activation of T cell

**Figure 1.9 Schematic diagrams illustrating the two-signal postulate.**

Signal 1: involves antigen (Ag) presentation in association with MHCII by and antigen presenting cell (APC) to a T cell receptor. Their interaction alone is insufficient to illicit a T cell immune response. Signal 2: involves co-stimulatory molecule CD80/86 expression in conjunction with MHCII activation. This results in an interaction with naïve T cells leading to activation and proliferation. In addition co-stimulation is achieved by additional interaction with adhesion molecule CD40 (not shown) (Adapted from Chandran *et al.*, 2003 *Surgeon* 1: 63-75). TCR = T cell receptor.



**Figure 1.10 Schematic diagrams illustrating interaction between accessory molecules on APC and T cells.**

Interaction between accessory molecules, such as ICAM-1 with LFA-1, increases the association between the APC and the T cell. (Adapted from Chandran *et al.*, 2003 *Surgeon 1*: 63-75). LFA = lymphocyte function-associated antigen.

in protein levels of the glutamate receptor 2 subunit (GluR2) in AMPA receptors. This, they hypothesize, leads to a rise in cytosolic  $\text{Ca}^{2+}$  concentration toxic to neurons.

#### **1.4.8 Amyloid- $\beta$ -induced microglial activation; links to cognitive impairment**

One of the central issues in AD research is to establish the causes of the cognitive impairments associated with clinical dementia. Selkoe and colleagues (2002) would argue that AD is a form of synaptic failure as it produces a markedly pure impairment of memory beginning with subtle changes in hippocampal synaptic efficiency to outright neuronal degeneration. The gradual nature and subtlety in the nature of the cognitive decline associated with AD suggests that it is possible that an A $\beta$ -induced interruption in the functioning of synapses is interfering with the encoding of new declarative memories.

Davies and colleagues (1987) suggested evidence that many of the biochemical and morphological hallmarks of AD may contribute to the decline in synaptic integrity. A quantitative study of temporal cortical biopsies performed two to four years following the onset of AD revealed that there was 15-35% decrease in the numbers of synapses per cortical neuron. The severity of cognitive decline in AD sufferers has also been shown to correlate with changes in hippocampal concentrations of the presynaptic vesicle protein synaptophysin (Dickson *et al.*, 1995; Sze *et al.*, 1997). In parallel, Lynch and colleagues (1994), reported alterations in the presynaptic proteins, synapsin, synaptophysin and synaptotagmin following induction of LTP and a decrease in some of these proteins with age (Dhanrajan *et al.*, 2004) further strengthening the case for a link between alterations in cognitive capacity and synaptic composition. Data for transgenic rodent models support these findings. Naslund and colleagues (2000) reported a significant depletion in the number of presynaptic terminals in two to four month old APP transgenic mice with a concomitant rise in their soluble A $\beta$  levels; these findings were observed prior to any evidence of plaque formation. Many investigators (e.g. McLean *et al.*, 1999; Lue *et al.*, 1999) have also found that the memory and cognitive impairments seen in AD patients correlates much better with post mortem cortical A $\beta$  levels rather than with plaque load.



The A $\beta$  hypothesis of AD puts A $\beta$  to the fore as its primary cause (Hardy and Selkoe, 2002) and there is much evidence to suggest that A $\beta$  impairs induction of LTP. *In vivo* experiments (Cullen *et al.*, 1996) using A $\beta_{1-40}$  showed reductions in baseline synaptic transmission in the CA1 area of the rat hippocampus. This effect was prevented by treatment with an NMDA receptor antagonist, 3,3-(2 Carboxypiperazine-4-yl)-Propyl-1-Phosphonate (CPP). Cullen and colleagues (1997) have shown that acute icv injection of either A $\beta$  species ( $_{1-40}$  or  $_{1-42}$ ) inhibits LTP in the intact hippocampus. Another study, (Itoh *et al.*, 1999), showed that continuous icv infusion of A $\beta_{1-40}$  for 10 -11 days inhibited LTP in CA1.

#### **1.4.8.1 Acute microglial activation is beneficial to the CNS**

Discrete or temporary microglial activation in the CNS is considered to be beneficial to the surrounding tissue in that it facilitates the clearance of potential harmful foreign peptides or damaged resident cells. Microglia undergo phenotypic activation in response to A $\beta$  deposition in AD affected brains and this results in an up-scaling in the production of inflammatory molecules and phagocytic activity.

Recent experiments, conducted by Hayashi and colleagues (2006), showed that following global ischemia, hippocampal slices prepared from control rats displayed a significant reduction in synaptic responses and a marked reduction of LTP in the CA3-CA1 Schaffer collateral synapses. They also demonstrated that intra-arterial injection of microglia into rats reduced significantly the deleterious effects of ischemia-induced functional deficits of hippocampal CA1 neurons. In addition, these authors also found that the microglial injections prevented the ischemia-induced decline of brain-derived neurotrophic factor (BDNF) levels in CA1. This, they argue, could contribute to the protective effect of the arterial-injection of microglia against ischemia-induced neuronal degeneration.

#### **1.4.8.2 Sustained microglial-associated inflammation impairs long-term potentiation and promotes neuronal loss.**

Chronic inflammation and sustained microglial activation are, however, deleterious to the CNS. Both are key features of the AD affected-brain with particular

emphasis on the hippocampus and temporal lobe areas. There are strong links between changes in inflammatory profile in this brain region and impairment in LTP (Lynch, 2004). This association has been underscored by experiments showing that rats treated acutely with A $\beta$ <sub>1-40</sub> (Minogue *et al.*, 2003) displayed an impairment in maintenance of LTP and this was associated with a concomitant rise in IL-1 $\beta$ , this association has been corroborated by other data (Murray and Lynch, 1998; Lynch and Lynch, 2002; Martin *et al.*, 2002). Hauss-Wegrzyniak and colleagues (1998 and 2002) showed that persistent inflammation induced by chronic LPS administration caused robust activation of microglia in rat entorhinal cortex. They reported that such microglial activation was accompanied by loss of hippocampal pyramidal neurons and entorhinal cells. Following electrophysiological analysis they conclude that the loss of afferents from the entorhinal cortex may account for the need for increased stimulus strength to induce LTP in the LPS infused rats. Quantitative volumetric testing showed these results to parallel a 13% and 9% loss in volume both in the hippocampus and temporal lobe region respectively.

Several studies have shown deleterious effects of A $\beta$  (A $\beta$ <sub>25-35</sub> or A $\beta$ <sub>1-42</sub>) on both long-term and short-term memory after repeated hippocampal injection or i.c.v. infusion (Chen *et al.*, 1996; Nakamura *et al.*, 2001; Yamada *et al.*, 1999). These behavioral effects were associated with progressive elevations in norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid or decreases in cholinergic activity, in the absence of long-lasting amyloid deposits, suggesting that the repeated administration of A $\beta$  can affect a larger range of neurotransmitter system and memory function. This bigger and less specific effect of repeated injections has been observed in other experiments using the less aggregative form of peptide, A $\beta$ <sub>1-40</sub>, injected into the dorsal hippocampus, that failed to induce behavioral effects on performance related to short-term working memory. However, multiple repeated injections or i.c.v. infusion for several days produced performance decrements on spatial and working memory tasks several weeks later and positive thioflavin S staining around the area of cannulae termination (Cleary *et al.*, 1995; Nitta *et al.*, 1994, 1997).

Stephan and colleagues (2001) showed that multiple injections of a combination of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-43</sub> into the dentate gyrus of rats produced aggregated amyloid deposits, with cell loss and inflammation in the surrounding tissue. In addition, these rats showed

deficits in working memory and impaired LTP in dentate gyrus. Assessment of rats injected with the A $\beta$  peptides alone showed an intense level of inflammation around amyloid deposits exemplified by OX42-positive microglia. The authors also showed that chronic treatment of rats with the non-steroidal anti-inflammatory drug (NSAID) (Indomethacin) following A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-43</sub> treatment completely reversed the dysfunctional synaptic plasticity previously observed.

## 1.5 Apoptosis

Cell suicide, termed apoptosis, is recognized as an important physiological process that contributes to normal development and disease. The preservation of cellular homeostasis is fundamental for tissue integrity. Apoptosis is a highly conserved process that has evolved overtime to maintain cell number and cell positioning within various tissue compartments. Kerr and colleagues first described apoptosis in 1972; they identified it as a common type of cell death associated with distinctive morphological features. During development cells are often produced in excess and eventually undergo a 'programmed cell death' thereby contributing to the sculpting of organs and tissues (Meier *et al.*, 2000). Apoptosis is delicately regulated and failure of this regulation contributes to pathological conditions such as developmental defects and neurodegenerative disease (Thompson, 1995). Much work has gone into understanding the molecular mechanisms of the apoptotic signaling pathways including the initiation, mediation, execution and regulation of apoptosis. The first evidence for an apoptotic like process came from studies of the nematode *C.elegans*. Researchers found that controlled cell death depended on the presence and activation of cell death abnormal-3 (CED-3) (a caspase homologue) and CED-4, an apoptosis protease activity factor-1 (Apaf-1) homologue that binds to and activates CED-3. The initiation and regulation of apoptosis in higher organisms is governed by corresponding components found in *C.elegans* but with greater complexity (Ellis *et al.*, 1996; Metzstein *et al.*, 1998).

### 1.5.1 Key features of apoptosis

The characteristic features of an apoptotic cell include cell membrane blebbing, cell shrinkage, chromatin condensation and deoxyribonucleic acid (DNA) fragmentation

with the eventual engulfment of the damaged cell by macrophages, thereby avoiding an inflammatory response in the surrounding tissues (Figure 1.11). In contrast to apoptosis cells can undergo destruction by necrosis. In this case, cells suffer a major insult and the cellular contents are released uncontrolled into the cells' environment which results in damage to surrounding cells and a strong inflammatory response in the corresponding tissue (Leist and Jaattela, 2001).

### **1.5.1.1 Intrinsic and Extrinsic apoptotic signaling**

Mammalian cells are influenced by a wide variety of external signals which may, in the right circumstances, trigger one of two major apoptotic pathways. The first is the extrinsic or death receptor pathway; the second, the intrinsic or mitochondrial pathway (Figure 1.12). The extrinsic pathway is driven by the binding of death-inducing ligands to cell surface receptors, the intrinsic by signals such as DNA damage or oxidative stress which initiate apoptosis via the mitochondria (Green and Kroemer, 2004). The latter pathway is utilised by most neurons in most situations. The extrinsic pathway is governed by cell surface receptors such as Fas (cell death receptor), or tumour necrosis factor inducing ligand (TRAIL) receptors. Ligand stimulation results in oligomerization of the receptors and recruitment of an adaptor protein called the Fas-associated death domain (FADD) and caspase-8. FADD and caspase-8 form a complex that signals the inducement of cell death known as death inducing signal complex (DISC). Activation of caspase-8 at the DISC is followed by activation of effector caspases, including caspase-3 (Ashkenazi and Dixit, 1998).

The intrinsic pathway is mediated by diverse apoptotic stimuli, which converge on the mitochondria. Release of cytochrome c from the mitochondria to the cytoplasm initiates a caspase cascade. Cytosolic cytochrome c binds to Apaf-1 and procaspase-9, generating an intracellular equivalent of DISC known as the 'apoptosome'. Within the confines of the apoptosome, caspase-9 is cleaved and activated, leading to the processing of caspase-3 (Shi, 2002). This results in the activation of several other proteases and nucleases that drive the terminal events of programmed cell death.

## 1.5.2 Caspases

Caspases are a group of proteins, which comprise one of the main effectors of apoptosis. Their activation is considered to be a hallmark of the process. To date about 14 caspases have been identified and all share a few common features. All are synthesized as inactive zymogens containing a pro-domain. These zymogens are cleaved to form active enzymes following the initiation of apoptosis. All caspases act as signaling mediators. They propagate their signals by cleaving their cellular protein substrates at aspartate-Xxx (Asp-Xxx) bonds. More than 100 substrates have been identified thus far.

### 1.5.2.1 Caspase-8, Caspase-3; Initiators and executioners of apoptosis

In a review of the apoptotic process, Degterev and colleagues (2003), have subdivided caspase substrates based on their cellular function 1) mediators of apoptosis, 2) structural proteins, 3) cellular DNA repair proteins and 4) cell cycle-related proteins. Based on function, caspases can be classified as, firstly, inflammatory e.g. caspase-1. Caspase-1 plays a role in inflammation rather than apoptosis, secondly, initiator caspases e.g. caspase-8 or caspase-9 and thirdly, effector caspases e.g. caspase-3. Initiator caspases mediate the interaction with upstream adaptor molecules and effector caspases cleave cellular substrates downstream (Degterev *et al.*, 2003).

Effector caspases are usually more abundant and active than upstream initiator caspases. Genetic studies in CPP32- and Apaf-1-deficient mice have shown that deletion of caspase-3 results in gross developmental malformation and premature death (Kuida *et al.*, 1998; Honarpour *et al.*, 2000). Caspase-3 knockout mice do not respond appropriately to intrinsic and / or extrinsic apoptotic stimuli (Kuida *et al.*, 1996; Woo *et al.*, 1998). Caspase-3 is therefore recognised as the pivotal executioner caspase as other effector caspases such as caspase-6 or -7 have displayed redundancy in most apoptotic pathways (Zhang *et al.*, 2000). Not only this, caspase-3 also proves a vital point of convergence for the extrinsic and intrinsic apoptotic pathways (Figure 1.13).

### 1.5.2.2 Amyloid precursor protein mis-processing and apoptosis

Neuronal loss is prominent in AD, and yet its mechanisms remain unresolved. Apoptotic cell death has been implicated on the basis of studies demonstrating DNA fragmentation and up-regulation of pro-apoptotic proteins in the AD brain. However, DNA fragmentation is too frequent an occurrence in neurons to be considered definitive proof of apoptosis. Using affinity-purified anti-serum, Stadelmann and colleagues (1999), reported that activated caspase-3 was increased in AD brains with an absence of similar evidence in age-matched controls. Apoptotic neurons were identified at a frequency of 1 in 1100 to 5000. This frequency, it was postulated, is compatible to the rate of neuronal degeneration seen during the progression of AD.

Familial forms of AD (fAD) are caused by mutations of the APP gene and by mutations of the genes encoding presenilin 1 and 2 (PSen1 and PSen2). Thus far 18 AD-related mutations have been reported in the APP gene (21q21), 142 mutations in the PSen1 gene (14q24.3) and 10 mutations in the Psen2 gene (1q31-q42) (Jansenn *et al.*, 2003; Papassotiropoulos *et al.*, 2006). Carriers of these mutations develop a form of AD with an earlier onset and shorter disease duration than sporadic cases (Lippa *et al.*, 1996). These mutations alter APP processing with respect to enhanced A $\beta$  production and have been linked to an increased vulnerability to cell death (Eckert *et al.*, 2001). Secretase cleavage of the double mutation form of APP leads to a 3-6-fold increase in A $\beta$  production of both A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> (Citron *et al.*, 1992; Cai *et al.*, 1993). Similarly, research shows that increased oxidative stress can enhance the rapid progression of the Swedish form of fAD. Marques and colleagues (2003) report on the effect of the Swedish double mutation (K670M/N671L) in APP on oxidative stress-induced cell death mechanisms in PC12 cells. The authors' findings revealed an elevated activity of caspase-3, after treatment with H<sub>2</sub>O<sub>2</sub> in cells containing the mutation. The elevation in caspase-3 activity was, the authors believed, the result of activation of both the intrinsic and extrinsic apoptotic pathways and included the enhancement of caspase-8 activation. These results may indicate that the aggressive neurodegeneration seen in early onset fAD patients could be attributable to increased vulnerability of neurons, through activation of apoptotic pathways as a consequence of elevated levels of oxidative stress.

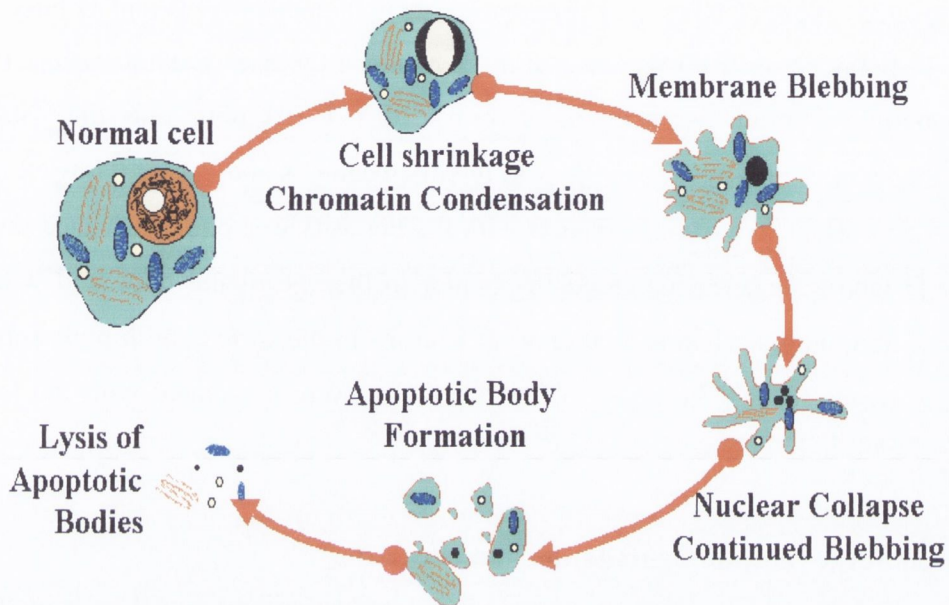
### 1.5.2.3 Caspase activation can be amyloid- $\beta$ species specific

Using mouse models, Galvan and colleagues (2006) have shown that, by blocking caspase action on proteins associated with AD, the progression of the disease is significantly attenuated. Their results show that it is specific aspartate residues that are the targets of the caspases and that they are essential for the development of AD type symptoms in their model. Awasthi and colleagues (2005) revealed that different A $\beta$  species were capable of activating different caspase cascade initiators in cultured neuronal cells. Specifically, A $\beta_{1-42}$  activated caspase-9 and A $\beta_{1-40}$  activated caspase-8, both A $\beta$  species utilized the same executioner caspase, caspase-3. The same experiments revealed the difference in toxicity to neurons of both A $\beta$  species. The authors measured the proliferative response of neurons to various A $\beta$  species. A $\beta_{1-42}$  was the most potent inducer of cell stress causing lysosomal membrane injury. A $\beta_{1-40}$ , while still toxic, was much less so than its longer counterpart.

Although caspases are recognised as playing a major role in programmed cell death the contribution of other forms of cell death to the progression of AD cannot be ignored. Autophagy is a process of self-cannibalization through a lysosomal degradation pathway. The term “autophagic cell death” describes a form of programmed cell death morphologically distinct from apoptosis and presumed to result from excessive levels of cellular autophagy (Schweichel and Merker, 1973). In classical apoptosis, or type I programmed cell death, there is early collapse of cytoskeletal elements but preservation of organelles until late in the process. In contrast, in autophagic, or type II, programmed cell death, there is early degradation of organelles but preservation of cytoskeletal elements until late stages. Whereas apoptotic cell death is caspase-dependent and characterized by internucleosomal DNA cleavage, caspase activation and DNA fragmentation occur very late (if at all) in autophagic cell death. In contrast with necrosis, both apoptotic and autophagic cell death are characterized by the lack of a tissue inflammatory response (Levine and Yuan, 2005).

One feature that distinguishes apoptosis from autophagic cell death is the source of the lysosomal enzymes used for most of the dying cells' degradation. Apoptotic cells

## Apoptosis (Programmed Cell Death)



**Figure 1.11 Apoptosis; Programmed Cell Death**

Although many pathways and signals lead to apoptosis, there is only one mechanism, which actually causes the death of the cell in this process; after the cell has received the appropriate stimulus, and the necessary controls exerted, a cell will undergo the organised degradation of cellular organelles by activated proteolytic caspases.

Microbiologybytes Web Site [Internet]. University of Otago, Dunedin, New Zealand. © Kalmakoff & Ward 2003; Updated Jan 28 2007 [cited 24 Jan 2008]. Available from <http://www.microbiologybytes.com/virology/kalmakoff/baculo/baculohostinteract.html>



use phagocytic cell lysosomes for this process, whereas cells with autophagic morphology use the dying cells' endogenous lysosomal machinery (Shintani and Klionsky, 2004). Although it is possible that the increased lysosomal activity contributes to removal of neurotoxic materials, the loss of lysosomal integrity may cause cell death in neurodegenerative disorders, or there may be a combination of both effects.

Elevated levels of lysosomal proteinases such as cathepsins B and D have been observed in brain tissue from patients with Alzheimer's disease (Cataldo *et al.*, 1997). Immunolabelling of brain tissue prepared from APP x Psen1 transgenic mice showed increased deposition of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> in the hippocampus and cortex (Howlett *et al.*, 2008). Also present in close association with the deposits was phosphorylated tau and cathepsin D whose instance increased in parallel to the age of the mice and with A $\beta$  deposition. Immunohistochemical labeling of neurons in the cortex and hippocampus of these mice suggested that the areas of A $\beta$  deposition were associated with the loss of neurons.

### **1.5.3 Membrane receptor aggregation and clustering**

Research has shown that receptor-mediated activation and signal transduction in cells is governed by at least two principles: 1) receptors regulate enzymatic activity and 2) receptor and intracellular signaling molecules reorganize on stimulation. These principles determine the response of the cell to a stimulus. Receptor molecules aggregate on stimulation and increase their density by concentrating themselves in a small area of the cell membrane. This process usually follows ligand binding. Clustering of receptor molecules correlates with a realignment of intracellular signaling molecules e.g. FADD, caspase-8 and caspase-3 as highlighted above.

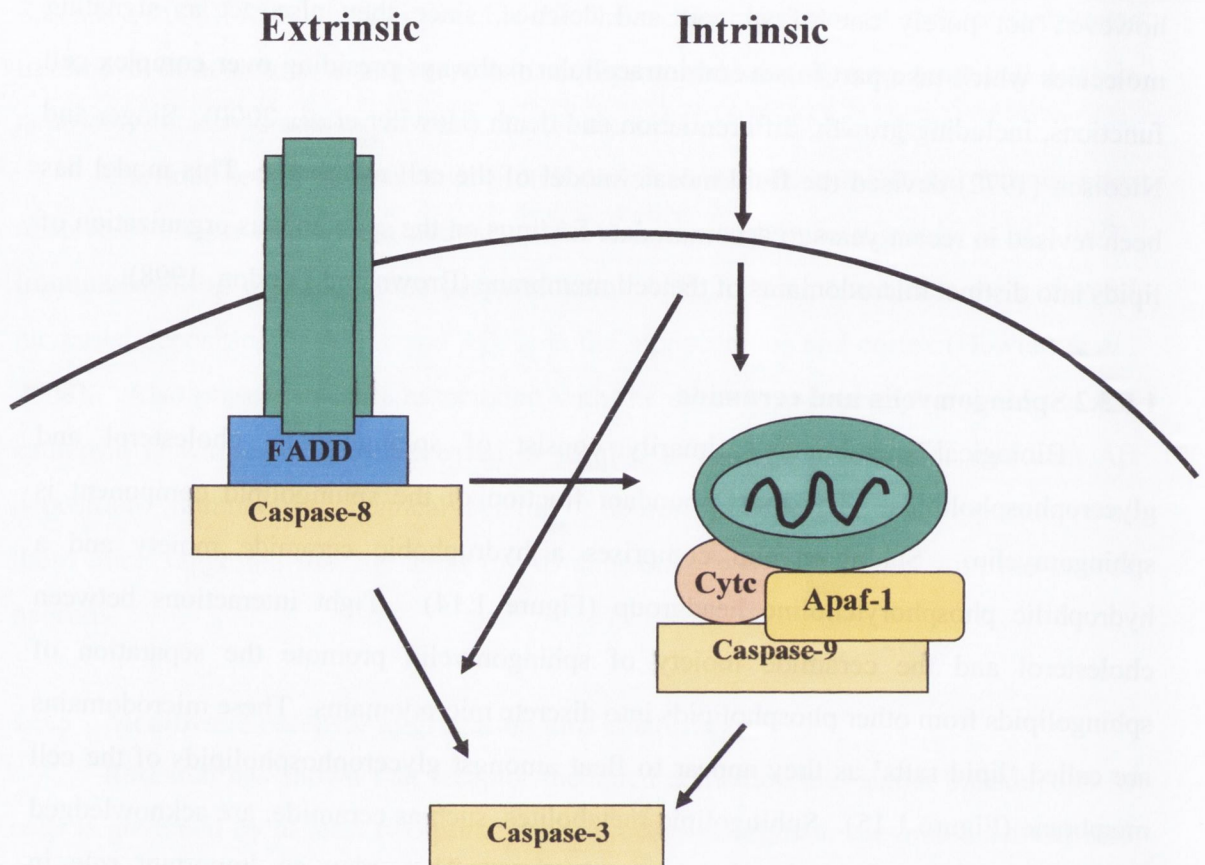
#### **1.5.3.1 Sphingolipids and membrane organization**

When a cell becomes stressed it is more susceptible to damage. Any number of toxic substances can affect the integrity of its surrounding membrane, attacking it and breaking down its constituent parts. Sphingolipids are a component of all membranes but are particularly abundant in the myelin sheath surrounding neurons. The sphingolipids in normal circumstances serve as mechanical stabilizers anchoring cell membrane

components, for example receptor complexes, as well as providing a layer of chemical resistance to the outer leaflet of the plasma membrane. The role of sphingolipids is, however, not purely one of support and defence, since they also act as signaling molecules which take part in several intracellular pathways presiding over complex cell functions, including growth, differentiation and death (Huwiler *et al.*, 2000). Singer and Nicolson (1972) devised the fluid mosaic model of the cell membrane. This model has been revised in recent years to accommodate findings on the spontaneous organization of lipids into distinct microdomains of the cell membrane (Brown and London, 1998).

### 1.5.3.2 Sphingomyelin and ceramide

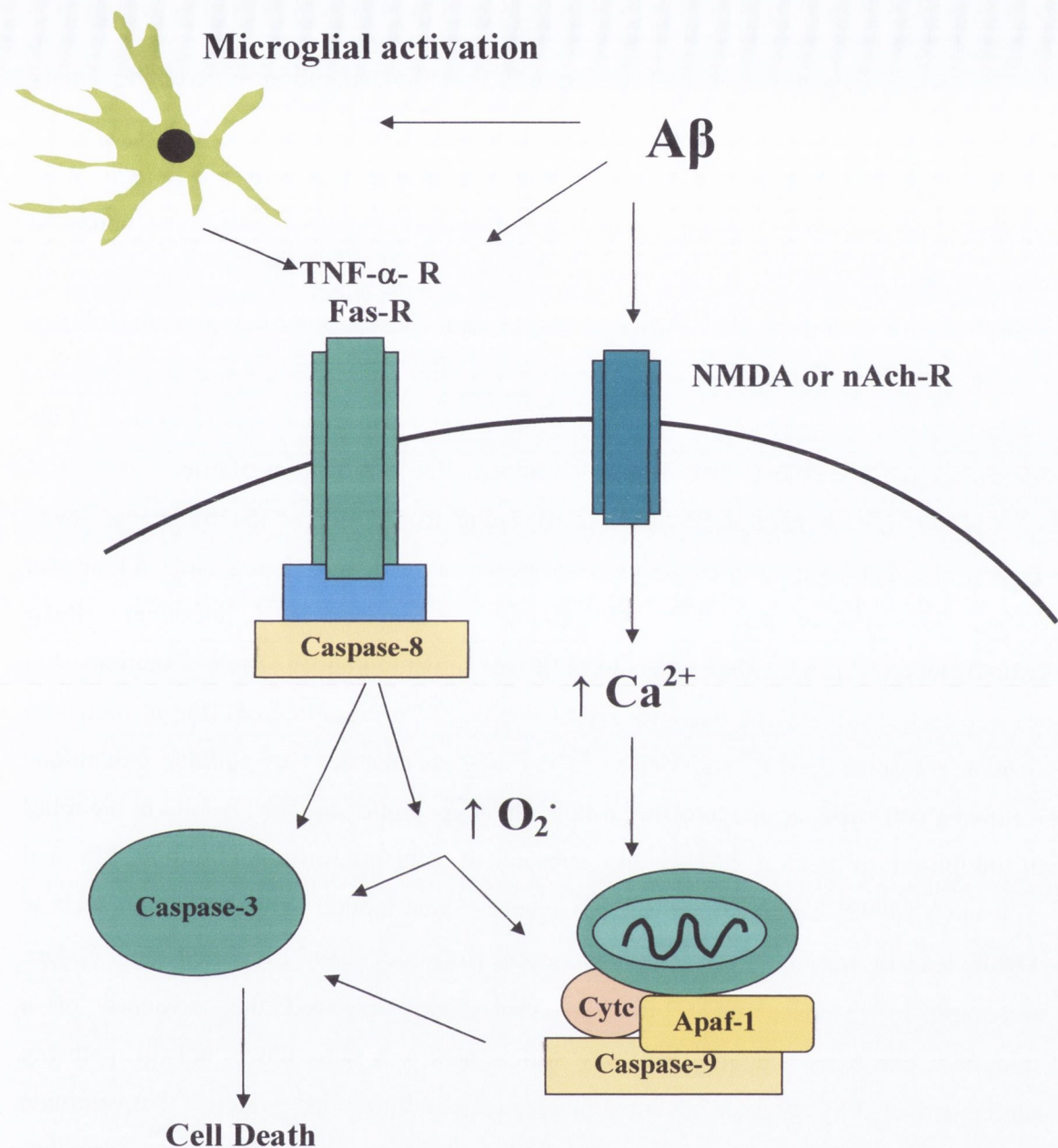
Biological membranes primarily consist of sphingolipids, cholesterol and glycerophospholipids. The most abundant fraction of the sphingolipid component is sphingomyelin. Sphingomyelin comprises a hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup (Figure 1.14). Tight interactions between cholesterol and the ceramide moiety of sphingomyelin promote the separation of sphingolipids from other phospholipids into discrete microdomains. These microdomains are called 'lipid rafts' as they appear to float amongst glycerophospholipids of the cell membrane (Figure 1.15). Sphingolipid metabolites, such as ceramide, are acknowledged as important components in signal transduction. They play an important role in apoptosis, cell cycle arrest and differentiation (Hannun and Luberto, 2000; Pettus *et al.*, 2002). Ceramides are rarely found at greater than trace levels in tissues, although they can exert important biological effects. Ceramide is a hydrophobic lipid that does not form membrane bilayers in aqueous environments. Ceramide formed from the hydrolysis of sphingomyelin accumulates in the membrane interior (Sillence, 2001). They are not soluble in water and ceramide-enriched membrane platforms serve 'to lubricate' a cells membrane allowing for the reorganization and clustering of receptors constitutively expressed in membranes molecules (Grassme *et al.*, 2002) (Figure 1.15). However, many receptor molecules are located outside rafts when quiescent but become trapped within ceramide-enriched platforms on activation. It's thought that sequestration may be mediated by conformational changes in the receptor molecules on ligand binding ending with the preferential interaction of the receptor with ceramide (Gulbins and Li, 2006).



**Figure 1.12 Apoptotic signaling pathways**

Caspases play an important role as signal transducers (caspase-8, -9) and as terminal executioners (caspase-3) in apoptosis. FADD = Fas associated death domain, Cyt c = Cytochrome C, Apaf-1 = apoptotic protease activating factor-1.

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**Figure 1.13 Aβ-induced stimulation of apoptotic pathways converge at caspase-3 activation**

Aggregated forms of amyloid- interact with several different neuronal cell-surface receptors and with microglia, triggering signal transduction cascades that result in caspase activation, free-radical generation and Ca<sup>2+</sup> influx. Adapted from Yuan and Yanker (2000) *Nature* **407**; 802-809. Aβ = amyloid-β, TNFαR = Tumour necrosis factor α receptor, NMDA = N-methyl-D-aspartate, nAch-R = nicotinic acetylcholine receptor, Cyt c = Cytochrome C, Apaf-1 = apoptotic protease activating factor-1.

### 1.5.3.3 The sphingomyelin cycle and cell death

Two main pathways have been identified for the generation of ceramide: firstly hydrolysis of sphingomyelin and secondly, *de novo* biosynthesis. The first occurs by the action of sphingomyelinases (SMases), which are classified as acid and neutral. Acid SMase is endosomal-lysosomal associated and neutral SMase is membrane bound in neurons (Adam-Klages *et al.*, 1998). SMases are specialized enzymes with phospholipase C activity that hydrolyse the phosphodiester bond of sphingomyelin to release ceramide (Figure 1.14). Zumbansen and Stoffel (2002) describe two isoforms of neutral SMase: neutral sphingomyelinase 1 and 2 and showed that neutral SMase2 is the form responsible for most of the production of ceramide in the brains of mice.

The role of neutral SMase is only being teased out at the molecular level. Transgenic acid SMase<sup>-/-</sup> mice show that one or more distinct genes code for neutral SMase (Horinuchi *et al.*, 1995). Glutathione is recognised as an important cellular antioxidant and has been shown to control SMase activation under certain conditions (Liu *et al.*, 1998). Glutathione becomes rapidly depleted during apoptosis due to oxidation (van den Dobbelsteen *et al.*, 1996). This suggests that loss of cellular glutathione following cell stress or due to efflux following an apoptotic stimulus, results in the relief of inhibition of neutral SMase and subsequent generation of ceramide. Liu and colleagues (1998) have also shown that agents, which induce oxidative stress, such as TNF $\alpha$ , lead to prolonged ceramide generation. Kolesnick (1987) reported rapid SMase activation in response to 1,2-diacylglycerols, and proposed the existence of a sphingomyelin-based signaling pathway. Subsequently, it was shown that this pathway can be activated by receptor-mediated mechanisms and provided evidence that ceramide is a second messenger (Hannun *et al.*, 1989). Indeed, sphingolipid metabolism has proved to be a dynamic process, and sphingolipid metabolites, including ceramide, are now recognized as messengers playing essential roles in cell growth, survival, and death (Mathias *et al.*, 1998; Hannun *et al.*, 2001).

### 1.5.4 Ceramide production facilitates the cellular stress response

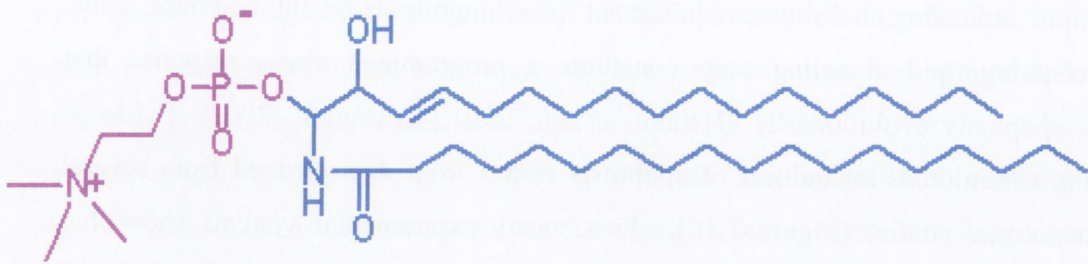
Ceramide may serve as a stress response signal even in organisms with the simplest of physiology such as *Saccharomyces cerevisiae*. Yeast mutants incapable of

rapid *de novo* synthesis of this sphingolipid class fail to adapt and to regrow at elevated temperatures, and instead undergo growth arrest. This defect is bypassed by exogenous sphingolipid, indicating an obligate requirement for sphingolipids for this response. Thus, ceramide/sphingolipid signaling may constitute a programmed stress response that predates apoptosis evolutionarily (Hannun *et al.*, 2001; Kolesnick, 2002). Evidence supporting ceramide as an inducer of apoptosis comes from data gleaned from several cell systems and studies (Figure 1.16). First, many experimental systems show that agonist- and stress-induced elevation of ceramide levels precede the biochemical and morphologic manifestations of apoptosis. Second, increasing cellular ceramide by adding natural ceramide or exogenous SMase mimics effects of stress on apoptosis induction. Third, genetic models, including acid SMase<sup>-/-</sup> mice show the predicted cellular abnormalities associated with the stress responses. As other sphingolipid metabolites have been shown to be non-cytotoxic (Mathias *et al.*, 1998; Hannun *et al.*, 2001), it seems that ceramide, *per se*, is necessary and may be sufficient for some forms of cell stress-associated apoptosis.

### 1.5.5 Ceramide; a promoter of apoptosis

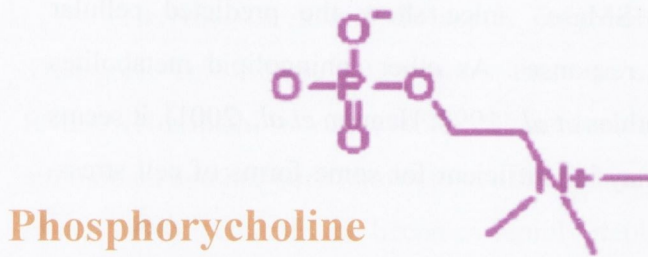
Ceramide, a product of SMase activation, functions as a lipid second messenger and has been shown to increase the cellular oxidative state of cells and it is implicated in apoptotic paradigms (Coroneos *et al.*, 1995). Ceramide stresses a cell to the point where it induces mitochondrial dysfunction and nuclear fragmentation (Hannun and Luberto, 2000). A $\beta$  and ceramide share cell death signaling characteristics, A $\beta$ -induced apoptosis involves TNF receptor 1 (TNFR1) and p75 cell surface receptors that relay death signals through the sphingomyelin-ceramide pathway (Hayakawa *et al.*, 1996). Söderberg and colleagues (1992) reported lower sphingomyelin levels and higher ceramide levels in post-mortem AD brains, implying that increased sphingomyelin degradation and ceramide accumulation contribute to AD pathogenesis. Alessenko and colleagues (2004) found that icv administration of A $\beta$  significantly increased SMase activity and ceramide in rat hippocampus, these effects were still detectable 7 days post injection.

Jana and Pahan (2004) have shown a two-fold increase in ceramide levels within 15 minutes of A $\beta$ <sub>1-42</sub> treatment in human primary neurons and this rises to a twelve-fold

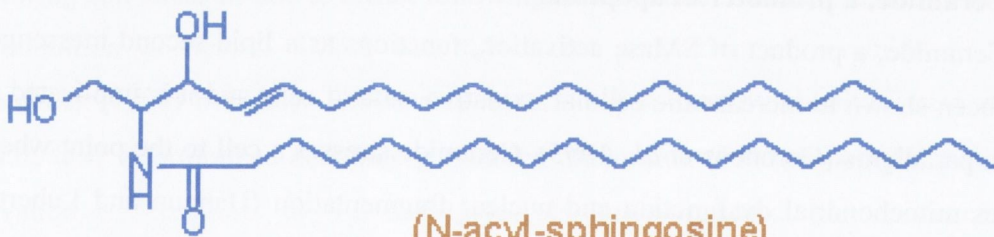


Sphingomyelin

Sphingomyelinase



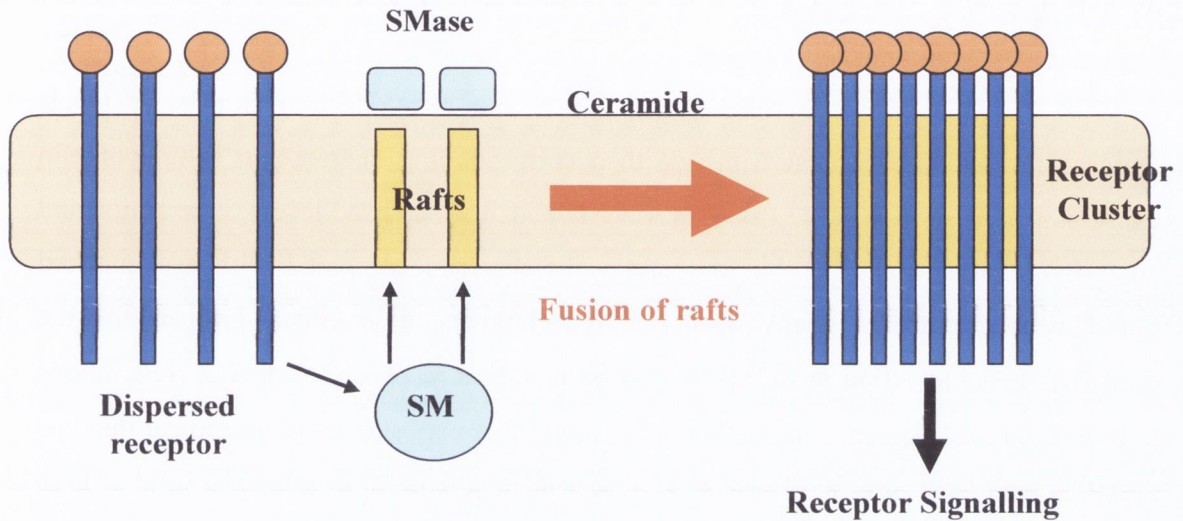
Phosphorycholine



(N-acyl-sphingosine)  
Ceramide

Figure 1.14 Schematic representation of sphingolipid intermediary metabolism

(Adapted from Kolesnick (2002) *J Clin Invest.* **110**:3-8)



**Figure 1.15 Model of the formation and function of ceramide enriched membrane platforms.**

Stimulation of cells results in a translocation of SMase onto the extracellular leaflet of the cell membrane, the release of ceramide and a ceramide-mediated re-organization of small membrane rafts to larger platforms. These platforms serve to cluster receptor molecules, recruit intracellular signaling molecules or ion channels to the receptor. (Adapted from Szabó *et al.*, 2004, *Eur J Physiol.* **448**: 304-312). SMase = sphingomyelinase, SM = sphingomyelin.



increase after 10 hours. The increase in ceramide production was dose-dependant with increases produced by A $\beta$  concentrations as low as 0.25 $\mu$ M. In the same study, antisense oligonucleotides were utilized to elucidate the role of SMase activity in A $\beta$ <sub>1-42</sub> induced apoptosis. It was found that control neurons displayed few apoptotic bodies compared to those treated with A $\beta$  peptide, addition of the antisense oligomers markedly blocked this compromise of neuronal cell viability.

In 2004, Cutler and colleagues reported that ceramide and cholesterol levels increase in the brains of aged mice with a corresponding decrease in sphingomyelin expression. In autopsy tissue from several AD patients increased levels of ceramide and cholesterol and decreased sphingomyelin were observed compared with age matched non-demented controls. Finally, using culture techniques, these authors surmised that the oxidative stress resulting from A $\beta$  deposition could be responsible for the irregularities seen in lipid metabolism. The authors also found that exposure of hippocampal neurons to A $\beta$ <sub>1-42</sub> led to increases in ceramide, cholesterol and decreases in sphingomyelin. These findings were accompanied by oxidative stress that resulted in the death of the neurons within 24h of exposure to A $\beta$ .

#### **1.5.6 Amyloid- $\beta$ -lipid interactions; their contribution to neuronal toxicity**

An early clue that alterations in lipid metabolism function in AD came from genetic studies that linked polymorphisms in the apolipoprotein E (ApoE) gene to the risk of late-onset AD. ApoE is essential for the normal catabolism of lipoprotein constituents. Defects in ApoE result in increased plasma cholesterol. In the nervous system non-neuronal cell types, most notably astroglia and microglia, are the primary producers of ApoE, while neurons preferentially express the receptors for ApoE. The ApoE allele, ApoE  $\epsilon$ 4, has been shown to cause an increased susceptibility to AD. 40%-65% of AD patients have at least one copy of the  $\epsilon$ 4 allele. The binding of various forms of A $\beta$  to plasma membranes has been studied in an effort to assess the direct toxicity of A $\beta$  to neurons, and the pathway leading to activation of a local inflammation phase involving microglia (for review see Verdier and Penke, 2004). ApoE forms part of the cholesterol transport complex and is thought to bind A $\beta$  causing it to clump thus affecting its

clearance from the brain (Fagan *et al.*, 2000; Tokuda *et al.*, 2000). Bales and colleagues (1999) have shown that ApoE<sup>-/-</sup> mice display markedly reduced A $\beta$  deposition and scant fA $\beta$  load. A $\beta$ , too, displays an affinity for cholesterol itself (Kakio *et al.*, 2002). A $\beta$  binding of ganglioside clusters (cholesterol/sphingomyelin) facilitates A $\beta$  translocation to phosphatidylcholine membranes. This process competes with A $\beta$  oligomerization in lipid rafts. The ganglioside clusters are thought to act as chaperones generating a membrane-active form of A $\beta$  with seeding ability (Kakio *et al.*, 2003). Grimm and colleagues (2005) report that APP processing is sensitive to cholesterol and sphingomyelin membrane concentrations, they found that sphingomyelin levels were higher and neutral SMase activity lower in mouse embryonic fibroblasts (MEFs) lacking PSen1 and PSen2. The authors found that neutral SMase activity was increased in cells exposed to A $\beta$  and decreased in cells lacking APP. Surprisingly, relatively low (nanomolar) concentrations of A $\beta$ <sub>1-42</sub> increased the activity of purified neutral SMase, whereas A $\beta$ <sub>1-40</sub> was less effective.

Hydroxymethylglutaryl-CoA reductase (HMGR) and SMase are the main enzymes that regulate cholesterol and sphingomyelin levels respectively. Grimm and colleagues (2005) have also shown that control of cholesterol and sphingomyelin metabolism involves APP processing directly. A $\beta$ <sub>1-42</sub> directly activates neutral SMase lowering sphingomyelin levels and A $\beta$ <sub>1-40</sub> reduces cholesterol synthesis by inhibition of HMGR. These processes are strictly  $\gamma$ -secretase dependant. They also report that PSen mutations also serve to decrease sphingomyelin levels. These findings suggest a biological function for APP processing and a functional basis for the link between AD and lipids (Figure 1.17 and 1.18). A $\beta$ -induced toxicity may not just be due to its aggregation but may also be induced by mechanical means. Eckert and colleagues (2000) found that A $\beta$  peptide generation disturbs membrane structure and function in that it promotes membrane fusion, thus hampering membrane fluidity, and ion channel formation. Alternatively, A $\beta$  aggregation can promote the formation of its own ion channels in the lipid bilayer of neurons. These channels are large and voltage-independent and poorly selective amongst ions. The Ca<sup>2+</sup> influx facilitated by these channels destabilizes neuronal Ca<sup>2+</sup> balance inducing neurotoxicity (Arispe *et al.*, 1993; Kourie *et al.*, 2001).

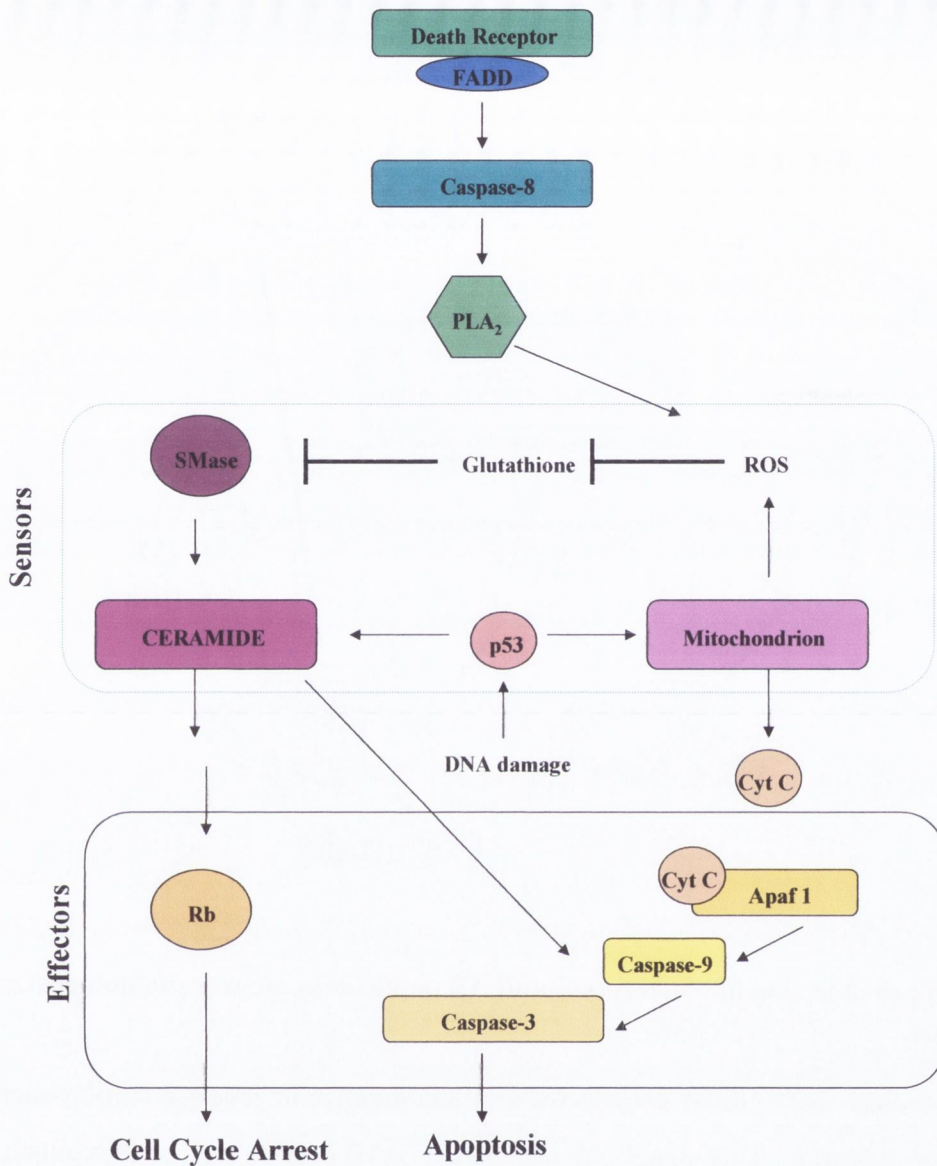
### **1.5.7 Inflammatory signaling and cell death in neurons**

Chronic inflammation is thought to contribute to the pathogenesis of AD. One of the most extensively studied pro-inflammatory cytokines in terms of the workings of the CNS is IL-1 $\beta$ . A post-mortem gene array study involving A $\beta$ <sub>1-42</sub> stimulation of human brain microglia (Walker *et al.*, 2001) showed that IL-1 $\beta$  precursor expression was increased 3.2 fold. IL-1ra, a naturally occurring antagonist to IL-1 prevents neuronal damage by inhibiting endogenous IL-1 $\beta$  (Loddick and Rothwell, 1996). Downstream of IL-1 $\beta$  stimulation is the mitogen-activated protein kinase, JNK. JNK, acts on specific substrates responsible for its physiological function and apoptotic action in the CNS, it enhances any IL-1 $\beta$  induced pro-inflammatory pro- apoptotic signal by phosphorylating a variety of cytoskeletal proteins such as, the transcription factor, p53. p53 serves to promote apoptosis as it suppresses the expression of anti-apoptotic Bcl-2 (Miyashita *et al.*, 1994; Maundrell *et al.*, 1997).

Marques and colleagues (2003) report that elevated levels of caspase-2 / -8 and -3 activity in cells containing the double Swedish mutation found in fAD (FAD - K670M/N671L) are accompanied by enhanced activation of the aforementioned JNK pathway. This suggests that increased vulnerability of neurons in AD patients could in part be due to activation of IL-1 $\beta$  or pro-inflammatory driven apoptotic pathways. Similarly, findings in this lab show an increase in JNK phosphorylation in the hippocampus of adult rats following acute A $\beta$  administration; an age related increase in activation of JNK has also been observed (Minogue, 2005). Morishima and colleagues (2001) characterised a mechanism by which A $\beta$ -induced inflammation could possibly lead to neuronal death. The authors found that cortical neurons exposed to A $\beta$  activated JNK. JNK is required for the phosphorylation of c-jun transcription factor, which in turn stimulated the expression of the death inducer Fas ligand. Fas ligand binding to its receptor ultimately leads to caspase activation and cell death.

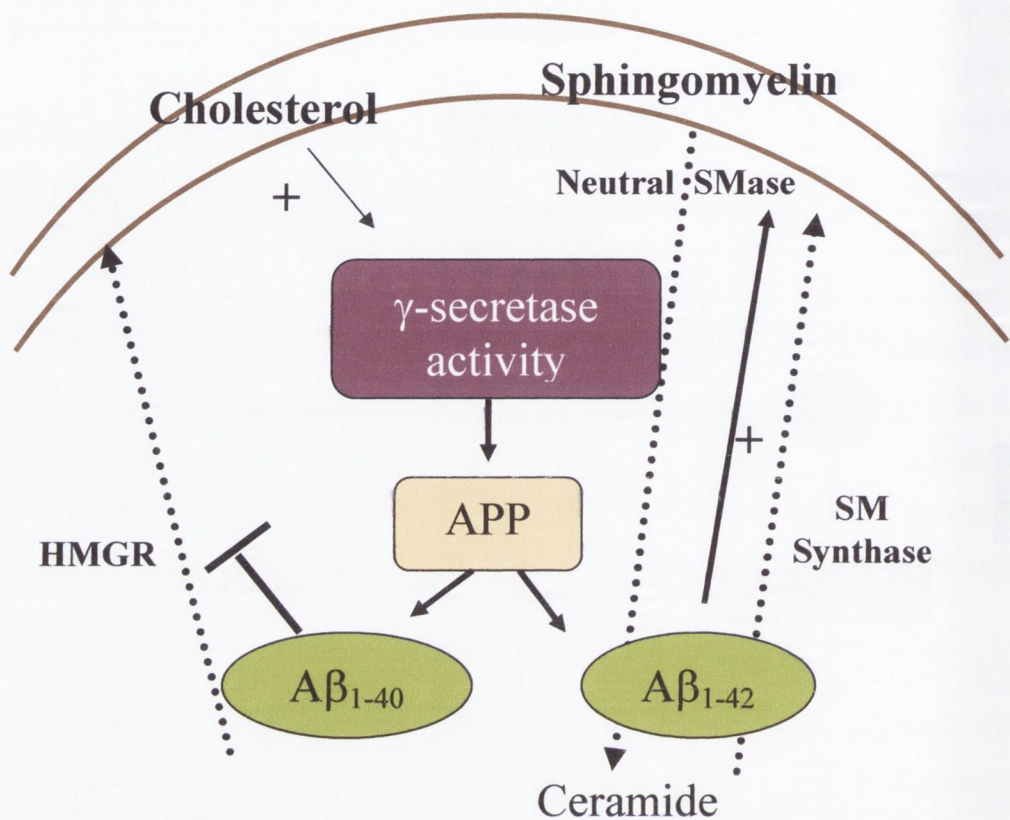
### **1.5.8 Ceramide-associated inflammatory signaling in the central nervous system**

Sanchez-Alavez and colleagues (2006) found that IL-1 $\beta$  action on neurons can be divided into fast (ceramide-mediated) and slow nuclear factor- $\kappa$ B mediated (NF- $\kappa$ B) and



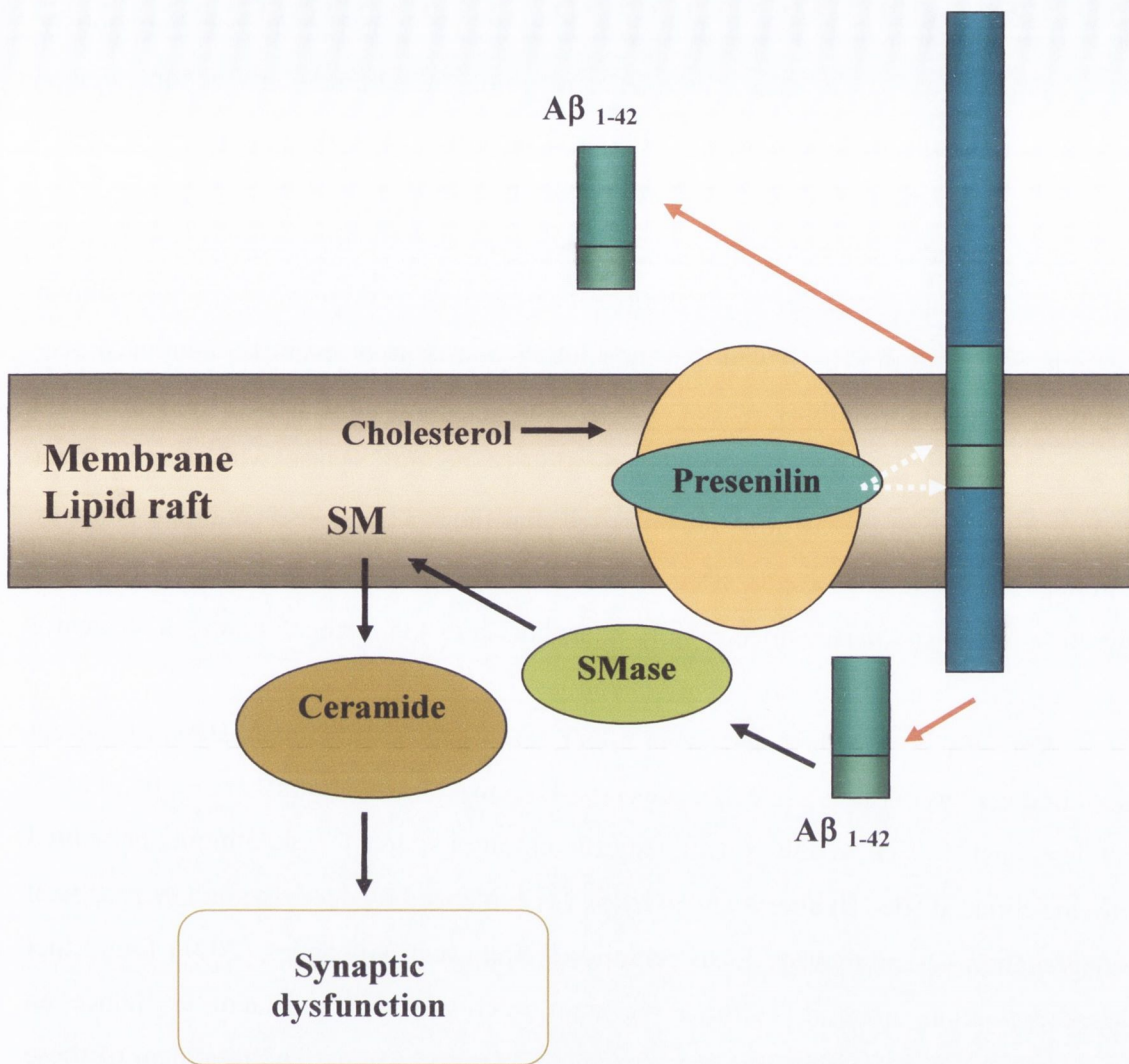
**Figure 1.16 Hypothetical scheme of events regulating the response of the stressed cell.**

This schema postulates roles for ceramide. In response to stress or injury, proximal caspases become activated if a death receptor is involved or p53 if DNA damage occurs. Both signal types induce ceramide production. FADD = Fas associated death domain, PLA<sub>2</sub> = Phospholipase A<sub>2</sub>, Smase = sphingomyelinase, ROS = Reactive oxygen species, p53 = Protein 53, Rb = retinoblastoma protein, Cyt c = Cytochrome C, Apaf-1 = apoptotic protease activating factor-1. . (Adapted from Dbaibo and Hannun (1998) *Apoptosis*.)



**Figure 1.17** A possible role for  $\gamma$ -secretase and  $A\beta$  peptides in membrane homeostasis.

This model predicts that cellular cholesterol and sphingomyelin levels control  $\gamma$ -secretase activity in APP cleavage. This signal is transmitted via  $A\beta$  peptide release to regulate lipid biosynthetic pathways.  $A\beta_{1-42}$  directly down regulates Sphingomyelin levels by activation of neutral SMase.  $A\beta_{1-40}$  down-regulates cholesterol synthesis by inhibiting HMGR activity. This system produces its own feedback mechanism, as the lipids that influence  $\gamma$ -secretase's environment also adjusts the rate of  $\gamma$ -secretase cleavage of APP. SMase = sphingomyelinase, SM = sphingomyelin, APP = amyloid precursor protein, HMGR = Hydroxymethylglutaryl-CoA reductase,  $A\beta$  = amyloid- $\beta$ . (Adapted from Grimm *et al.*, 2005, *Nat Cell Biol.* 7(11): 1118-23.



**Figure 1.18 Altered APP processing directly affects sphingolipid metabolism**

Altered APP processing directly affects cholesterol and sphingolipid metabolism. Figure 1.17 shows a positive feedback loop in AD in which lipid alterations increase Aβ<sub>1-42</sub> production and Aβ<sub>1-42</sub>, in turn, exacerbates the lipid abnormalities. SMase = sphingomyelinase, SM = sphingomyelin (Adapted from Grimm *et al.*, 2005, *Nat Cell Biol.* 7(11): 1118-23.

that IL-1 $\beta$ -induced action occurs downstream of IL-1R. IL-1 $\beta$  has been previously shown to increase neutral SMase activation resulting in ceramide production in non-neuronal cells (Kolesnick and Golde, 1994) and in mouse neuronal synaptosomes from wild-type, but not from IL-1<sup>-/-</sup> mice (Nalivaeva *et al.*, 2000).

On generation of ceramide in cell membranes, lipid rafts merge to form larger membrane domains (Bollinger *et al.*, 2005). The merging of these rafts has been shown to couple SMase to the IL-1R and that their interaction is involved in IL-1-induced proapoptotic signaling (Mathias *et al.*, 1993). In an interesting paper by Davis and colleagues (2006) IL-1 $\beta$ -mediated the activation of neutral SMase with a subsequent increase in ceramide production and proposed that the IL-1 $\beta$ -SMase-ceramide pathway comprises a second messenger system, activated by IL-1 $\beta$ , which leads to the rapid activation of Src (a kinase which rapidly phosphorylates ion channels) and subsequently negatively affects neuronal functioning in culture.

Not only does SMase activation affect neurons it has also been shown to affect microglial activity. Singh and colleagues (1998) showed in rat primary microglia, that *N*-acetylcysteine (NAC), an antioxidant and efficient thiol source for glutathione, prevented cytokine-induced (IL-1 $\beta$ ) alterations in redox (as evidenced by decreases in GSH) as well as degradation of sphingomyelin to ceramide. Yang and colleagues (2001) found that SMase activation alone in cultured rat brain microglia was sufficient to induce an increase in iNOS expression and hence promote NO production. The treatment of these cells with membrane permeable C2-ceramide had no such effect, suggesting that the redox sensitivity of brain microglia may play a critical role in the generation of ceramide leading to apoptosis of brain cells in neurodegenerative diseases.

### **1.5.9 Inhibition of SMase activation by GW4869**

Luberto and colleagues (2002) performed high throughput screening on neutral SMase in order to discover a suitable inhibitor of its functioning. They isolated GW4869 as a possible candidate. It functions as a non-competitive inhibitor and does not interfere with acid SMase activity. They found that GW4869 (20 $\mu$ M) completely inhibited neutral SMase activity following TNF- $\alpha$  treatment of MCF7 cells *in vitro*. This inhibition of the enzyme was accompanied by the inhibition of ceramide accumulation. GW4869 did not

alter cellular glutathione levels in response to TNF $\alpha$ , ruling it out as a modulator of GW action. Similarly it did not alter TNF- $\alpha$ -induced NF- $\kappa$ B translocation to the nucleus. Therefore it does not interfere with other TNF- $\alpha$  mediated signaling effects. The authors also showed that GW4869, in a dose dependant manner, was capable of significantly protecting MCF7 cells from apoptosis as measured by poly ADP ribose polymerase (PARP) degradation and trypan blue uptake. These effects were accompanied by a significant reduction in cytochrome c release from mitochondria with a concomitant decrease in caspase-9 activation. These findings localize GW4869 activity upstream of mitochondrial activation of the apoptotic machinery. It is still not known exactly what the targets of ceramide are when generated through the actions of neutral SMase pathway. Birbes and colleagues (2001) suggest that mitochondrial neutral SMase targets its own membranes and that the subsequent accumulation of endogenous ceramide is sufficient to induce cell death. A number of direct effectors have also been proposed in this process. Ceramide has been found to specifically activate a mitochondrial protein phosphatase 2A, which efficiently de-phosphorylates the anti-apoptotic protein, Bcl-2, leading to cell death (Ruvolo *et al.*, 1999). Recent studies have also shown that ceramide induces conformational changes in Bax protein causing it to alter mitochondrial function (Birbes *et al.*, 2005).

#### **1.5.10 secretory Phospholipase A<sub>2</sub> , its pathophysiological significance in Alzheimer's disease**

Using **secretory Phospholipase A<sub>2</sub>** (PLA<sub>2</sub>) inhibitors such as quinacrine, Emmerling and colleagues (1993) first linked PLA<sub>2</sub> activation to an increase in APP secretion. PLA<sub>2</sub> are a family of enzymes that catalyze the hydrolysis of phospholipids at the sn-2 position leading to the generation of free fatty acids such as arachidonic acid (AA). The secreted form of mammalian sPLA<sub>2</sub> plays an important role in the pathogenesis of inflammatory diseases.

Induction of the synthesis of sPLA<sub>2</sub> is generally initiated by a limited number of cytokines (TNF $\alpha$ , IL-1 $\beta$  and IL-6) via paracrine and/or autocrine processes (Touqui & Alaoui-El-Azher, 2001). The phospholipases are functionally coupled with cyclooxygenase (COX) and lipoxygenase (LOX) pathways to produce eicosanoids such



as the prostaglandins (PG) and leukotrienes from AA (Murakami *et al.*, 2000). AA is a potent second messenger and it operates in both an auto / paracrine manner in the CNS. sPLA<sub>2</sub> release of AA is dependant on the activational state of the cell. Under non-inflammatory conditions cell membranes are naturally resistant to the hydrolysing activity of the enzyme (Mounier *et al.*, 1994).

Eicosanoids are biologically very active proteins. The PGs, secreted by inflamed tissue and vascular endothelium induce vasoconstriction. PGs can be produced within the CNS or augmented by entry from the periphery as shown by Dascombe and Milton (1979). COX-2 up-regulation has been observed in transgenic mouse models of AD (Xiang *et al.*, 2002). Again using transgenic mice, Andreasson and colleagues (2001) have shown that neuronal over-expression of the COX2 isozyme in brain leads to neurodegeneration and age-related cognitive deficits. It has also been suggested that NSAIDs such as ibuprofen preferentially decrease A $\beta$ <sub>1-42</sub> via an effect that is independent of COX (Weggen *et al.*, 2001). Montine and colleagues (1999) have shown a specific approximately fivefold increase in CSF prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in patients with early AD, none of whom were taking NSAIDs or aspirin. Findings such as these have fueled substantial interest in polyunsaturated fatty acid (PUFA) oxidation, either enzyme-catalyzed or free radical-mediated, in the molecular pathogenesis of AD.

sPLA<sub>2</sub> – lipid interactions may also contribute to A $\beta$ -induced neurotoxicity. sPLA<sub>2</sub> promotes the generation of pro-inflammatory mediators by the interactions of its heparin-binding domains with heparan sulphate proteoglycans (HSPG). HSPGs are abundant in membrane lipid rafts (Murakami *et al.*, 2001) and function as a shuttling pathway. On binding of heparan, sPLA<sub>2</sub> is internalised, re-routed and concentrated into restricted compartments enriched in AA-containing phospholipids. The HSPG shuttling pathway allows sPLA<sub>2</sub> to come in contact with COX and LOX thereby creating conditions suitable for the efficient production of eicosanoids. HSPG have also been described as binding sites for A $\beta$ . The concentration of A $\beta$  and sPLA<sub>2</sub> at these sites, and their subsequent close proximity, could account for some of the pro-inflammatory effects driven by the peptide. HSPGs themselves are considered central to the pathology of AD in that they play a role in plaque formation, APP processing and fibril formation (McLaurin *et al.*, 1999). HSPGs bind a region on A $\beta$  known as the HHQK site. This

small region, which corresponds to the 13-16 domain of A $\beta$ , is the region of the peptide that binds to microglia. A $\beta$  binding to microglia is sensitive to competition with heparan sulphate, which suggests that plaque-microglia interactions are mediated by membrane-associated heparan sulphate (Giulian *et al.*, 1998). Strategies that exploit HHQK-like agents, may offer a specific therapy to block plaque-induced microgliosis and, in this way, slow the neuronal loss and dementia of AD.

## **1.6 Therapeutic approaches to Alzheimer's disease**

### **1.6.1 Acetylcholine esterase inhibitors**

There is extensive support for the involvement of cholinergic mechanisms in the biochemical and behavioural effects of A $\beta$  (Auld *et al.*, 2002). Recent studies have found that alterations in acetylcholine and related signaling can reverse the inhibitory effect of A $\beta$  on LTP, thereby providing a mechanistic basis for this important therapeutic target. Current medications used in the treatment of AD include AChE inhibitors for mild to moderate cases, and memantine, an NMDA-receptor antagonist for the treatment of moderate to severe AD dementia. All these drugs produced modest symptomatic improvements in some patients (Scarpini *et al.*, 2003; Cummings, 2004).

According to the cholinergic hypothesis of AD, the destruction of cholinergic neurons in the basal forebrain and the resulting deficit in central cholinergic transmission contribute substantially to the cognitive symptoms observed in AD patients (Bartus *et al.*, 1982; Cummings and Back, 1998). After its release into the synaptic cleft, acetylcholine is degraded rapidly by cholinesterase-driven hydrolysis – the most common enzymes in the human brain associated with this are AChE and butyrylcholinesterase (BChE) (Mesulam *et al.*, 2002). Inhibition of these enzymes leads to an increase in acetylcholine concentration in the synaptic cleft and subsequently enhances cholinergic transmission thereby ameliorating cholinergic deficits. There are 3 widely used cholinesterase inhibitors, galantamine, donepezil and rivastigmine and all are used to treat mild to moderate cognitive deficits. Rivastigmine also inhibits BChE, which accounts for 10% of cholinesterase activity in normal human brain and appears to be associated solely with glia (reviewed in Scarpini *et al.*, 2003). Analysis of the available randomized double-

blind, placebo-controlled studies support the use of any of the 3 cholinesterase inhibitors (Birks *et al.*, 2000; Birks *et al.*, 2003; Loy and Schneider, 2006), all performed equally in that treatment effects observed after 6 months were comparable for the 3 substances.

### **1.6.2 N-methyl-D-aspartate receptor antagonism**

NMDA receptor antagonists may protect the brain's nerve cells against excess amounts of glutamate; a messenger chemical released in large amounts by cells damaged by AD or certain other neurological disorders. Glutamate neurotoxicity is mediated via excessive activation of NMDA receptors and is believed to play a role in the cell death observed in AD affected brains (Hynd *et al.*, 2004).

Glutamate represents the main excitatory neurotransmitter in the CNS (Kornhuber and Weller, 1997) and its effects can be mediated by metabotropic receptors (G-protein coupled) or ionotropic receptors, which are ligand gated ion channels.

NMDA receptors fall into the latter category. Excessive activation of these receptors is thought to cause excessive concentrations of intracellular  $Ca^{2+}$ , which triggers downstream events leading to neurodegeneration. Consequently antagonists of this process have the potential for protecting neurons from glutamate-mediated neurotoxicity (Sucher *et al.*, 1991). Use of non-competitive inhibitors such as dextrorphan (Faden *et al.*, 1989) or MK801 (Mukhin *et al.*, 1997) have shown considerable neuroprotective activity after traumatic injury *in vivo* and *in vitro*, however, concerns have been raised about the possible side effects with such compounds, including neuronal vacuolization (Olney *et al.*, 1989) and psychotropic action (Kornhuber and Weller, 1997).

Memantine is, however, a non-competitive inhibitor, which has been shown not to affect NMDA receptor functioning. Areosa Sastre and colleagues (2005) reported that memantine was well tolerated and beneficial to those with moderate to severe AD. However, it has recently been reported that combination therapies to include AChE inhibitors (Galantamine) and NMDA receptor antagonists including memantine are significantly more effective than mono-therapy in improving or maintaining cognition, functionality, and behavior in patients with mild to moderate AD (Grossberg *et al.*, 2006).

### 1.6.3 Modulation of secretase activity

Several pharmaceutical companies have actively researched the production of small molecule compounds that can reduce A $\beta$  production by interfering with secretase activity. Following phase II clinical trials using  $\gamma$ -secretase compounds, Eli Lilly reported a reduction in A $\beta$  levels in plasma but not in CSF at concentrations that did not produce side effects (Siemers *et al.*, 2005). But concerns regarding the potential side effects that arise from interference with other  $\gamma$ -secretase targets such as Notch-1 (Geling *et al.*, 2002) have grown. As an alternative to  $\gamma$ -secretase inhibition, augmentation of the activities of  $\alpha$ -secretase was assessed as  $\alpha$ -secretase cleavage of APP precludes the generation of A $\beta$  since the  $\alpha$ -secretase cleavage site is located within the A $\beta$  sequence (Esch *et al.*, 1990). Cleavage of APP with  $\alpha$ -secretase can be stimulated by muscarinic acetylcholine-receptor agonists and were shown to reduce A $\beta$  generation in culture (Wolf *et al.*, 1995). Agonists such as these may play a role not only in the symptomatic treatment of the disease but show unique disease-modifying properties in that they modify enzymes which initiate A $\beta$  generation thus they may help prevent the instigation or progression of the disease with time (Fisher *et al.*, 2002).

### 1.6.4 Inhibition of amyloid- $\beta$ aggregation

Preventing the toxic aggregation of A $\beta$  by small molecules represents another approach to the development of AD therapeutics. Neurochem Inc. completed phase II clinical trials on a glycosaminoglycan mimetic (Alzhemed), which were designed to bind A $\beta$  peptides and thereby limit the formation of aggregates – the compound awaits phase III testing (Citron, 2004). Metal ions like Cu<sup>2+</sup> and Zn<sup>2+</sup> may be involved in the mediation of A $\beta$  toxicity (Atwood *et al.*, 1998). APP mutant mice displayed a significant decrease in A $\beta$  load following 9 weeks of treatment with clioquinol, an antibiotic and Cu/Zn chelator that crosses the BBB (Cherney *et al.*, 2001). Phase III clinical trials of clioquinol have been put on hold as toxic impurities have been found in the compound thought to occur during the manufacturing process (Boggs, 2005).

Recent work has indicated the potential usefulness of immunization strategies in the attenuation of symptoms of AD. Active (vaccination) and passive immunotherapy

with A $\beta$  are effective in transgenic mouse models of AD (Schenk *et al.*, 2004). Similar immunization strategies have been shown to improve cognitive deficits in APP<sup>-/-</sup> mice (Janus *et al.*, 2000) and to neutralize infused A $\beta$ O with a concomitant improvement in synaptic plasticity (Klyubin *et al.*, 2005). As vaccination was shown to be benign and effective in laboratory animals much enthusiasm surrounded its entry into phase I and II trials in human beings: around 200 patients in phase I tolerated the vaccine without incident, but about 6% of 300 patients in the phase II study developed acute autoimmune meningoencephalitis, causing the trial to be stopped (Orgogozo *et al.*, 2003). The patients have continued to be studied to characterize their cognitive function over a long period. Autopsy studies in patients registered for this trial and who have subsequently died showed a T cell-mediated autoimmune response to A $\beta$  but also showed extensive neocortical areas devoid of amyloid plaques and associated dystrophic neurites (Nicoll *et al.*, 2003; Ferrer *et al.*, 2004). The possibility for further immunization studies in humans remains open, but further studies involving a clear understanding of the mechanism of antibody-mediated targeting and clearance of A $\beta$  species need to be reconsidered before further clinical trials can be countenanced.

### 1.6.5 Other approaches

Markers of inflammation are typically observed in association with AD neuropathology (McGeer, Rogers and McGeer, 1984). Studies have indicated that long-term usage of NSAIDs may have a preventive effect against the development of AD (Szekely *et al.*, 2004) suggesting that neuroinflammation may contribute to neurodegeneration. Selective COX-2 inhibitor rofecoxib and the non-selective NSAID, naproxen, were tested in the treatment of mild to moderate AD, but neither drug slowed the rate of cognitive decline when compared with placebo-treated controls (Aisen *et al.*, 2003). On the other hand some NSAIDs such as ibuprofen modify  $\gamma$ -secretase activation by specifically reducing the production of A $\beta$ <sub>1-42</sub> alone (Weggen *et al.*, 2001), its activity did not affect Notch cleavage thereby showing that some anti-inflammatory treatments can preferentially reduce the generation of the highly amyloidogenic species A $\beta$ <sub>1-42</sub> without the potential side effects related to the complete inhibition of  $\gamma$ -secretase. APP<sup>-/-</sup> mice, have proven to be ibuprofen sensitive and display a reduced amyloid load and

microglial activation post treatment (Lim *et al.*, 2000). Other anti-inflammatory agents would therefore be of considerable interest for prevention of treatment of the cognitive decline associated with AD.

## **1.7 VP025; a novel anti-inflammatory agent**

VP025 is a proprietary preparation of phospholipid nanoparticles incorporating phosphatidylglycerol (PtG). Recognition of specific phospholipids by cells of the innate immune system has been identified as having important signaling function. For example, there is evidence that phosphatidylserine (PS), which is normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma membrane leaflet during apoptosis to serve as a trigger for recognition of apoptotic cells by phagocytes thus facilitating the clearance of cell debris and the containment of potentially deleterious pro-inflammatory responses (Martin *et al.*, 1996).

Phospholipids other than PS have also been implicated in anti-inflammatory reactions; particularly relating to the evasion by pathogens of attack by the immune system e.g. phosphatidylglycerol has been implicated in this phenomenon (Bayer *et al.*, 2006). Previously published results from other studies using different nanoparticles (phosphatidylserine) indicate their ability to block the inflammatory changes in the hippocampus associated with LPS (Nolan *et al.*, 2004). Recent unpublished results from this lab show that VP025 treatment of aged rats results in reversal of age-related inhibition of LTP accompanied by a significant decrease in JNK expression and IL-1 $\beta$  concentration in aged rat hippocampus. Not only this, VP025 treatment reverses the age-induced decrease in the pro-survival kinase ERK, indicating that, in addition to its neuroprotective effects VP025 can modulate processes which promote neuronal functioning.

For example, VP025 has been shown in a number of animal models of neurological disease, such as amyotrophic lateral sclerosis (ALS) and PD, or Age- or LPS-associated neuroinflammation in rats, to have both neuroimmunomodulatory and neuroprotective effects. These effects are thought to stem from modulation of microglial activation and/or apoptotic signalling.

In ALS based *in vitro* experiments, co-cultures of microglia and motoneurons treated with LPS or ALS IgG, showed that the effect of both treatments were significantly attenuated by VP025 so that VP025 prevented motoneuron cell death when compared with PBS-treated controls. *In vivo* experiments, which utilised a similar VP025 pre-treatment protocol to that used in this work, showed that VP025 treatment of SOD1 mice delayed disease onset by ~8 days, and death by ~18 days, in ALS affected animals. Immunohistochemistry showed that spinal chord tissue prepared from these animals had significantly less microglial activation compared with PBS-treated controls (Beers *et al.*, 2004 a, b).

VP025 pre-treatment has also had a positive effect in a rat model of Parkinson's disease, the 6-hydroxydopamine (6-OHDA) lesion of the medial forebrain bundle (Crotty *et al.*, 2008). Crotty and colleagues (2008) found that nigrostriatal dopaminergic neurons assessed 7 and 21 days post-lesion by amphetamine-induced rotational testing, displayed normal functioning in that rotational counts were significantly less in rats pre-treated with VP025 compared with PBS pre-treated 6-OHDA-lesioned rats. Neurochemical analysis, 10 and 28 days after lesion induction, showed that VP025 prevented the 6-OHDA-induced reduction in concentrations of striatal dopamine and its metabolites. In addition immunocytochemical analysis of the ipsilateral substantia nigra showed that VP025 significantly inhibited 6-OHDA-induced loss of dopaminergic neurons as well as preventing activation of microglia (MHCII) and p38 activation in these cells.

With respect to how peripherally administered VP025 may elicit its effects, unpublished confocal microscopy data has shown that VP025 is phagocytosed by human monocytic U937 cells showing that VP025 may readily be taken up by macrophage at the site of injection (Helen Skerit, Royal College of Surgeons Ireland (RCSI), by personal communication). Further research has shown VP025 capable of modulating T cell function in the periphery, in that it manipulates IL-2 and IFN- $\gamma$  production in paramethoxyamphetamine (PMA) stimulated T cells prepared from human donors (Helen Skerit, RCSI, by personal communication). Based on this evidence one could speculate that it's possible that peripheral administration of VP025 changes the intracellular signalling mechanisms of peripheral immune cells, leading to an alteration in their

surface morphology, their immune modulatory capacity, and the way they interact with each other – some of these elements will be explored in this work.

## 1.8 Objectives

The main objectives of this study are:

- To assess the effect of acute and long-term A $\beta$  administration on LTP in rat hippocampus.
- To assess the effect of acute and long-term A $\beta$  administration in the hippocampus and cortex with specific reference to A $\beta$ -related changes in microglial activation or neuronal viability.
- To assess the ability of VP025 to protect the hippocampus against the deleterious effects of A $\beta$  treatment, specifically any A $\beta$ -related attenuation in LTP.
- To assess the ability of VP025 to reverse any A $\beta$ -related changes in hippocampal or cortical tissue, specifically any A $\beta$ -associated increase in microglial activation, inflammation or neuronal cell death.



## **Chapter 2**

### **Materials and Methods**

## 2.1 Materials

Actin antibody	Santa Cruz
Agarose	Sigma
Amyloid- $\beta$	Biosource
Anti-mouse IgG	Sigma
Arabino-furanoside	Sigma
B-27	Gibco
Bis-Tris gels	Invitrogen
Bovine serum albumin	Sigma
Calcium chloride	Lennox
Caspase-3 assay kit	Biomol
Caspase-8 assay kit	Biomol
CD86 antibody	R&D systems
Ceramide	Sigma
Chloroform	Sigma
Coomassie G-250 solution	Pierce
Dental cement	Stoelten
Deoxynucleoside triphosphate	Promega
DEPC	Sigma
Dimethyl sulphoxide	Sigma
Dithiothreitol	Invitrogen
DNase	Sigma
DNase-RNase free water	Sigma
ECL	GE Healthcare
EDTA	Sigma
ELISA substrate solution	R&D Systems
Ethanol	Sigma
Ethidium bromide	Sigma
First-strand buffer	Invitrogen
Gel dye	Promega
Glutamax	Gibco
Glycerol	Sigma

Goat anti-rat antibody	Vector
GW4869	Sigma
HEPES	Sigma
ICAM-1 antibody	Santa Cruz
IL-1 $\beta$ ELISA kit	R&D systems
Immuno plates	Nunc
Isopropanol	Sigma
Loading dye	Promega
Magnesium chloride	Sigma
Magnesium sulphate	Sigma
MTS cell proliferation assay	Promega
Neurobasal media	Gibco
Normal goat serum	Vector
Oligo (dT)	Invitrogen
Osmotic minipumps	Alzet
PBS (sterile)	Sigma
Penicillin	Gibco
Potassium chloride	Sigma
Potassium hydrogen phosphate	Sigma
Potassium hydrogen phosphate	Sigma
Ribonuclease inhibitor	Invitrogen
Rimadil	Pfizer
Rompun	Bayer
Sample buffer & Reducing agent	Invitrogen
Sodium chloride	Sigma
Sodium hydrogen carbonate	Sigma
Sodium hydrogen phosphate	Sigma
Sphingomyelinase assay kit	Molecular Probes
sPLA <sub>2</sub> activity assay kit	R&D Systems
Streptomycin	Gibco
Superscript II	Invitrogen
Taqman universal PCR master mix	Applied Biosystems
Thioflavin-T	Sigma

Tissue chopper	McIlwain
Tris-HCL	Sigma
Trypsin	Sigma
Trypsin inhibitor	Sigma
Tween-20	Lennox
Urethane	Sigma
Vetalar	Pharmacia
VP025	Vasogen

## 2.2 Chronic amyloid- $\beta$ administration study; preparation

### 2.2.1 Preparation of artificial cerebrospinal fluid

aCSF was the vehicle used to deliver treatment and control peptides from the osmotic mini-pumps. aCSF comprised two solutions, both were made up under sterile conditions to 50ml in sterile pyrogen free water to a ten times stock concentration. The first solution contained: sodium chloride (NaCl) (149mM, Sigma, UK), potassium chloride (KCl) (3mM, Sigma, UK), calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) (1.9mM, BDH, UK), magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) (0.8mM, Sigma, UK), the second: disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) (0.8mM, Sigma, UK), sodium hydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) (0.2mM, Sigma, UK). When required both solutions were diluted by a factor of 10, filtered sterilized and combined on a 1:1 ratio for use. Solutions stored at  $-4^\circ\text{C}$ .

### 2.2.2 Preparation of amyloid- $\beta$ peptides

$\text{A}\beta$  peptides ( $\text{A}\beta_{1-40}$ ,  $\text{A}\beta_{1-42}$ , Biosource, Belgium) and reverse control peptide ( $\text{A}\beta_{40-1}$ , Biosource, Belgium) were aggregated according to manufacturer's instructions. The  $\text{A}\beta_{1-40}$  and  $\text{A}\beta_{1-42}$  lyophilised peptides were dissolved in sterile  $\text{dH}_2\text{O}$  to a concentration of 6mg/ml and then further diluted to a 1mg/ml stock solution with calcium free sterile phosphate buffered saline (PBS) (Sigma, UK). The  $\text{A}\beta_{1-40}$  peptide was aggregated for 24h at 25 degrees Celsius ( $^\circ\text{C}$ ), the  $\text{A}\beta_{1-42}$  peptide at  $37^\circ\text{C}$  for 48h. The control peptide,  $\text{A}\beta_{40-1}$ , was dissolved in sterile  $\text{dH}_2\text{O}$  to a concentration of 1mg/ml and incubated at  $25^\circ\text{C}$  for 24 hrs. Samples of all  $\text{A}\beta$  stock solutions (50 $\mu\text{l}$ ) were drawn off at the time of dissolving, and again 24 and 48h later. These aliquots were set aside for analysis of the fibrillar nature of the  $\text{A}\beta$  stock (see section 2.2.3). The remaining stock solutions were aliquoted and stored at  $-20^\circ\text{C}$  until required.

In the experiment in which  $\text{A}\beta_{1-40}$  was delivered acutely icv,  $\text{A}\beta$  was diluted in sterile water to a final concentration of 200 $\mu\text{M}$  and 5 $\mu\text{l}$  were injected, saline (5 $\mu\text{l}$ ) was used as control in this experiment. The study, which involved  $\text{A}\beta_{1-40}$  and  $\text{A}\beta_{1-42}$  being acutely co-administered, both  $\text{A}\beta_{1-40}$  and  $\text{A}\beta_{1-42}$  were diluted in aCSF to give a combined final concentration of 45 $\mu\text{M}$  or 200 $\mu\text{M}$ , again 5 $\mu\text{l}$  of both concentrations were injected,

the inert reverse peptide A $\beta$ <sub>40-1</sub>, at a concentration of 200 $\mu$ M (5 $\mu$ l), was used as control in this experiment.

Experiments in which A $\beta$  was delivered chronically by osmotic mini-pump, A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> were diluted in aCSF to a final concentration of 26.9 $\mu$ M (0.7 $\mu$ g/ $\mu$ l) and 36.9 $\mu$ M (1 $\mu$ g/ $\mu$ l). Pumps were loaded with 23.33 $\mu$ l and 33.33 $\mu$ l of each peptide respectively. The control peptide (A $\beta$ <sub>40-1</sub>) was loaded at a molecular concentration of 63.8 $\mu$ M. All pumps were filled to a final 200 $\mu$ l volume using aCSF.

The administration concentration (200 $\mu$ M) for both acute A $\beta$  administration studies (single (1-40) and combined A $\beta$  species (1-40/1-42)), was chosen as A $\beta$ <sub>1-40</sub> (200 $\mu$ M) had previously been shown to inhibit LTP, as well as increase markers of A $\beta$ -induced microglial stress, such as JNK activation (Minogue *et al.*, 2005). It was not known prior to acute A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> at 200 $\mu$ M, whether this concentration of combined A $\beta$  peptides would elicit similar effects. The lower concentration of 45 $\mu$ M was also used in the acute A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> study to see whether a dose response could be elicited with respect to A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>'s effect on LTP.

For the chronic administration studies the concentrations of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> (26.9 and 36.9 $\mu$ M respectively) were derived from a number of papers, which had previously shown co-infusion of A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> to induce cognitive deficits, microglial activation and neurotoxicity in rat brain at these concentrations (Frautschy *et al.*, 1998; 2001). A $\beta$ <sub>1-42</sub> was utilized as it is considered to be the more toxic of the two A $\beta$  species. The difference in biological activity between the two oligomeric species of A $\beta$  is thought to lie in the addition of a C-terminal Ile-Ala dipeptide. This difference alters the  $\beta$ -sheet folding of the larger 1-42 peptide lending itself to great toxicity (Klein *et al.*, 2004). The A $\beta$ <sub>1-42</sub> peptide is however prone to greater aggregation than A $\beta$ <sub>1-40</sub> and excessive aggregation of A $\beta$ <sub>1-42</sub> in the pump may have lead to failure of the A $\beta$  treatment reaching the ventricles. Thus A $\beta$ <sub>1-40</sub>, the most prevalent A $\beta$  species in the brain, was co-infused with A $\beta$ <sub>1-42</sub>. Both peptides have been shown to have inhibitory effects on LTP (Cullen *et al.*, 1997).

### 2.2.3 Confirmation of the fibrillar nature of the amyloid- $\beta$ peptide

Using sample peptides prepared for the chronic A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> administration studies (section 2.2.2), the presence of fibrillar A $\beta$  in the infused preparations was

demonstrated using a thioflavin T (ThT) fluorescent assay. The binding of ThT (10  $\mu$ l; 100  $\mu$ M; Sigma, UK) to fibrillar A $\beta$  was monitored by an increase in ThT fluorescence (435nm excitation, 485nm emission). ThT fluorescence was increased maximally by 209% over the 48 h incubation period. The A $\beta$  preparation (0 and 48 hrs aggregation) was also assessed for oligomeric forms by gel electrophoresis and Coomassie blue staining. Briefly, samples (10  $\mu$ l) were boiled for 2min, loaded onto 12% sodium dodecyl sulphate (SDS) gels and the A $\beta$  species were separated by application of a 32 mA current. Gels were rinsed in water, incubated overnight with the Coomassie G-250 solution (15 ml; GelCode Blue Stain Reagent, Pierce, USA) and photographed (Gel-Doc-It Bioimaging System, Ultraviolet Products Inc., UK). The predominant A $\beta$  species in the non-incubated preparation (56% of the total) was the 13.5-kDa species and its presence decreased linearly with incubation time so that after 48h it comprised 36% of the total. Therefore the injected A $\beta$  was maximally aggregated and contained this oligomer (see Figure 2.1).

#### **2.2.4 Preparation and priming of osmotic mini-pumps**

The osmotic mini-pump (model 2004, Alzet, USA) delivers solutions continuously for 28 days. The pumps work by osmotic displacement. The entry of water causes the osmotic chamber to expand, thereby compressing the flexible reservoir enabling delivery of the test compound at the site of implantation. All pumps and catheters/cannulae were exposed to a sterilizing dose of radiation during the filling and priming process. The pump rate used was 0.25 $\mu$ l/h ( $\pm$  0.05 $\mu$ l at 37°C) and reservoir volume was 200 $\mu$ l. The complete pump system (pump/flow moderator and catheter/cannula) was filled and primed according to the manufacturers' instructions. Briefly, using a 1ml syringe (with blunt-tipped 27 gauge filling needle) each pump/catheter unit was slowly filled with A $\beta$ <sub>40-1</sub> or A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> treatment solution to minimize the occurrence of air bubbles. The filled pumps were then placed in a sterile saline filled 50ml falcon tube at 37°C for 40h prior to implantation to allow for osmotic priming to occur. Priming allows the pump to begin operating before implantation and minimizes the chance of occlusions or clots forming in the catheter.

#### **2.2.5 Preparation of VP025 for *in vivo* work**

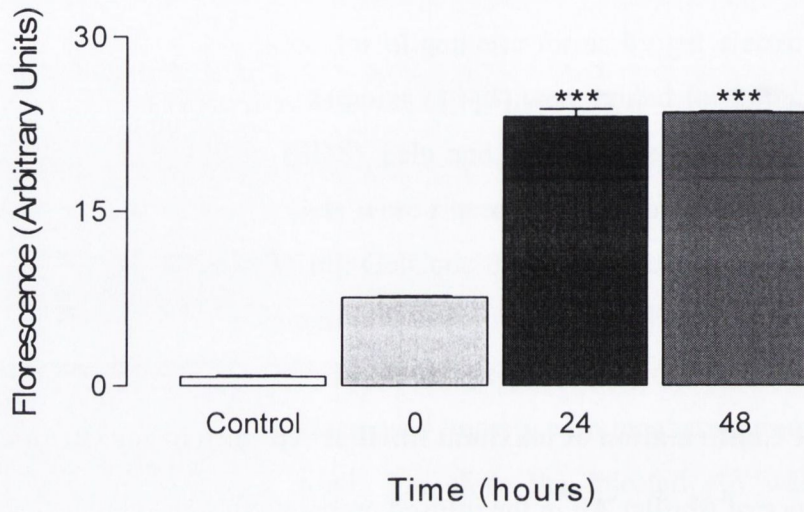
VP025 is a novel preparation of phospholipid nanoparticles incorporating phosphatidylglycerol (Vasogen Inc., Canada). Serial dilutions of VP025 stock

### **Figure 2.1 Confirmation of maximal fibrillar A $\beta$ content for chronic infusion**

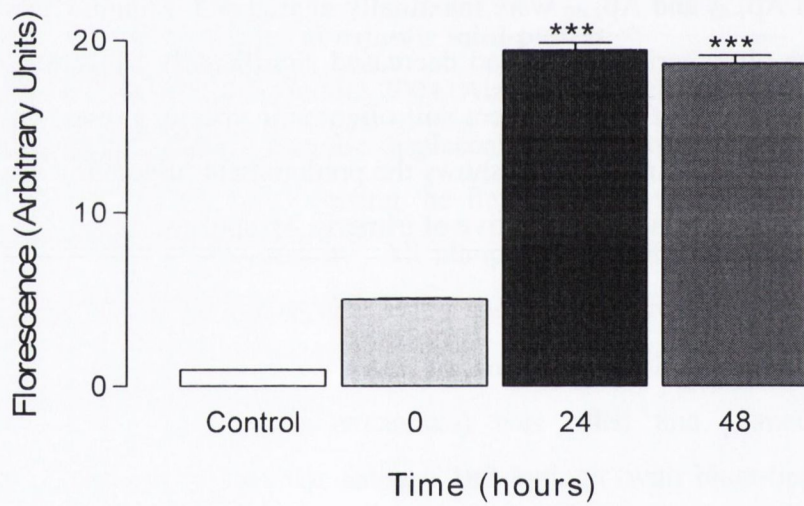
The presence of fibrillar A $\beta$  in the infused preparations was demonstrated using a thioflavin T fluorescent assay . Figure 3.1a and 3.1b show that following 24h incubation A $\beta_{1-42}$  and A $\beta_{1-40}$  were maximally aggregated. Figure 3.1c shows that the relative oligomeric content had decreased significantly by some 20% over 48h aggregation but that there were still oligomeric species present in the pump infusate – denistometric analysis shows the predominant oligomeric species to be ~13.5 kDa in size which is indicative of trimeric A $\beta$  species.



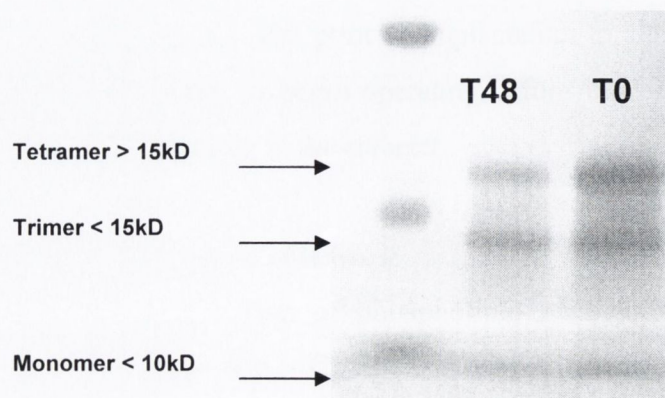
a)  $A\beta_{1-42}$  displays maximal aggregation at 24h



b)  $A\beta_{1-40}$  displays maximal aggregation at 24h



c)  $A\beta_{1-42}$  oligomeric content drops significantly with aggregation



solution were made as follows. VP025 stock solution (10 $\mu$ l;  $1.1 \times 10^{14}$  particles/ml, batch number: VSG200401) was diluted in 10ml sterile PBS to  $1.1 \times 10^{11}$  particles/ml. The  $1.1 \times 10^{11}$  solution (10 $\mu$ l) was further diluted in 10ml of PBS yielding 10ml at  $1.1 \times 10^8$ . An aliquot of the  $1.1 \times 10^8$  solution (2.727 ml) was added to 22.273ml of sterile PBS giving 25ml of VP025 solution at a final injecting concentration of  $1.2 \times 10^7$  particles/ml.

## **2.3 Animals**

### **2.3.1 Housing of animals**

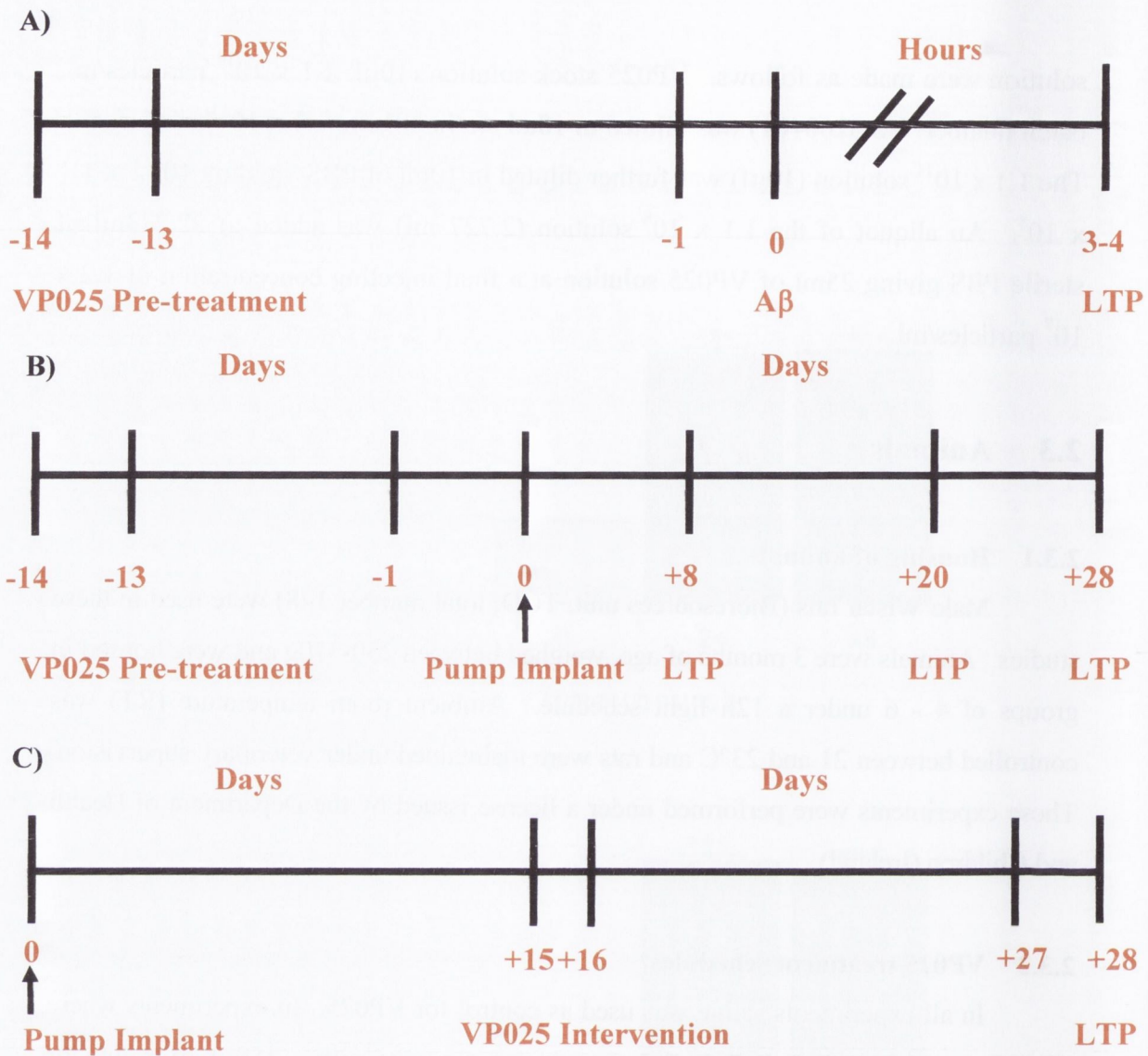
Male Wistar rats (Bioresources unit, TCD; total number 148) were used in these studies. Animals were 3 months of age, weighed between 250-370g and were housed in groups of 4 - 6 under a 12h light schedule. Ambient room temperature (RT) was controlled between 21 and 23°C and rats were maintained under veterinary supervision. These experiments were performed under a license issued by the Department of Health and Children (Ireland).

### **2.3.2 VP025 treatment schedules**

In all experiments saline was used as control for VP025. In experiments where VP025 was administered prior to surgery rats received either 150 $\mu$ l of saline or VP025 ( $1.2 \times 10^7$  particles/ml) by intramuscular injection (im) 14, 13 and 1 day prior to surgery. Where VP025 was used as an intervention treatment rats received either 150 $\mu$ l of saline or VP025 ( $1.2 \times 10^7$  particles/ml) im 15, 16 and 27 days following surgery. The hind leg to which VP025 was administered alternated over the 3 days (Figure 2.1).

### **2.3.3 Pre-surgical preparation of animals**

In the experiment in which A $\beta$ <sub>1-40</sub> or A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> were delivered by acute icv injection, rats were anaesthetized by intraperitoneal (ip) injection with urethane (Sigma, UK; 1.5g/kg; 33% w/v). In the experiments in which A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> was delivered chronically using osmotic mini-pumps rats were anesthetized by ip injection with ketamine (Vetalar; 75mg/kg; Pharmacia, Germany) and xylazine (Rompun; 10mg/kg;



**Figure 2.2 Experimental outlines and VP025 treatment schedules**

A1) Acute  $A\beta_{1-40}$  administration - Rats were pre-treated with saline or VP025 14, 13 and 1 day prior to icv injection with saline or  $A\beta_{1-40}$ (200 $\mu$ M), LTP was assessed after 3h.

A2)  $A\beta_{40-1}$  (200 $\mu$ M) or  $A\beta_{1-40}/A\beta_{1-42}$  (45 and 200 $\mu$ M), were administered icv and 4h later rats were assessed for their ability to maintain LTP, VP025 was not administered in this experiment.

B) Chronic  $A\beta_{1-40}/A\beta_{1-42}$  infusion study – Rats were pre-treated with saline- or VP025- 14, 13 and 1 day prior to being implanted with osmotic minipumps containing  $A\beta_{1-40}/A\beta_{1-42}$  or  $A\beta_{40-1}$ , rats were infused for 8, 20 or 28 days prior to LTP assessment.

C) Chronic  $A\beta_{1-40}/A\beta^+$  infusion study –Rats were implanted with osmotic minipumps containing  $A\beta_{1-40}/A\beta_{1-42}$  or  $A\beta_{40-1}$ , VP025 intervention treatment began 15 days following implantation.

Bayer, Germany). The absence of pedal reflex was used to establish the existence of deep anaesthesia.

### **2.3.4 Acute amyloid- $\beta$ administration; icv procedure**

Following anesthetization (section 2.3.3) rats were placed in a stereotaxic instrument with the incisor bar 2 millimetres (mm) below the ears to ensure the skull was flat. The scalp was incised and retracted and a hole drilled (diameter 0.45mm) 2.5 mm posterior to bregma to allow for  $A\beta_{1-40}$  or  $A\beta_{1-40}/A\beta_{1-42}$  administration to the lateral ventricle. In the first acute  $A\beta$  administration study, rats were injected (icv) with sterile  $dH_2O$  as control, or  $A\beta_{1-40}$  (5 $\mu$ l, 200 $\mu$ M, Biosource, Belgium), the rats were subsequently rested for 3h prior to being assessed for their ability to sustain LTP. In the second acute  $A\beta$  administration study, rats were injected with control peptide,  $A\beta_{40-1}$ , or  $A\beta_{1-40}/A\beta_{1-42}$  at two concentrations 45 and 200 $\mu$ M (5 $\mu$ l, Biosource, Belgium), rats in this study were assessed for the ability to sustain LTP 4h following  $A\beta$  treatment (Figure 2.1).

### **2.3.5 Chronic amyloid- $\beta$ administration; implantation procedure**

Following anesthetization (section 2.3.3) rats were placed in a stereotaxic instrument with the incisor bar 2mm below the ears to ensure the skull was flat. The scalp was incised and retracted. A hole was drilled (diameter 0.45mm) at coordinates 0.9mm posterior to bregma and 1.3mm lateral to the midline to allow for icv  $A\beta_{1-40}/A\beta_{1-42}$  administration to the fourth ventricle. An osmotic mini-pump (Alzet, model 2004) containing  $A\beta_{1-40}/A\beta_{1-42}$  (26.9 $\mu$ M and 36.9 $\mu$ M) or reverse peptide  $A\beta_{40-1}$  (63.8 $\mu$ M) was positioned in a subcutaneous pocket between the scapulae. The pump was attached via polyvinylchloride tubing (Alzet, 0.69mm diameter) to a chronic indwelling cannula (Alzet, Infusion Kit II), which was positioned stereotaxically to allow the cannula tip to extend 3.5mm ventral to the dura through the pre-existing drilled hole. The cannula was affixed to the skull using cryanoacetate gel and was secured in place by a smooth covering of dental cement (Stoelten, USA). Post-operative care included a subcutaneous injection of analgesia (Rimadil; 5mg/kg; Pfizer, Ireland). The rats were closely monitored during recovery and kept under a heat lamp until they were active. Food and liquid intake as well as behaviour were monitored closely for the implant period i.e. 8, 20 or 28 days (Figure 2.2).

## **2.4 Induction of long-term potentiation *in vivo***

### **2.4.1 Preparation of animals**

Following urethane anaesthetization (section 2.3.3) rats were placed in a stereotaxic instrument with the incisor bar set 2mm below the ear bars to ensure the skull was flat. The scalp was incised along the midline and retracted, and a drill was used to remove a window of the skull to expose the brain. The dura was peeled away to allow penetration of and correct placement of electrodes.

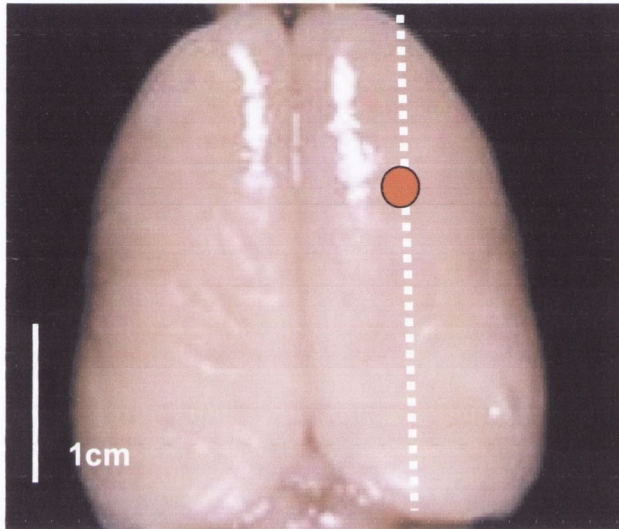
### **2.4.2 Electrode implantation and excitatory postsynaptic recordings**

To assess the ability of rats to sustain LTP a bipolar stimulating electrode and a unipolar recording electrode were stereotaxically positioned in the perforant path (4.4mm lateral to lambda) and dorsal cell body region of the dentate gyrus (2.5mm lateral and 3.9mm posterior to Bregma) respectively. Following a period of stabilization, test shocks were delivered at 30 second (s) intervals and responses recorded over 10min to establish stable baseline recordings. This was followed by delivery of 3 trains of stimuli (250Hz for 200ms; 30s intertrain interval). Recording at test shock frequency resumed for 45min. The slope of the EPSP was used as a measure of excitatory synaptic transmission in the dentate gyrus. A post tetanic change in the EPSP slope was used to indicate excitatory synaptic transmission and changes were expressed as a percentage of control baseline recordings prior to tetanus.

## **2.5 Preparation of tissue**

### **2.5.1 Dissection**

At the end of the LTP recording period, rats were killed by decapitation, the brains were rapidly removed, placed on ice, and one quarter of the brain was frozen for later preparation of cryostat sections. The portion of the brain taken for cryostat sections were taken from the outer half of the hemisphere into which the cannula had been implanted (Figure 2.3). Both hippocampus and cortex were dissected from the remaining brain. A small portion of both cortex and whole hippocampus (included CA1 and dentate gyrus) were flash-frozen in liquid N<sub>2</sub> for later analysis of messenger



**Figure 2.3. Dorsal view of the whole adult rat brain**

The red dot indicates the approximate area of cannulae implantation. The white dashed line indicates the portion of quarter brain taken cryostat sectioning. Image adapted from a photograph by Adam C. Puche, copyright Adam C. Puche. [Updated Oct 2003; cited 20/02/2008]. Available from: [www.apuche.org/OIA/Anatomical-Page-03.htm](http://www.apuche.org/OIA/Anatomical-Page-03.htm)

RNA (mRNA). Slices (350 x 350  $\mu\text{m}$ ) were prepared from the rest of the tissue using a McIlwain tissue chopper. These slices were prepared for storage in Krebs buffer (NaCl 136mM, potassium chloride (KCl) 2.54mM, potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) 1.18mM, magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) 1.18mM, sodium hydrocarbonate ( $\text{NaHCO}_3$ ) 16mM, glucose 10mM) with added  $\text{CaCl}_2$  (1.13 mM) and 10% dimethyl sulphoxide (DMSO) and stored at  $-80^\circ\text{C}$  until required for analysis.

### **2.5.2 Protein quantification**

Protein quantification was assessed using the bicinchoninic acid (BCA) assay. It provides a sensitive assay for proteins that is not affected by the presence of detergents in the protein solution. The protein to be analysed reacts with copper ( $\text{Cu}^{2+}$  ions) in an alkaline solution to make  $\text{Cu}^{1+}$  ions. These ions then chelate with the BCA converting the green solution of free BCA to a deep purple colour indicating the formation of the copper-BCA complex. The complex is linear with increasing protein concentration over a working range of  $20\mu\text{g/ml}$  to  $2000\mu\text{g/ml}$ . Protein standards were prepared from a stock solution of  $2000\mu\text{g/ml}$  bovine serum albumin (BSA). This was diluted in sample buffer (e.g. Krebs solution) to prepare a range of standards from 0 to  $2000\mu\text{g/ml}$ . Standard and samples ( $25\mu\text{l}$ ) were added in triplicate to a 96-well plate (Sarstedt, Ireland), BCA reagent was added, samples were incubated at  $37^\circ\text{C}$  for 30min and absorbance assessed at 540nm using a 96-well plate reader (Labsystems Multiskan RC). A regression line was plotted (GraphPad Prism, USA) and the concentration of protein per sample extrapolated from it.

### **2.5.3 Preparation of hippocampal and cortical homogenate**

Samples of whole-cell homogenate for immunoblotting, Enzyme Linked ImmunoSorbent assay (ELISA) and enzymatic activity analyses were prepared from whole brain tissue. In brief, hippocampal or cortical slices were thawed rapidly, washed three times and homogenised (x30 strokes) in Krebs solution containing  $\text{Ca}^{2+}$  ( $600\mu\text{l}$ – $1\text{ml}$ ) using a 1ml glass homogeniser (Jencons, Bedfordshire, UK). The homogenized tissue was divided into aliquots according to assay requirements. The whole-cell homogenates were stored at  $-80^\circ\text{C}$  until required. Protein concentrations were determined by the BCA (Pierce, USA) method using BSA as a standard (section 2.5.2).

#### **2.5.4 Messenger ribonucleic acid isolation**

For all RNA work, standard precautions were taken to keep reagents, consumables and equipment free from RNases (Sambrook and Russell, 2001). Hippocampal tissue was thawed on ice and homogenized in ~600ml of TRI Reagent (Sigma, UK) using a sterile homogenizer. The homogenized tissue was then removed to autoclaved 1.5ml eppendorf tubes, which were centrifuged (12,000g, 10min, 4°C) to remove debris from the supernatant. Supernatant was transferred to clean 1.5ml eppendorf tubes and allowed to stand at RT for 5min. Chloroform (150µl; Sigma, UK) was added, samples were shaken vigorously for 15s and allowed to stand (15min, RT). Samples were centrifuged (12,000g, 15min, 4°C) and the clear aqueous layer placed in fresh 1.5ml eppendorf tubes to which ~350µl of isopropanol (Sigma, UK) had been added. The tubes were mixed vigorously and allowed to stand for 10min at RT. The samples were centrifuged (12,000g; 10min; 4°C) to yield a white mRNA pellet and supernatant, which was removed and discarded. Pellets were washed with ~ 1ml of 75% ethanol (EtOH) (Sigma, UK) vortex-mixed and centrifuged (7,500g, 5min, 4°C). The alcohol supernatant was carefully removed; the pellet air dried in a sterile fume hood and resuspended in 30µl of DNase-RNase free water (Sigma, UK). All hippocampal mRNA samples were stored at -80°C until required.

### **2.6 Reverse transcriptase-polymerase chain reaction (RT-PCR) of amyloid-β treated hippocampal ribonucleic acid and copy deoxyribonucleic acid product**

#### **2.6.1 Ribonucleic acid integrity checks**

Gel electrophoresis is used to separate RNA products according to their size. In order to visualize RNA, ethidium bromide (EtBr), which interchelates between the nucleotides of the RNA and fluoresces when illuminated by ultraviolet light, was added to the gel. Agarose (1.3g; Sigma, UK) was dissolved in 130ml of 1 times Tris-borate-ethylenediaminetetraacetate (EDTA) (TBE) buffer by heating in a microwave giving a 1% agarose solution. Once the agarose had cooled sufficiently (~40°C), EtBr (1.3µl, 10mg/ml stock; Sigma, UK) was added to a final concentration of 0.5µg/ml.



The contents of the flask were poured into a mini-gel electrophoresis tank, which was fitted with a well comb and dividers, and allowed to set.

Once solid, the comb and supports were removed and TBE was added to the tank until the surface of the gel was submerged. Equal quantities (3 $\mu$ l) of mRNA samples were added to 2 $\mu$ l of gel dye (Promega, UK) and 2 $\mu$ l of diethyl pyrocarbonate (DEPC; Sigma, UK)-treated sterile water. Aliquots of sample mixture (3 $\mu$ l) were loaded to each well and gels were run at 90 volts for 40min. RNA was photographed and quantified using densitometry (Labworks, UVP BioImaging Systems, UK). The resulting images were used to assess the presence and the quantity of RNA in each sample. Successful preparation of mRNA is displayed by separation of the mRNA into two predominant bands of small ~2kb and large ~5kb ribosomal RNA (28S:18S rRNA), the ratio of intensities of these bands should be 1-2:1 (Sambrook *et al.*, 2001).

### **2.6.2 Reverse transcriptase-polymerase chain reaction copy deoxyribonucleic acid synthesis from amyloid- $\beta$ treated hippocampal messenger ribonucleic acid.**

The amount of hippocampal RNA used as template for first strand or copy DNA (cDNA) synthesis was sample specific. The maximum RNA template used for synthesis was 10 $\mu$ l. The amount loaded was determined by examining each samples RNA (28S:18S rRNA) band intensity relative to the other samples. If less than 10 $\mu$ l RNA was required the template shortfall was made up using DEPC-treated sterile water. The RT-PCR protocol is divided into two discrete steps; (i) RT (reverse transcription) to convert hippocampal mRNA to cDNA and (ii) PCR to amplify a fragment of the hippocampal cDNA using specific oligonucleotides (primers). Briefly, the mRNA was reverse transcribed in 0.2ml microcentrifuge tubes to produce first strand cDNA. To achieve this, Oligo(dT) (1 $\mu$ l; Invitrogen, UK) and deoxynucleoside triphosphate (dNTP) (1 $\mu$ l; Promega, UK) were added to each mRNA sample (10 $\mu$ l), giving a total reaction volume of 12 $\mu$ l, using a cDNA thermocycler (Biometra, Germany) the mixture was incubated at 65°C for 5min and then placed on ice. The following reagents were added to each tube: 4 $\mu$ l 5x First-Strand Buffer (Invitrogen, UK), 2 $\mu$ l of dithiothreitol (DTT, 0.1mM, Invitrogen, UK), 1 $\mu$ l ribonuclease inhibitor (Invitrogen, UK) for a final volume of 19 $\mu$ l. Samples were mixed gently and incubated at 42°C for 2min. Following heating, Superscript II (1 $\mu$ l;

Invitrogen, UK) was added to each tube and mixed by pipetting giving a total volume of 20µl of hippocampal cDNA. The tubes were incubated for 50min at 42°C and the reaction inactivated by heating to 70°C for 15min. All hippocampal cDNA products were stored at -20°C until required.

### **2.6.3 Differential expression analysis of $\beta$ -actin/OX-6 in amyloid- $\beta$ treated rat hippocampus**

Expression of MHCII mRNA was assessed by probing for OX-6. The same basic protocol, for PCR amplification of  $\beta$ -actin or MHCII PCR product, was used throughout the study. All reaction components and cDNA samples were thawed and kept on ice and all non-DNA containing components were irradiated to ensure sterility. Master Mix (DNase-RNase free water, buffer, dNTPs, and *Taq* DNA polymerase) was made first (number of reactions plus one), with *Taq* DNA polymerase added last. The Master Mix was vortex-mixed and kept on ice. Template, in the form of DNase-RNase free water as negative control, or sample cDNA (2µl) was then added to the PCR tubes. The Master Mix (23µl) was added to the negative control tube and sample tubes. The PCR tubes were vortex-mixed, placed in the thermocycler (PTC-2000, MJ Research, USA) and the PCR programme started (Table 2.1 (a) (b)).

### **2.6.4 Agarose gel electrophoresis of hippocampal $\beta$ -actin/OX-6 deoxyribonucleic acid**

Gel electrophoresis analysis was also used to separate DNA products according to size. In order to visualize PCR DNA products EtBr is added to the gel (0.5µg/ml; Sigma, UK, 10mg/ml stock). It interchelates between the nucleotides of the DNA and fluoresces when illuminated with ultraviolet light. PCR DNA products are electrophoresed on 1.5% agarose TBE gels. To this end agarose (2g; Sigma, UK) was heat-dissolved in 130ml of 1x tris borate-EDTA (TBE) buffer. A 100bp ladder (New England Biolabs, UK) was used to cover the size range of the expected PCR product. PCR products (7µl) were added to a loading dye (2µl; Promega, UK) and 1µl of DEPC (Sigma, UK) treated sterile water. PCR product solution (8µl) was loaded into each well and the PCR products were allowed to run on the gel until the DNA bands of the ladders had separated sufficiently ~ 90min at 90 volts. PCR products were

photographed and quantified using densitometry (Labworks, UVP BioImaging Systems, UK). The target gene was normalized to mRNA expression of the  $\beta$ -actin housekeeping gene.

### **2.6.5 Quantitative polymerase chain reaction (QPCR)**

QPCR primers and probes were delivered as “TaqMan® Gene Expression Assays” for the rat genes listed in Table 2.2 (Applied Biosystems, Darmstadt, Germany). QPCR was performed on Applied Biosystems ABI Prism 7300 Sequence Detection System v1.3.1 in 96-well format and 25 $\mu$ l reaction volume per well. cDNA (200 picograms(pg)/well) was mixed with Taqman Universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany) and the respective target gene assay. Rat  $\beta$ -actin RNA (# 123456, Applied Biosystems, Darmstadt, Germany) was used as reference. Each sample was measured in duplicate in a single QPCR run. Forty cycles were run with the following conditions: 2min at 50°C, 10min at 95°C and for each cycle 15s at 95°C for denaturation and 1min at 60°C for transcription. Analysis of gene expression values was performed using the efficiency-corrected comparative CT method, determining target gene expression relative to  $\beta$ -actin endogenous control expression and relative to the control sample.

## **2.7 SDS-polyacrylamide gel electrophoresis**

### **2.7.1 Preparation of whole cell lysate for assessment of cluster of differentiation 86 and intracellular adhesion molecule-1 expression**

CD86 and ICAM-1 protein expression was analysed in samples prepared from hippocampal tissue homogenates diluted to equalize for protein concentration. Aliquots (200 $\mu$ l; ~0.7mg/ml) were added to 67 $\mu$ l of lithium dodecyl sulfate (LDS) sample buffer (lauryl alcohol sulphate 7%; lithium salt 13%) and 26.8 $\mu$ l of Reducing Agent (DTT 15%), (NuPAGE®, Invitrogen, Ireland) and heated to 70°C for 10min.

### **2.7.2 Preparation of polyacrylamide gels; cluster of differentiation 86 and intracellular adhesion molecule-1 western immunoblotting**

Samples (10 $\mu$ l) representative of each treatment group were loaded onto 10% NuPAGE® Novex Bis-Tris gels (Invitrogen, Ireland) and were separated by

application of a constant voltage (130v; 90min), transferred onto nitrocellulose strips (30volts; 60min). Proteins were immunoblotted for 2h at RT with antibodies (1:200 – (rabbit anti-rat) or 1:200 (mouse anti-rat)) in Tris-buffered saline (TBS)-Tween containing 2% BSA, R&D systems, USA; Santa Cruz Biotechnology Inc., USA) that specifically target CD86 and ICAM-1 respectively. Membranes were washed three times in TBS-Tween, incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (1:1000). The blots were then stripped with ‘Reblot’ (1:10 dilution; Chemicon International, USA) and stained for actin expression to ensure equal loading of protein on all Bis-Tris Gels. Actin expression was assessed using a mouse monoclonal IgG<sub>1</sub> antibody (1:10,000 in TBS-Tween containing 0.1% BSA) corresponding to an amino acid sequence mapping at the C terminus of actin of human origin (Santa Cruz Biotechnology Inc.,USA). Immunoreactive bands were detected using peroxidase-conjugated anti-mouse IgG (Sigma) and ECL (GE Healthcare, UK) chemiluminescence. Western Blot products were photographed and quantified using densitometry (Labworks, UVP BioImaging Systems, UK) and data are expressed as a ratio of the expression of specific protein:  $\beta$ -actin.

## **2.8 Analysis of hippocampal interleukin-1 $\beta$ concentration *ex vivo* by enzyme linked immunosorbent assay**

### **2.8.1 Preparation of hippocampal samples**

Samples of whole-cell homogenate for ELISA were prepared from hippocampal tissue. In brief, hippocampal slices were thawed rapidly, washed three times and homogenised (30 strokes) in Krebs solution (~600 $\mu$ l) using a 1ml glass homogeniser (Jencons, Bedfordshire, UK). Protein concentrations were assessed (see section 2.5) and samples were equalised for protein concentration with Krebs solution. The tissue was divided into aliquots and frozen at -80°C until required for analysis.

### **2.8.2 Analysis of interleukin-1 $\beta$ concentration by enzyme linked immunosorbent assay**

The ELISA method was used to determine the concentration of IL-1 $\beta$  (Duo Set, R&D Systems, UK). Plates (96 well; Nunc – Immuno plate with MaxiSorp

surface) were coated with capture antibody (100 $\mu$ l; 1 $\mu$ g/ml; goat anti-rat IL-1 $\beta$  in 0.1M PBS; 137mM NaCl, 2.7mM KCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5mM KH<sub>2</sub>PO<sub>4</sub> pH 7.3) and incubated overnight at 4°C, washed several times with PBS containing 0.05% Tween-20 (PBS-T), blocked for 1h at RT with 300 $\mu$ l of diluent (0.1M PBS, pH 7.3, with 1% BSA, and incubated with 100 $\mu$ l IL-1 $\beta$  standards (0 – 1000pg/ml) or samples for 2h at RT.

Plates were washed three times in PBS-T and samples were incubated with 100 $\mu$ l of secondary antibody (biotinylated goat anti-rat IL-1 $\beta$ ; final concentration 350ng/ml in blocking buffer with 200 $\mu$ l of normal goat serum (Vector Laboratories, UK) for 2h at RT. Plates were washed again three times in PBS-T and incubated in 100 $\mu$ l detection agent (horse radish peroxidase (HRP)-conjugated streptavidin; 1:200 in diluent) for 20min at RT (in the dark). Substrate solution (100 $\mu$ l; R&D Systems, UK) was then added to each well and the reaction stopped by addition of 50 $\mu$ l of 1M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450nm within 30min (Labsystems Multiskan RC). A standard curve was constructed and results were expressed as pg IL-1 $\beta$ /mg tissue corrected for protein (section 2.5.2).

## **2.9 Analysis of cortical enzymatic activity**

### **2.9.1 Caspase-8**

Caspase-8 activity (Biomol, UK) was analyzed in cortical homogenate prepared from rats treated with A $\beta$ <sub>1-40/1-42</sub> for 8, 20 or 28 days. Samples were equalized (~0.7–0.9mg/ml) for protein concentration in assay reaction buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol; pH7.4). Caspase-8 stock solution was diluted 1/30 in assay buffer and 15 $\mu$ l was added in each well of a 96-well plate. Cortical sample or assay buffer (35 $\mu$ l) was added to test and control wells respectively giving a total reaction volume of 50 $\mu$ l. Samples were incubated at RT for 10min. A dilution of the Ac-IETD-p-nitroanilide (pNA) substrate was prepared to twice the desired concentration (400 $\mu$ M) for a final concentration of 200 $\mu$ M. The reaction was started by the addition of 50 $\mu$ l of the 400 $\mu$ M Ac-IETD-pNA substrate solution. Absorbance was measured continuously, from 0-9min, at 405nm. Background absorbance was corrected for by subtracting the values derived

(a)

**Master Mix Template Volume ( $\mu$ l)**

Promega 10x Buffer	2.5
Promega 50mM MgCl <sub>2</sub>	1.5
Promega 10mM dNTP	1
Primer (Fwd; $\beta$ -Actin / OX-6)	0.5 / 2
Primer (Rev; $\beta$ -Actin / OX-6)	0.5
Promega <i>Taq</i> polymerase	0.5
DNase-RNase-free water	16.5 / 15

**Total Volume** 23

**cDNA template** 2

**Final Reaction Volume** 25

Note: NTP = nucleotide triphosphate

**$\beta$ -actin / OX6 PCR conditions**

Step 1: 95 <sup>0</sup> C for 5min - Denaturation
Step 2: 65 <sup>0</sup> C for 1min - Annealing
Step 3: 72 <sup>0</sup> C for 2min - Elongation
Step 4: 94 <sup>0</sup> C for 1min - Amplification
Step 5: 65 <sup>0</sup> C for 1min
Step 6: 72 <sup>0</sup> C for 2min
Return to step 4 x 29 times

(b)

Primer	Primer Sequence	Size	Tanneal	Supplier	Product
5' $\beta$ -Actin	AGAAGAGCTATGAGCTGCCTGACTG	25	65 <sup>0</sup> C	Sigma-Genosys	236bp
3' $\beta$ -Actin	CTTCTGCATCCTGTCAGCGATGC	23			
5' OX-6	CAGTCACAGAAGGCGTTTATG	21	58 <sup>0</sup> C	Sigma-Genosys	245bp
3' OX-6	GATCGCAGGCCTTGAATGATG	21			

**Tables 2.1(a) (b)  $\beta$ -actin / OX-6 PCR cycling conditions, components and primer composition for RT-PCR**

Gene name	Taqman gene expression assay number	GenBank accession no.
MHCII	Rn01768597-m1	NM-198741.1

**Table 2.2 Genes used for QPCR and respective assay number and GenBank accession number**

from the non-caspase-3 control. Data were corrected for protein (section 2.5.2) and plotted as pNA substrate produced pmol/min/mg.

### 2.9.2 Caspase-3

Caspase-3 activity (Biomol, UK) was analyzed in cortical homogenate prepared from rats treated  $A\beta_{1-42}$  for 4h or  $A\beta_{1-40/1-42}$  for 8, 20 or 28 days. Samples were equalized for protein concentration in assay reaction buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol; pH7.4). Caspase-3 stock solution was diluted 1/30 in assay buffer and 15 $\mu$ l was added to each well of a 96-well plate. Cortical sample or assay buffer (35 $\mu$ l) was added to test and control wells respectively giving a total reaction volume of 50 $\mu$ l. Samples were incubated at RT for 10min. A dilution of the Ac-DEVD-pNA substrate was prepared to twice the desired concentration (400 $\mu$ M) for a final concentration of 200 $\mu$ M. The reaction was started by the addition of 50 $\mu$ l of the 400 $\mu$ M Ac-DEVD-pNA substrate solution. Absorbance was measured continuously, from 0-9min, at 405nm. Background absorbance was corrected for by subtracting the values derived from the non-caspase-3 control. Data were corrected for protein (section 2.5.2) and plotted as pNA substrate produced pmol/min/mg.

### 2.9.3 Sphingomyelinase

SMase activity (Molecular Probes, UK) was analysed in cortical homogenate prepared from rats treated with  $A\beta_{1-40/1-42}$  for 8, 20 and 28 days. The cortical homogenate was equalized (0.75mg/ml) for protein concentration in reaction buffer (0.5M Tris-HCl, 50mM  $MgCl_2$ ). SMase stock solution (10 U/ml) was diluted in reaction buffer to produce a 0.04 U/ml positive control; the reaction buffer without SMase was used as a negative control. A working solution was prepared as follows: 100 $\mu$ l (100 $\mu$ M) Amplex Red Reagent, 100 $\mu$ l (2U/ml) HRP, 100 $\mu$ l (0.2U/ml) choline oxidase, 200 $\mu$ l (8U/ml) alkaline phosphatase and 1ml (0.5mM) sphingomyelin. Working solution (100 $\mu$ l) was incubated with 100 $\mu$ l samples and controls (30min, 37°C). Fluorescence was measured, from 0 to 60min at excitation emission of 544nm and emission detection of 590nm. Background fluorescence was subtracted from the values derived from the non-sphingomyelinase control.

## **2.9.4 Secretory phospholipase A<sub>2</sub>**

sPLA<sub>2</sub> activity was assessed, by colourimetric assay (R&D Systems, UK), in cortical homogenate prepared from rats treated with A $\beta$ <sub>1-40/1-42</sub> for 8, 20 and 28 days. The tissue was equalized (0.75mg/ml) in Tris-reaction buffer (R&D Systems, UK). Standards (0-80U/ml) and samples (50 $\mu$ l) were incubated (30min, 37°C) with 100 $\mu$ l of reaction buffer to which 50 $\mu$ l of sPLA<sub>2</sub> substrate (lyophilized 2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC) resuspended in ddH<sub>2</sub>O) was added. Stop solution (25 $\mu$ l; EDTA in reaction buffer) was added to each well followed by 25 $\mu$ l of colour reagent (DTNB in EtOH) and the plate incubated for 10min at RT. Absorbance was read at 405nm.

## **2.10 Preparation of cultured cells**

### **2.10.1 Preparation of sterile coverslips**

To ensure sterility, glass coverslips (13mm; Chance Proper, UK) were soaked in 70% EtOH overnight, and exposed to ultraviolet light overnight. To provide cells with an appropriate surface on which to adhere, coverslips were coated with poly-L-lysine (40 $\mu$ g/ml in sterile dH<sub>2</sub>O; Sigma, UK) for 1h at 37°C. The coated coverslips were air-dried, placed in 24-well plates (Greiner, Austria) and stored at 4°C until required.

### **2.10.2 Preparation of primary cultures of cortical neurons**

Primary cortical neurons were cultured from 1-day-old Wistar rats (BioResources Unit, Trinity College, Dublin). The rat pups were killed by decapitation in a sterile laminar flow hood and the cerebral cortices were dissected free and placed in sterile PBS (Sigma, UK). The cortical tissue was chopped into 2 - 4 mm pieces using a sterile disposable scalpel (Schwann-Mann, UK) and then incubated in 2ml PBS, which contained 0.3% trypsin (Sigma, UK) for 20min at 37°C. Cortical tissue was gently triturated ~5 times in PBS containing 0.1% trypsin inhibitor (Sigma, UK), DNase (0.2mg/ml; Sigma, UK) and MgSO<sub>4</sub> (0.1M). Cell suspensions were passed through a sterile nylon mesh filter (40 $\mu$ m; Becton Dickinson Labware, France) and centrifuged (2000g; 3min; 20°C). The resulting pellet was resuspended



in neurobasal medium (NBM) supplemented with 10% heat inactivated horse serum (Gibco, UK), penicillin (100U/ml; Gibco, UK), streptomycin (100U/ml; Gibco, UK), glutamax (2mM; Gibco, UK) and B-27 (1:50 dilution; Gibco, UK). The resuspended cells were counted and equalised to a density of  $1 \times 10^6$  cells/ml and allowed to adhere to the glass coverslips for a minimum of 2h in a humidified incubator containing 5% CO<sub>2</sub>; 95% air at 37°C (Jencons, UK) before addition of 400µl pre-warmed supplemented media to each well. Cells were grown for 48h. Media was then replaced with NBM containing cytosine arabino-furanoside (5µg/ml; Sigma, UK) to prevent proliferation of non-neuronal cells. The use of cytosine arabino-furanoside as a method of glial depletion / neuronal enrichment *in vitro* has been shown to produce neurons in culture with a purity of around 89% (Masuko *et al.*, 1979). Arabino-furanoside supplemented media was replaced with following 24h incubation with NBM. For all experiments n = number of animals in each treatment group, all experiments were executed in duplicate.

## **2.11 Cell Treatments for *in vitro* work**

### **2.11.1 Amyloid-β<sub>1-42</sub>**

Aβ<sub>1-42</sub> (Biosource, USA) was aggregated as described in section 2.2.2. The peptide was diluted to a final concentration of 10µM in NBM and cells were treated with Aβ<sub>1-42</sub> for 24h. A concentration of 10µM Aβ<sub>1-42</sub> was chosen for these experiments as 2µM had previously proven ineffective in decreasing neuronal viability as measured with the MTS assay (data not shown). A brief review of recent literature, where Aβ was used in culture, led to 10µM being chosen as the lowest consistently effective treatment dose for Aβ<sub>1-42</sub> *in vitro* (Yao *et al.*, 2005; Hirata *et al.*, 2005; Biswas *et al.*, 2007).

### **2.11.2 Ceramide**

Ceramide (Sigma, UK) was reconstituted in DMSO (732µl) to a concentration of 20mM. Ceramide was diluted to a final concentration of 100µM in NBM for cell treatment and cells were treated for 24h. Ceramide at a concentration of 100µM was used in these experiments based on the findings of Kaipia and colleagues (1996) and Taniwaki and colleagues (1999).

### 2.11.3 GW4869

GW4869, a neutral sphingomyelinase inhibitor (Sigma, UK), was dissolved in DMSO (423 $\mu$ l) to a concentration of 20mM. GW4869 was diluted in NBM to a final concentration of 20 $\mu$ M. Cells were co-incubated with GW4869 in the presence of A $\beta$ <sub>1-42</sub> (10 $\mu$ M) for 24 h. The concentration of GW4869 used in these experiments was based on dose dependent studies where GW4869 was used against nSmase2 overexpressed in yeast cells, carried out by Marchesini and colleagues (2003).

**Note:** DMSO concentrations used in cell culture work were well below the accepted minimum level of DMSO-induced neurotoxicity, which is 0.1% (Dr. Aileen Lynch, personal communication. A review of current literature shows acceptable DMSO levels in culture to be as high as 0.01 and 0.1% (Schilling *et al.*, 1998, Xu *et al.*, 2000, Lannuzel *et al.*, 2003). In the experiments above a 1:200 and 1:1000 dilution of DMSO in dH<sub>2</sub>O was made (to dissolve ceramide and GW4869 respectively) so that the working concentration of DMSO in solution was 0.0005% for ceramide and 0.0001% for GW4869.

### 2.11.4 VP025

VP025 stock solution (10 $\mu$ l, 1.1x10<sup>14</sup> particles/ml, batch number: VSG200401) was diluted in 10ml sterile PBS (Sigma, UK) to 1.1 x 10<sup>11</sup> particles/ml. 2.8ml of the 1.1x10<sup>11</sup> solution was added to 22.2ml of sterile NBM giving 25ml of VP025 solution at a final treatment concentration of 1.2x10<sup>10</sup> particles/ml. Cells were treated with VP025 for 1h prior to treatment with A $\beta$ <sub>1-42</sub> or Ceramide.

## 2.12 Analysis of cell viability *in vitro*

### 2.12.1 MTS Assay

CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (Promega, Ireland) is a colorimetric method for determining the number of viable cells in culture. It contains MTS, a tetrazolium compound, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron-coupling reagent (phenazine ethosulfate). Following treatment of cultured neurons, MTS

(20 $\mu$ l/100 $\mu$ l NBM) was added to one well per treatment group per plate. The plate was re-incubated for 4h at 37°C. Absorbance was read on a plate reader at 490nm. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

### **2.12.2 Analysis of interleukin-1 $\beta$ concentration *in vitro***

An ELISA method was used to determine the concentration of IL-1 $\beta$  (R&D Systems, Duo Set, UK) in supernatant gleaned from cultured primary cortical neurons. In brief, 96-well plates (Nunc – Immuno plate with MaxiSorp surface) were coated with capture antibody (100 $\mu$ l; 1 $\mu$ g/ml; goat anti-rat IL-1 $\beta$  in 0.1M PBS(137mM NaCl, 2.7mM KCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5mM KH<sub>2</sub>PO<sub>4</sub> pH 7.3) and incubated overnight at 4°C, washed several times with PBS containing 0.05% Tween-20 (PBS-T), blocked for 1h at RT with 300 $\mu$ l of diluent (0.1M PBS, pH 7.3, with 1% BSA), and incubated with 100 $\mu$ l IL-1 $\beta$  standards (0 - 1000 pg/ml) or samples for 2h at RT. Following this period the plates were washed x3 in PBS-T and the standards and samples were incubated with 100 $\mu$ l of secondary antibody (biotinylated goat anti-ratIL-1 $\beta$ ; final concentration 350ng/ml in blocking buffer with 200 $\mu$ l of normal goat serum (Vector Laboratories, UK) for 2h at RT. Plates were washed again three times in PBS-T and incubated in 100 $\mu$ l detection agent (HRP-conjugated streptavidin; 1:200 in diluent) for 20min at RT (in the dark). Substrate solution (100 $\mu$ l; R&D systems, UK) was then added to each well and the reaction stopped by addition of 50 $\mu$ l of 1M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450nm within 30min (Labsystems Multiskan RC). A standard curve was constructed and results were expressed as pg IL-1 $\beta$ /ml supernatant.

### **2.12.3 Preparation of neuronal cell lysate for assessment of caspase-3 activity**

Culture plate containing primary cortical neurons cultured from 1-day-old Wistar rats treated with A $\beta$ <sub>1-40/1-42</sub> (10 $\mu$ M) or ceramide (100 $\mu$ M) in the presence or absence of GW4869 (20 $\mu$ M) for 24h were washed once with ice cold PBS, complete lysis buffer (60 $\mu$ l; 50mM NaCl; 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>; 50mM NaF; 1mM Na<sub>3</sub>VO<sub>4</sub>; 1mM PMSF; Protease Inhibitor cocktail (Aprotinin 2 $\mu$ g/ml, Leupeptin 2 $\mu$ g/ml, Pepstatin 2 $\mu$ g/ml) and IGEPAL (1%)) was then added to each. Cells were scraped from their

coverslips and the lysate collected in eppendorf tubes. Lysate was stored at -80°C until required.

#### **2.12.4 Analysis of caspase-3 activity**

Caspase-3 activity (Biomol, UK) was analyzed in cell lysate prepared from primary cortical neurons cultured from 1-day-old Wistar rats treated with A $\beta$ <sub>1-40/1-42</sub> (10 $\mu$ M) or ceramide (100 $\mu$ M) in the presence or absence of GW4869 (20 $\mu$ M) for 24h. The Caspase-3 activity assay was carried out as described in section 2.9.2.

#### **2.13 Statistical analysis**

Data were analysed, as appropriate; using either Student's t-test for independent means or a two-way analysis of variance (ANOVA) followed by Student Newman-Keuls or Bonferoni post hoc tests. Data are expressed as means with standard errors (SEM) and deemed statistically significant when  $p < 0.05$ .

## **Chapter 3**

LTP has received a great deal of attention, due to its probable role both in memory and learning and in forming and selecting connections in the developing nervous system (Zucker *et al.*, 1999). It has also become the focus of much research, in recognition of its essential roles in shaping the information processing characteristics of neurons (Larkman and Jack, 1995). It has been most intensively studied in the hippocampus, a structure that has long been implicated in some forms of memory (Scoville and Millner, 1957). Among the features of AD are neuroinflammation, memory impairment and increased deposition of A $\beta$ . Evidence suggests that an A $\beta$ -induced impairment in LTP exists in hippocampus and that up-regulation of pro-inflammatory signaling molecules may provide the basis for this. This chapter investigates the effects of acute and long-term A $\beta$  administration on LTP in perforant-path granule cell synapses, it also assesses the effectiveness of VP025 (Vasogen Inc.) in abrogating A $\beta$ -induced changes. VP025 is an investigational drug based on synthetic lipid technology; it is composed of bilayered phospholipid particles containing phosphatidylglycerol. VP025 has been shown to reverse impairment in LTP following acute icv A $\beta$  administration in rat (Martin *et al.*, 2004).

The acute A $\beta$  administration studies comprised groups of 6-8 young male Wistar rats. The first of the acute A $\beta$  administration studies saw animals injected im with either 150 $\mu$ l of saline or VP025 ( $1.2 \times 10^7$  particles/ml) on days 14, 13 and 1 before assessing their ability to sustain LTP. On day 0 these animals were urethane-anaesthetized and were injected icv (5 $\mu$ l) with saline or A $\beta_{1-40}$  (200 $\mu$ M) and 3 hours later were assessed for their ability to maintain LTP. The second acute A $\beta$  administration study saw A $\beta_{1-40}$ /A $\beta_{1-42}$  injected at two concentrations (45 $\mu$ M; 200 $\mu$ M) and the control peptide A $\beta_{40-1}$  was administered at 200 $\mu$ M, in this instance LTP was assessed in perforant path-granule cell synapses 4 hours following A $\beta$  administration. VP025 was not administered in this experiment as it was conducted to examine whether A $\beta_{1-40}$  and A $\beta_{1-42}$  when given in combination would impair LTP to a greater or lesser extent than A $\beta_{1-40}$  administered alone or whether the effects of A $\beta_{1-40}$ /A $\beta_{1-42}$  on LTP could be elucidated in a dose dependent manner.

Further studies saw groups of 6 rats similarly pre-treated with either saline or VP025. These animals were anaesthetized to allow implantation of Alzet minipumps (Model 2004) and were thereafter chronically infused icv with a combination of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  (26.9 $\mu$ M and 36.9 $\mu$ M respectively) or control peptide,  $A\beta_{40-1}$  (63.8 $\mu$ M) for 8, 20 or 28 days prior to LTP assessment. A final study comprised groups of 6 rats anaesthetized to allow implantation of Alzet minipumps (Model 2004). These rats were thereafter chronically infused icv with a combination of  $A\beta_{1-40}/A\beta_{1-42}$  (26.9 $\mu$ M and 36.9 $\mu$ M respectively) or control peptide,  $A\beta_{40-1}$  (63.8 $\mu$ M) for 28 days. During the infusion period the rats were injected im with 150 $\mu$ l of saline or VP025 (1.2 x 10<sup>7</sup> particles/ml) on days 15, 16 and 27 before assessing their ability to sustain LTP. In all studies rats were sacrificed with cortex and hippocampus taken for later analysis. These protocols are outlined in detail in chapter 2, sections 2.2.7, 2.2.9 and 2.2.10.

The data show that both acute and chronic  $A\beta$  treatment impaired LTP and this impairment was reversed in all cases by prior treatment or intervention with VP025. These data are consistent with the idea that  $A\beta$  has deleterious effects on neuronal function and that the hippocampus is susceptible to  $A\beta$  administration, not only this the data shows an incremental decrease in EPSP following tetanus with increasing exposure time to  $A\beta$ . These data also show a differing sensitivity in LTP to alternate peptide species and treatment concentrations. These findings support earlier observations demonstrating the anti-inflammatory and neuroprotective effects of VP025.

### **3.1 VP025 pre-treatment abrogates the amyloid- $\beta$ -induced deficit in long-term potentiation in rat dentate gyrus following acute amyloid- $\beta_{1-40}$ administration**

Delivery of a train of high frequency stimulation to the perforant path of saline-treated rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.1). An immediate increase in mean EPSP slope was also observed in saline-pretreated rats which received a single intracerebroventricular injection of  $A\beta_{1-40}$  (200 $\mu$ M) but, in this case, the increase was not sustained. Analysis of the data following stimulation revealed a significant difference in the response of saline pre-treated / saline-treated and saline pre-treated /  $A\beta_{1-40}$ -treated rats. VP025 pre-treatment of saline-treated rats exerted no marked effect on LTP so that mean EPSP slope following tetanic stimulation was similar to that in saline pre-treated / saline-treated animals. However VP025 pre-treatment attenuated the  $A\beta_{1-40}$ -induced decrease in LTP so that mean EPSP slope following tetanic stimulation in this group was significantly different from the group treated with  $A\beta_{1-40}$  alone and similar to the saline treated group.

To further analyse the effects of treatments, changes in mean population EPSP slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the first 2min after stimulation was  $151.87 \pm 2.08\%$  in saline pre-treated / saline-treated rats and significantly decreased in saline pre-treated /  $A\beta_{1-40}$ -treated rats ( $129.54 \pm 2.05\%$ ; Figure 3.2a). There was a similar decrease in  $A\beta_{1-40}$ -treated rats which received VP025 ( $129.54 \pm 0.84\%$ ; Figure 3.2a), and a less marked change in saline-treated rats which received VP025. Thus VP025 exerted no modulatory effect on the early change in EPSP slope induced by  $A\beta$  treatment.

The  $A\beta$ -associated effect persisted so that the mean percentage change in population EPSP slope in saline pre-treated /  $A\beta_{1-40}$ -treated rats in the last 5min of the recording period was  $102.5 \pm 0.53\%$ , compared with  $115.4 \pm 0.49\%$  in saline pre-treated / saline-treated rats (Figure 3.2b). Whereas treatment with VP025 exerted no significant effect in saline-treated animals ( $114.59 \pm 0.61\%$ ), it significantly attenuated the  $A\beta$ -induced decrease ( $123.15 \pm$



0.64%; Figure 3.2b). Data are expressed as the mean  $\pm$  SEM; n = 8. (This work was carried out in conjunction with Dr. Darren Martin and Michelle Walsh).

### **3.2 Acute amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ administration impairs long-term potentiation maintenance**

Delivery of a train of high frequency stimulation to the perforant path of A $\beta_{40-1}$  rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.3). An immediate increase in mean EPSP slope was also observed in rats, which received an intracerebroventricular injection of A $\beta_{1-40}$ /A $\beta_{1-42}$  at two different doses (45 $\mu$ M; 200 $\mu$ M), but in this case, the increase was not sustained to the same level as control-treated animals. Statistical analysis of the data following tetanic stimulation revealed a significant difference between the response in control and A $\beta$ -treated rats.

To further analyse the effects of treatments, changes in mean population EPSP slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the 2min after stimulation was  $148.7 \pm 0.94\%$  in A $\beta_{40-1}$ -treated rats. The mean percentage increase in EPSP slope in the 2min after stimulation was unaffected by A $\beta_{1-40}$ /A $\beta_{1-42}$  treatment ( $141.9 \pm 4.91\%$ ; 45 $\mu$ M;  $152.1 \pm 2.66\%$ ; 200  $\mu$ M; Figure 3.4a). The mean percentage change in EPSP slope in A $\beta_{1-40}$ /A $\beta_{1-42}$ -treated rats in the final 5min of recording period was  $118.7 \pm 0.46\%$  (45 $\mu$ M) and  $118.6 \pm 0.89\%$  (200 $\mu$ M), compared with  $129.0 \pm 0.91\%$  in A $\beta_{40-1}$ -treated rats (Figure 3.4b). Data are expressed as the means  $\pm$  SEM; n = 2 - 4. (These data were obtained in collaboration with Alessia Piazza and in this instance assistance with the LTP was provided by Dr. Thelma Cowley).

### **3.3 VP025 pre-treatment abrogates the deficit in long-term potentiation induced by treatment with amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ for 8 days**

Delivery of a train of high frequency stimulation to the perforant path of saline pre-treated A $\beta_{40-1}$ -treated rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.5). An immediate increase in mean EPSP slope was

also observed in saline pre-treated rats administered  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 days but, in this case, the increase was not sustained. Statistical analysis of the data following tetanic stimulation revealed a significant difference between the responses in saline pre-treated  $A\beta_{40-1}$ -treated and saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Pre-treatment of  $A\beta_{40-1}$ -treated rats with VP025 exerted no marked effect on LTP so that mean EPSP slope following tetanic stimulation was similar to that in saline pre-treated  $A\beta_{40-1}$ -treated animals. However VP025 attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease in LTP so that mean EPSP slope following tetanic stimulation in this group was significantly different from the saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated animals and similar to the  $A\beta_{40-1}$  treated groups.

To further analyse the effects of treatments, changes in mean population EPSP slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the first 2min after stimulation was  $135.4 \pm 1.047\%$  in  $A\beta_{40-1}$ -treated rats and this was significantly decreased in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $108.4 \pm 1.56\%$ ; Figure 3.6a). There was no significant decrease in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats which received VP025 ( $128.3 \pm 1.35\%$ ; Figure 3.6a) when compared to  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats which received saline or in  $A\beta_{40-1}$ -treated rats which received VP025 when compared to  $A\beta_{40-1}$ -treated rats which received saline. Thus VP025 exerted a modulatory effect on the early change in EPSP slope induced by  $A\beta_{1-40}/A\beta_{1-42}$  treatment.

The  $A\beta$ -associated effect persisted so that the mean percentage change in population EPSP slope in saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period was  $89.2 \pm 0.67\%$ , compared with  $112.8 \pm 1.12\%$  in saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (Figure 3.6b). Whereas treatment with VP025 exerted no significant effect in  $A\beta_{40-1}$ -treated animals ( $114.4 \pm 0.79\%$ ), it significantly attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease ( $109.3 \pm 0.59\%$ ; Figure 3.6b) when compared to  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats that received saline pre-treatment. Data are expressed as the mean  $\pm$  SEM;  $n = 4 - 6$ .

### **3.4 VP025 pre-treatment abrogates the deficit in long-term potentiation induced by treatment with amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ for 20 days**

Delivery of a train of high frequency stimulation to the perforant path of saline pre-treated  $A\beta_{40-1}$ -treated rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.7). An immediate increase in mean EPSP slope was also observed in saline pre-treated rats administered  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 20 days but, in this case, the increase was not sustained. Statistical analysis of the data following tetanic stimulation revealed a significant difference between the response in  $A\beta_{40-1}$ -treated and  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Pre-treatment of  $A\beta_{40-1}$ -treated rats with VP025 exerted no marked effect on LTP so that mean EPSP slope following tetanic stimulation was similar to that in saline pre-treated  $A\beta_{40-1}$ -treated animals. However, VP025 did not attenuate the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease in LTP so that mean EPSP slope following tetanic stimulation in this group was not significantly different from the saline pre-treated group treated with  $A\beta_{1-40}/A\beta_{1-42}$ .

To further analyse the effects of treatments, changes in mean population EPSP slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the first 2min after stimulation was  $142.5 \pm 1.03\%$  in saline pre-treated  $A\beta_{40-1}$ -treated rats and this was significantly decreased in saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $112.9 \pm 2.93\%$ ; Figure 3.8a). There was no significant decrease in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats which received VP025 ( $135.7 \pm 1.02\%$ ; Figure 3.8a) when compared to  $A\beta_{40-1}$ -treated rats pre-treated with saline. There was a significant difference in  $A\beta_{40-1}$ -treated rats pre-treated with VP025 when compared to  $A\beta_{40-1}$ -treated rats pre-treated with saline. ( $158.5 \pm 3.65\%$ ; Figure 3.8a). Thus VP025 exerted a modulatory effect on the early change in EPSP slope induced by  $A\beta$  treatment.

The  $A\beta_{1-40}/A\beta_{1-42}$ -associated effect persisted so that the mean percentage change in population EPSP slope in saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period was  $111.7 \pm 0.85\%$ , compared with  $123.6 \pm 2.98$  in saline pre-treated  $A\beta_{40-1}$ -treated rats (Figure 3.8b). Treatment with VP025 exerted no significant effect in  $A\beta_{40-1}$ -treated animals ( $127.3 \pm 2.41\%$ ), neither did it significantly attenuate the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease in EPSP slope ( $114.5 \pm 1.44\%$ ; Figure 3.8b) when compared to  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats pre-treated with saline. Data are expressed as means  $\pm$  SEM;  $n = 5-6$ .

### 3.5 VP025 pre-treatment abrogates the deficit in long-term potentiation induced by treatment with amyloid $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ for 28 days

Delivery of a train of high frequency stimulation to the perforant path of saline pre-treated  $A\beta_{40-1}$ -treated rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.9). An immediate increase in mean EPSP slope was also observed in saline pre-treated rats administered  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 28 days but, in this case, the increase was not sustained. Statistical analysis of the data following tetanic stimulation revealed a significant difference between the responses in saline pre-treated  $A\beta_{40-1}$ -treated and saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Pre-treatment of  $A\beta_{40-1}$ -treated rats with VP025 exerted a marked effect on LTP so that mean EPSP slope following tetanic stimulation was higher than that in saline-treated  $A\beta_{40-1}$ -treated animals. However VP025 also attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease in LTP so that mean EPSP slope following tetanic stimulation in this group was significantly different from saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated animals.

To further analyse the effects of treatments, changes in mean population EPSP slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the first 2min after stimulation was  $131.9 \pm 2.26\%$  in saline pre-treated  $A\beta_{40-1}$ -treated rats and significantly increased in saline-pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $155.3 \pm 6.47\%$ ; Figure 3.10a). There was also a significant increase in mean EPSP slope in  $A\beta_{40-1}$ -treated rats which received VP025 ( $136.4 \pm 1.99\%$ ; Figure 3.10a) when compared to saline pre-treated rats which received  $A\beta_{40-1}$  (Figure 3.10a). Thus VP025 exerted a modulatory effect on the early change in EPSP slope induced by  $A\beta$  treatment.

There was a significant decrease in the mean percentage change in population EPSP slope in saline pre-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period ( $85.36 \pm 1.70\%$ ) compared with  $113.6 \pm 0.77\%$  in saline pre-treated  $A\beta_{40-1}$ -treated rats (Figure 3.10b). Pre-treatment with VP025 reversed the decrease in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats so that the mean value was greater than in  $A\beta_{40-1}$ -treated rats ( $127 \pm 0.57\%$ ; Figure

3.10b). There was also a significant increase in mean EPSP slope in  $A\beta_{40-1}$ -treated rats which received VP025 when compared to saline pre-treated  $A\beta_{40-1}$ -treated animals. ( $123.2 \pm 1.01\%$ ; Figure 3.10b). Data are expressed as means  $\pm$  SEM;  $n = 5 - 6$ .

### **3.6 VP025 intervention abrogates the deficit in long-term potentiation induced by treatment with amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ for 28 days**

Delivery of a train of high frequency stimulation to the perforant path of saline pre-treated  $A\beta_{40-1}$ -treated rats resulted in an immediate and sustained increase in mean population EPSP slope (Figure 3.11). An immediate increase in mean EPSP slope was also observed in saline treated rats administered  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 28 days but, in this case, the increase was not sustained. Statistical analysis of the data following tetanic stimulation revealed a significant difference between the responses in saline treated  $A\beta_{40-1}$ -treated and saline treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Treatment of  $A\beta_{40-1}$ -treated rats with VP025 exerted no effect on LTP so that mean EPSP slope following tetanic stimulation was similar to that in saline-treated  $A\beta_{40-1}$ -treated animals. However VP025 intervention attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease in LTP so that mean EPSP slope following tetanic stimulation in this group was significantly different from the saline treated  $A\beta_{1-40}/A\beta_{1-42}$  treated group.

To further analyse the effects of treatments, changes in mean population slope in the 2min period immediately following tetanic stimulation and in the last 5min period of recording compared with the 5min before tetanic stimulation were assessed. The mean percentage increase in EPSP slope in the first 2min after stimulation was  $119.6 \pm 0.98\%$  in saline treated  $A\beta_{40-1}$ -treated rats and significantly decreased in saline treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $111.9 \pm 0.83\%$ ; Figure 3.12a). There was no significant increase in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats, which received VP025 ( $117.6 \pm 0.65\%$ ) when compared to saline treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Thus VP025 did not exert a modulatory effect on the early change in EPSP slope induced by  $A\beta$  treatment.

The  $A\beta$ -associated effect persisted so that the mean percentage change in population EPSP slope in saline treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period was  $95.64 \pm 0.92\%$ , compared with  $115.2 \pm 0.79\%$  in saline treated  $A\beta_{40-1}$ -treated

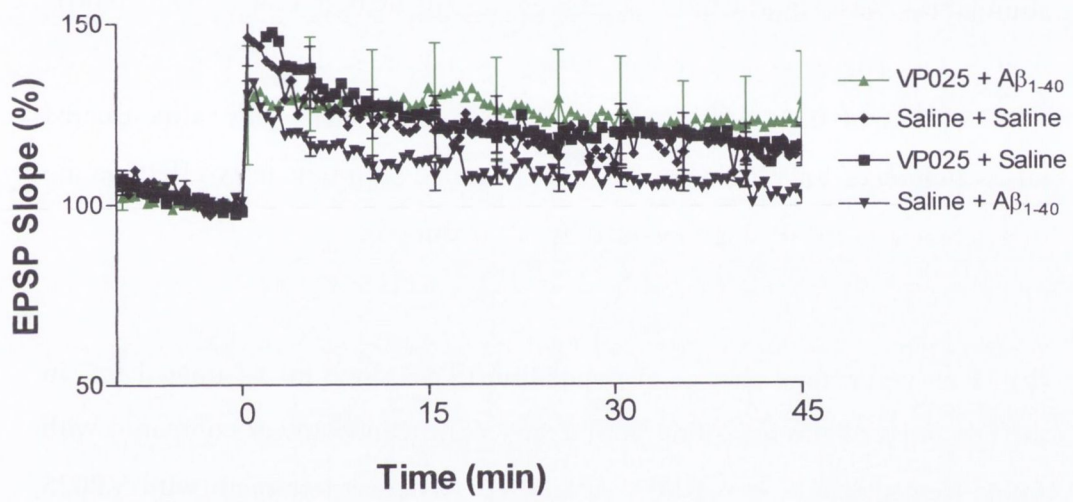
rats (Figure 3.12b). Whereas treatment with VP025 exerted no significant effect in  $A\beta_{40-1}$ -treated animals ( $116.5 \pm 0.71\%$ ), it significantly attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced decrease ( $116.8 \pm 0.35\%$ ; Figure 3.12b) when compared to saline-treated  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats. Data are expressed as means  $\pm$  SEM;  $n = 4 - 6$ .

### **3.7 A greater impairment in long-term potentiation in dentate gyrus is associated with increased exposure time to amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$**

Comparison of the change in mean EPSP slope in the last 5min of recording revealed that the most profound effect was observed in saline pre-treated rats which received  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days (Figure 3.13). The mean population EPSP slope in this group was significant compared with that in saline pre-treated rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days ( $*p < 0.05$ ; t-test) The data obtained from rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  confounded the notion that increased exposure time to  $A\beta$  had an additive detrimental effect on LTP in that the change in mean EPSP slope in the last 5min of recording in the dentate gyrus of saline pre-treated rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days was not significantly lower than in saline pre-treated rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days. Data are expressed as means  $\pm$  SEM;  $n = 3 - 5$ .

**Figure 3.1 The inhibition of LTP induced by acute  $A\beta_{1-40}$  treatment was reversed by VP025**

$A\beta_{1-40}$  administration (200 $\mu$ M) significantly inhibited LTP in perforant path granule cell synapses. Treatment with VP025 alone exerted no significant effect but significantly attenuated the  $A\beta$ -induced change (in green). Analysis was undertaken on all values following delivery of tetanus. Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM; n = 8. SEMs are included for every 10<sup>th</sup> response.



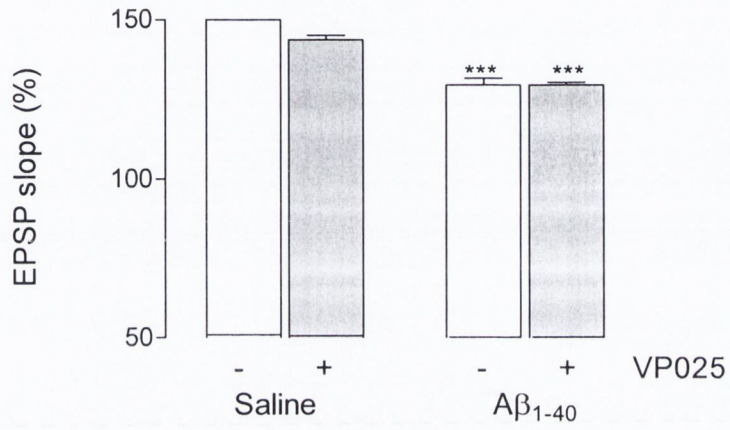


**Figure 3.2 VP025 reverses the percentage change in EPSP slope induced by acute A $\beta$ <sub>1-40</sub> treatment**

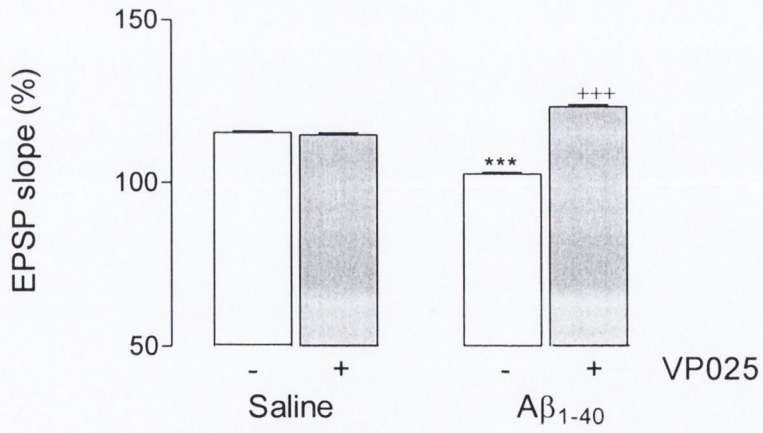
(a) The mean percentage increase in EPSP slope in the first 2min after stimulation was significantly decreased in A $\beta$ -treated rats (\*\*p < 0.001; ANOVA). There was a similar decrease in A $\beta$ -treated rats which received VP025 (\*\*p < 0.001; ANOVA), and a less marked change in saline-treated rats which received VP025. Thus VP025 exerted no modulatory effect on the early change in EPSP slope induced by A $\beta$  treatment.

(b) Mean percentage change in population EPSP slope in A $\beta$ -treated rats in the last 5min of the recording period was significantly lower compared with saline-treated rats (\*\*p < 0.001; ANOVA). Whereas treatment with VP025 exerted no significant effect in saline-treated animals it significantly attenuated the A $\beta$ -induced decrease (\*\*\*p < 0.001; ANOVA). Values are expressed as a percentage of the mean EPSP slope recorded in the 5 min immediately prior to tetanic stimulation and are the mean  $\pm$  SEM; n = 8.

a) 0-2 min post tetanus

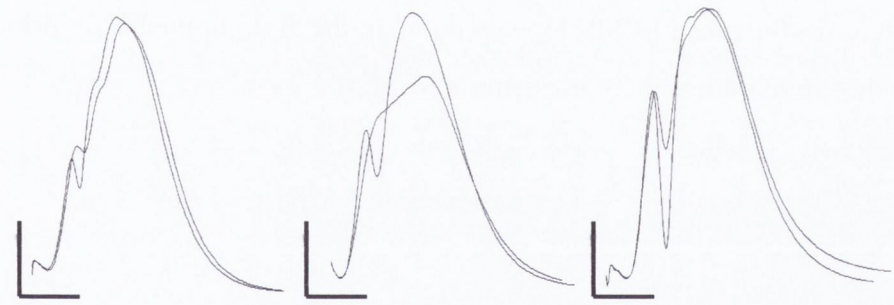
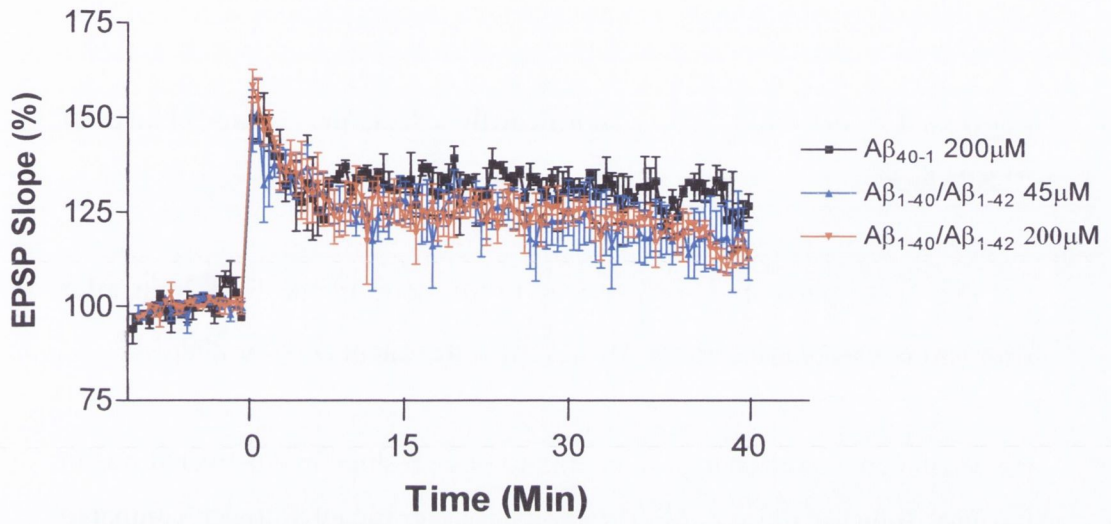


b) 40-45 min post tetanus



**Figure 3.3 Acute  $A\beta_{1-40}/A\beta_{1-42}$  treatment significantly inhibits LTP**

Icv  $A\beta_{1-40}/A\beta_{1-42}$  administration (45 and 200 $\mu$ M) significantly impaired LTP in perforant path granule cell synapses. Analysis was undertaken on all values following delivery of tetanus and revealed a significant difference between the response in  $A\beta_{40-1}$ -treated and  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (in red and blue). Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM; n = 2 - 4. SEMs are included for every 10th response. Representative traces, pre- and post-tetanus, for each of the treatment groups are presented with 1mV/5msec scale bar included for each pairing.



$A\beta_{40-1}$  (200 $\mu$ M)

$A\beta_{1-40}/A\beta_{1-42}$  (45 $\mu$ M)

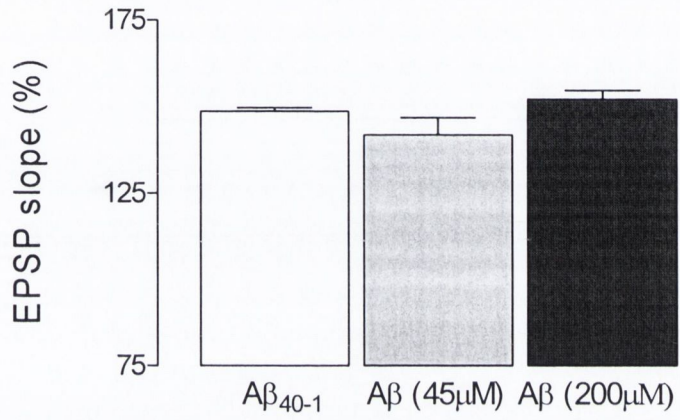
$A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M)

**Figure 3.4 Acute  $A\beta_{1-40}/A\beta_{1-42}$  significantly affects percentage change in EPSP slope**

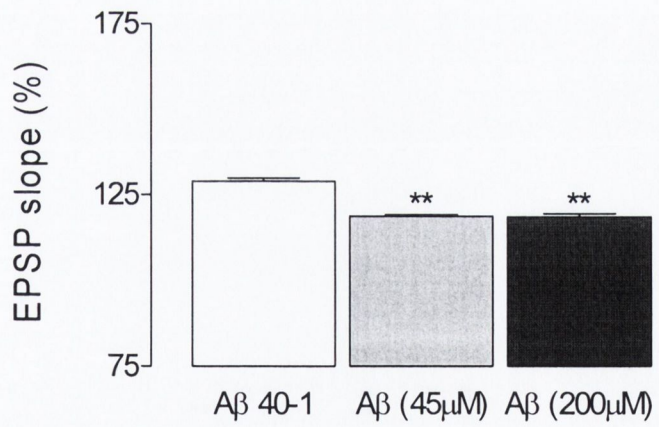
(a) The mean percentage increase in EPSP slope in the first 2min after stimulation was unaffected by  $A\beta_{1-40}/A\beta_{1-42}$  treatment at 45 or 200 $\mu$ M.

(b) Mean percentage change in population EPSP slope in  $A\beta$ -treated rats in the last 5min of the recording period was significantly lower compared with  $A\beta_{40-1}$ -treated rats (\*\* $p < 0.001$ ; ANOVA). Values are expressed as a percentage of the mean EPSP slope recorded in the 5min immediately prior to tetanic stimulation and are the mean  $\pm$  SEM;  $n = 2 - 4$ .

a) 0-2 min post tetanus

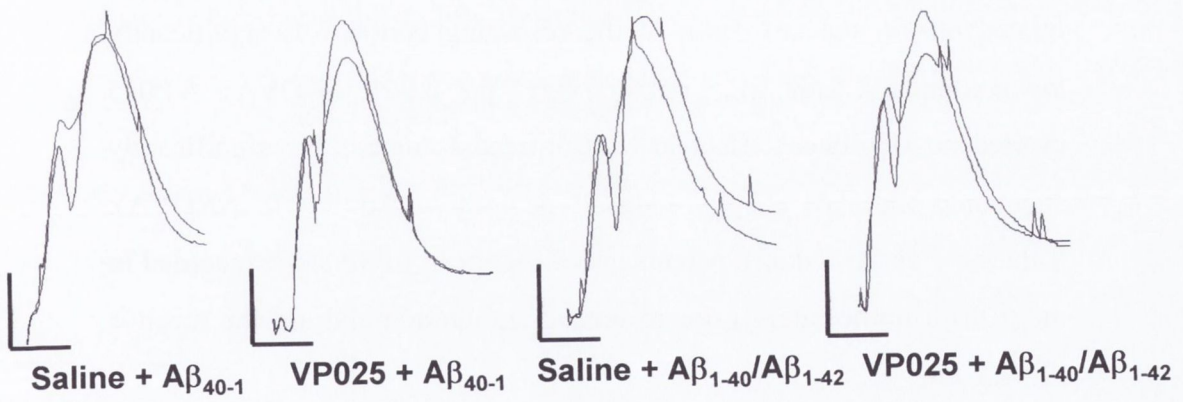
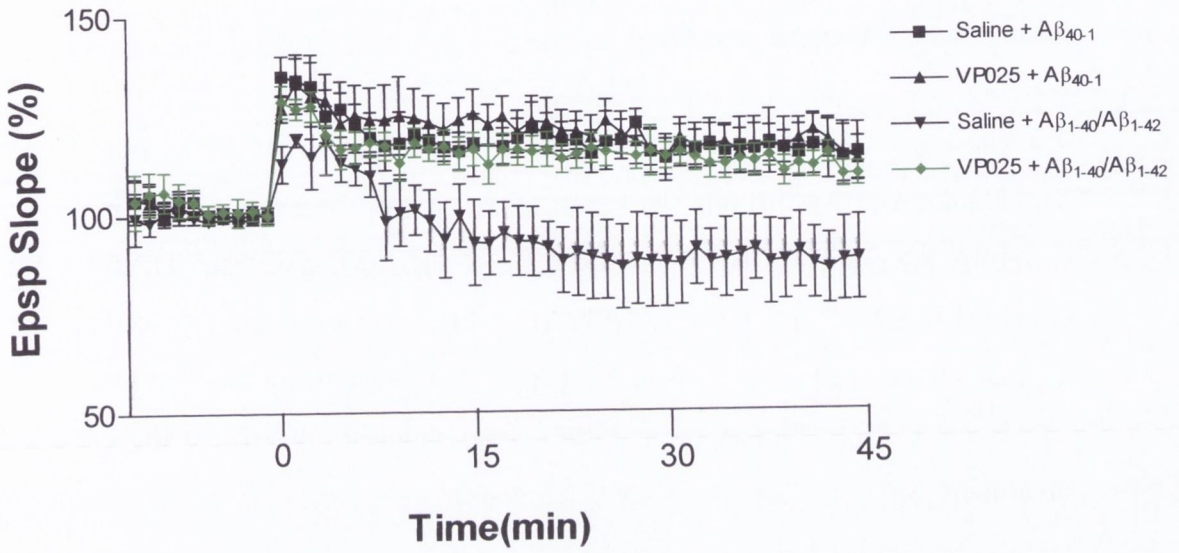


b) 40-45 min post tetanus



**Figure 3.5 VP025 reverses the inhibition of LTP induced by 8 days  
A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> treatment**

Chronic A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> administration (63.8 $\mu$ M) for 8 days significantly inhibited LTP in perforant path granule cell synapses in saline-treated rats. Treatment with VP025 alone exerted no significant effect but significantly attenuated the A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>-induced change (in green). Analysis was undertaken on all values following delivery of tetanus. Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM; n = 4 - 6. SEMs are included for every 10<sup>th</sup> response. Representative traces, pre- and post-tetanus, for each of the treatment groups are presented with 1mV/5msec scale bar included for each pairing.



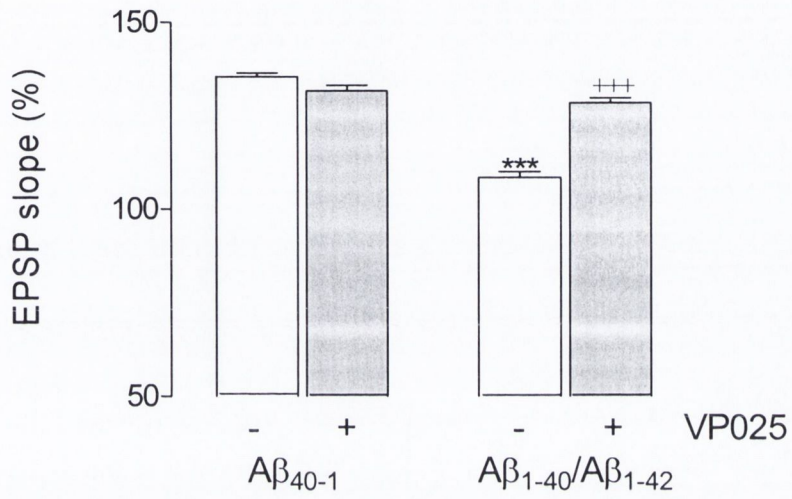


**Figure 3.6 VP025 reverses the percentage change in EPSP slope induced by 8 days A $\beta$  treatment**

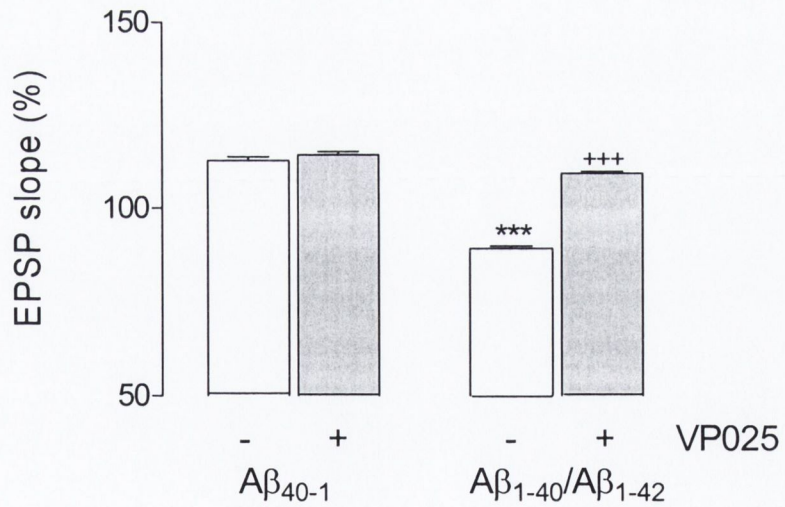
(a) The mean percentage increase in EPSP slope in the first 2min after stimulation was significantly decreased in A $\beta_{1-40}$ /A $\beta_{1-42}$ -treated rats ( $^{***}p < 0.001$ ; ANOVA). There was no decrease in A $\beta$ -treated rats which received VP025 ( $^{+++}p < 0.001$ ; ANOVA), and no change in A $\beta_{40-1}$ -treated rats which received VP025. Thus VP025 exerted a significant modulatory effect on the early change in EPSP slope induced by A $\beta_{1-40}$ /A $\beta_{1-42}$  treatment.

(b) Mean percentage change in population EPSP slope in A $\beta_{1-40}$ /A $\beta_{1-42}$  - treated rats in the last 5min of the recording period was significantly lower compared with A $\beta_{40-1}$ -treated rats ( $^{***}p < 0.001$ ; ANOVA). VP025 exerted no significant effect in control-treated animals but significantly attenuated the A $\beta_{1-40}$ /A $\beta_{1-42}$ -induced decrease ( $^{+++}p < 0.001$ ; ANOVA). Values are expressed as a percentage of the mean EPSP slope recorded in the 5 m in immediately prior to tetanic stimulation and are the mean  $\pm$  SEM; n = 4 - 6.

a) 0-2 min post tetanus

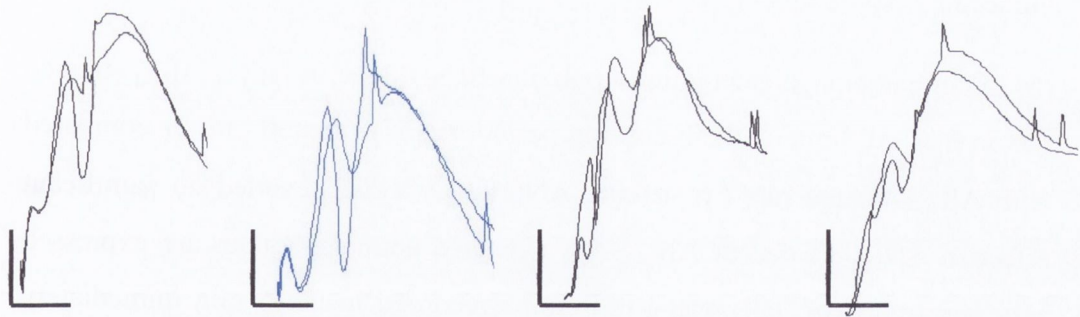
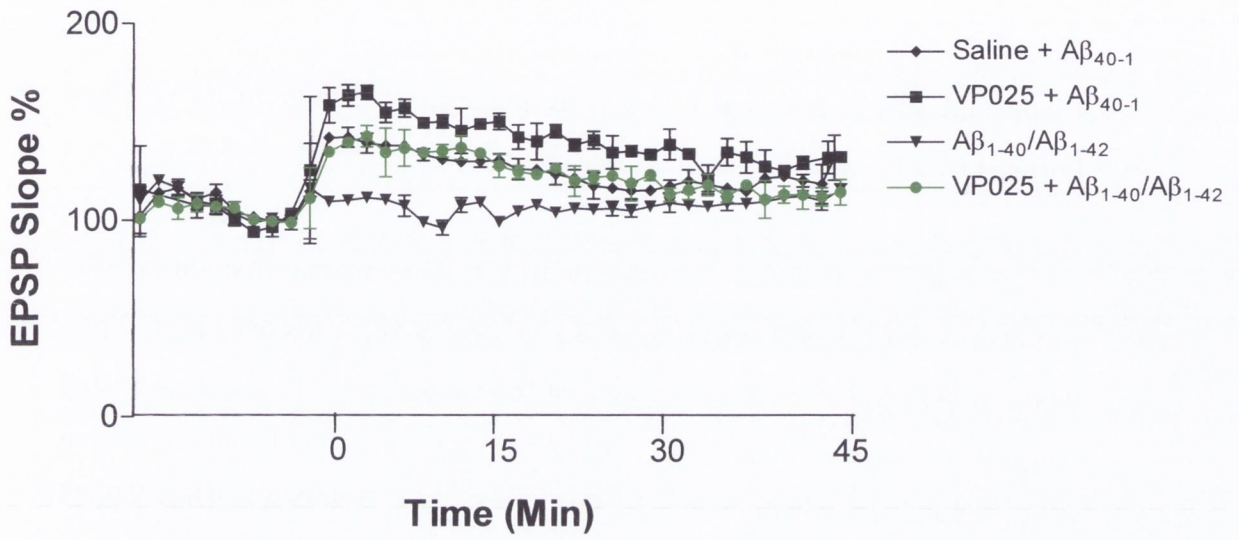


b) 40-45 min post tetanus



**Figure 3.7 VP025 does not reverse the inhibition of LTP induced by 20 days  $A\beta_{1-40}/A\beta_{1-42}$  treatment**

Chronic  $A\beta_{1-40}/A\beta_{1-42}$  administration ( $63.8\mu\text{M}$ ) for 20 days significantly inhibited LTP in perforant path granule cell synapses in saline pre-treated rats. Treatment of  $A\beta_{40-1}$ - or  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats with VP025 exerted no significant effect in this experiment (in green). Analysis was undertaken on all values following delivery of tetanus. Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM;  $n = 5 - 6$ . SEMs are included for every 10<sup>th</sup> response. Representative traces, pre- and post-tetanus, for each of the treatment groups are presented with 1mV/5msec scale bar included for each pairing.



Saline +  $A\beta_{40-1}$

VP025 +  $A\beta_{40-1}$

Saline +  $A\beta_{1-40}/A\beta_{1-42}$

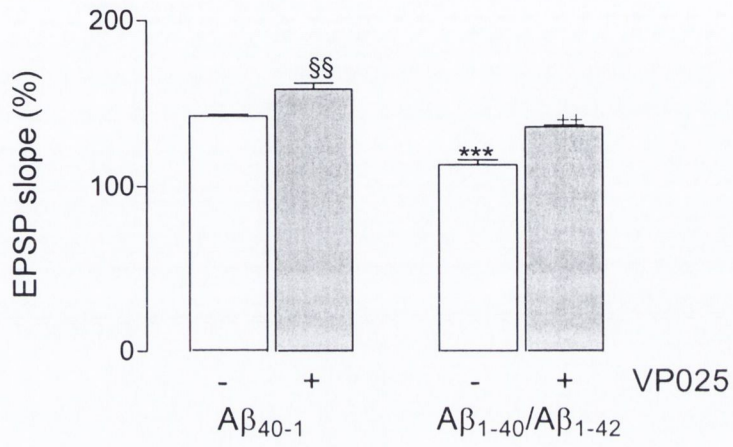
VP025 +  $A\beta_{1-40}/A\beta_{1-42}$

**Figure 3.8 VP025 does not reverse the percentage change in EPSP slope induced by 20 days  $A\beta_{1-40}/A\beta_{1-42}$  treatment**

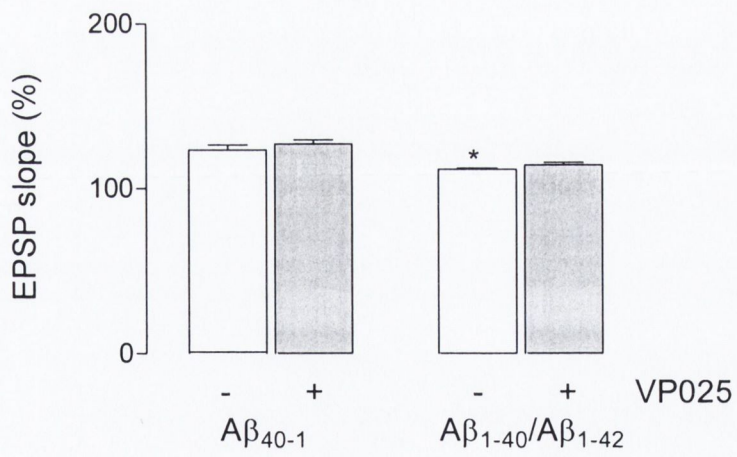
(a) The mean percentage increase in EPSP slope in the first 2min after stimulation was significantly decreased in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (\*\*\*p < 0.001; ANOVA; Figure 3.8a). There was no decrease in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats which received VP025 ( ++p < 0.01; ANOVA), and a significant increase in  $A\beta_{40-1}$ -treated rats which received VP025 (§§p < 0.01; ANOVA). Thus VP025 exerted a significant modulatory effect on the early change in EPSP slope induced by  $A\beta$  treatment.

(b) Mean percentage change in population EPSP slope in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period was significantly lower compared with  $A\beta_{40-1}$ -treated rats (\*p < 0.05; ANOVA). VP025 exerted no significant effect in  $A\beta_{40-1}$ -treated or  $A\beta_{1-40}/A\beta_{1-42}$ -treated animals. Values are expressed as a percentage of the mean EPSP slope recorded in the 5 min immediately prior to tetanic stimulation and are the mean  $\pm$  SEM; n = 5 - 6.

a) 0-2 min post tetanus

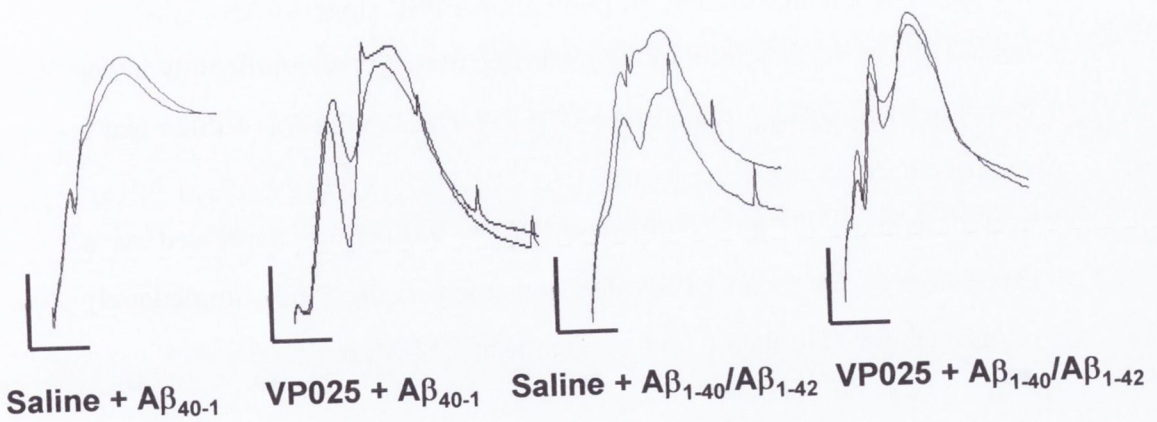
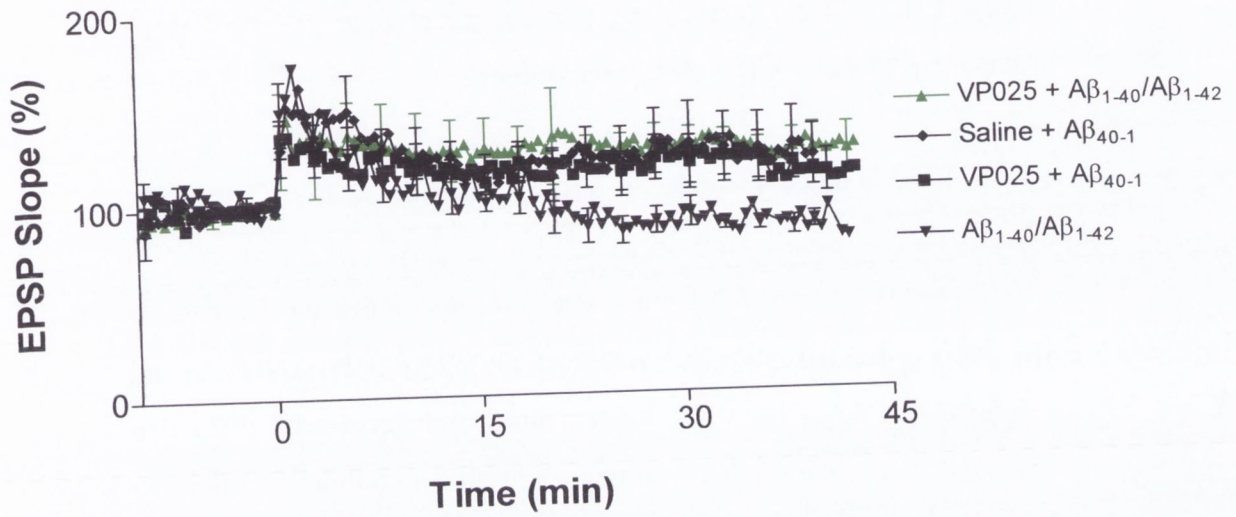


b) 40-45 min post tetanus



**Figure 3.9 VP025 reverses the inhibition of LTP induced by 28 days  $A\beta_{1-40}/A\beta_{1-42}$  treatment**

Chronic  $A\beta_{1-40}/A\beta_{1-42}$  administration ( $63.8\mu\text{M}$ ) for 28 days significantly inhibited LTP in perforant path granule cell synapses in saline pre-treated rats. Pre-treatment of  $A\beta_{40-1}$ -treated rats with VP025 exerted a marked effect on LTP so that mean EPSP slope following tetanic stimulation was higher than that in saline pre-treated  $A\beta_{40-1}$ -treated animals. VP025 also significantly attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced change in EPSP slope (in green). Analysis was undertaken on all values following delivery of tetanus. Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM;  $n = 5 - 6$ . SEMs are included for every 10<sup>th</sup> response. Representative traces, pre- and post-tetanus, for each of the treatment groups are presented with 1mV/5msec scale bar included for each pairing.



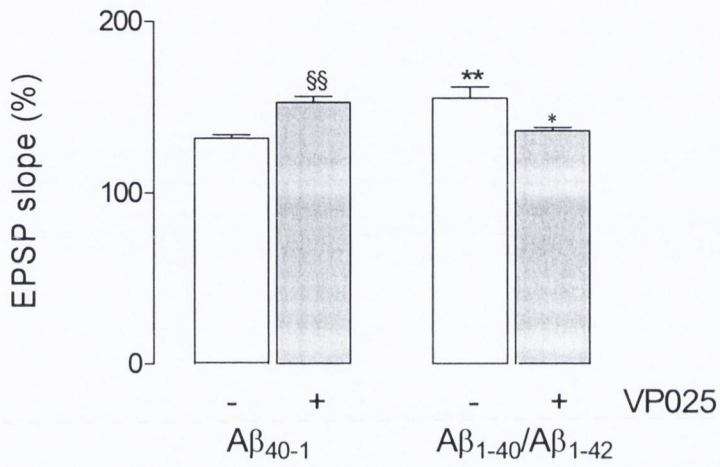


**Figure 3.10 VP025 reverses the percentage change in EPSP slope induced by 28 days  $A\beta_{1-40}/A\beta_{1-42}$  treatment**

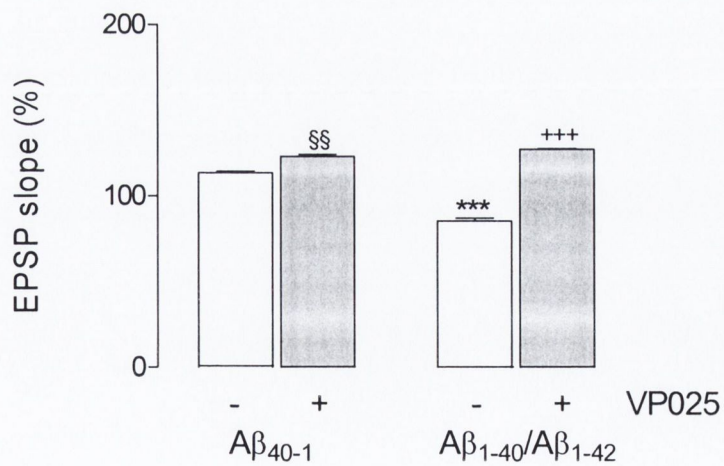
(a) Mean percentage increase in EPSP slope in the first 2min after stimulation was significantly increased in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (\*\*p < 0.01; ANOVA; Figure 3.10a). There was no significant decrease in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats which received VP025 (\*p 0.05; ANOVA) and a significant change in  $A\beta_{40-1}$ -treated rats which received VP025 (§§p 0.01; ANOVA). Thus VP025 exerted a significant modulatory effect on the early change in EPSP slope.

(b) Mean percentage change in population EPSP slope in  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats in the last 5min of the recording period was significantly lower compared with  $A\beta_{40-1}$ -treated rats (\*\*p < 0.001; ANOVA). VP025 had a significant effect in  $A\beta_{40-1}$ -treated (§§p 0.01; ANOVA) and  $A\beta_{1-40}/A\beta_{1-42}$ -treated animals (+++p < 0.001; ANOVA). Values are expressed as a percentage of the mean EPSP slope recorded in the 5 min immediately prior to tetanic stimulation and are the mean  $\pm$  SEM; n = 5 - 6.

a) 0-2 min post tetanus

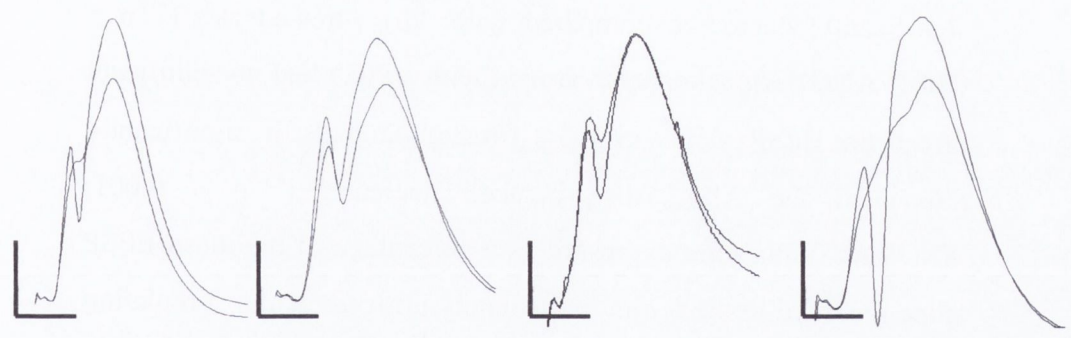
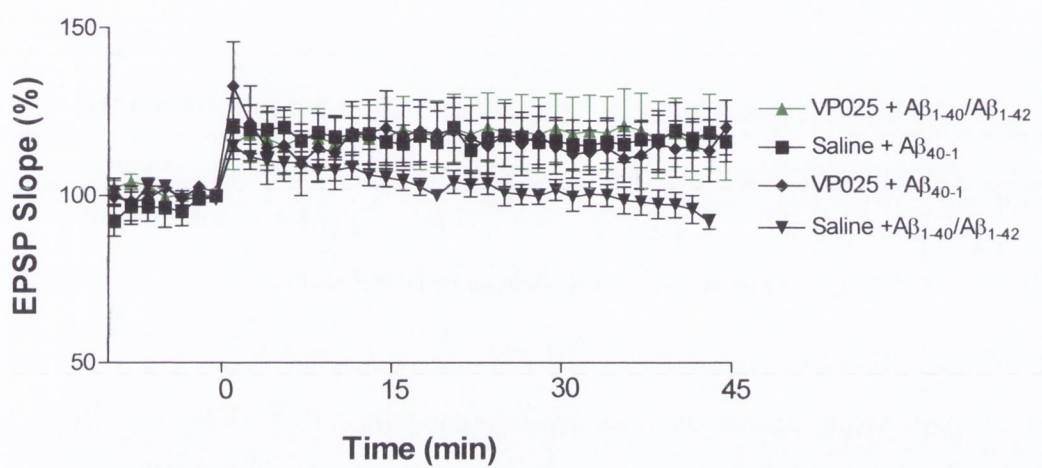


b) 40-45 min post tetanus



**Figure 3.11 VP025 intervention reverses the inhibition of LTP induced by 28 days  $A\beta_{1-40}/A\beta_{1-42}$  treatment**

Chronic  $A\beta_{1-40}/A\beta_{1-42}$  administration (63.8 $\mu$ M) for 28 days significantly inhibited LTP in perforant path granule cell synapses in saline pre-treated rats. Intervention treatment with VP025 alone exerted no significant effect but significantly attenuated the  $A\beta_{1-40}/A\beta_{1-42}$ -induced change (in green). Analysis was undertaken on all values following delivery of tetanus. Population EPSP slope was expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means  $\pm$  SEM; n = 4 - 6. SEMs are included for every 10<sup>th</sup> response. Representative traces, pre- and post-tetanus, for each of the treatment groups are presented with 1mV/5msec scale bar included for each pairing.



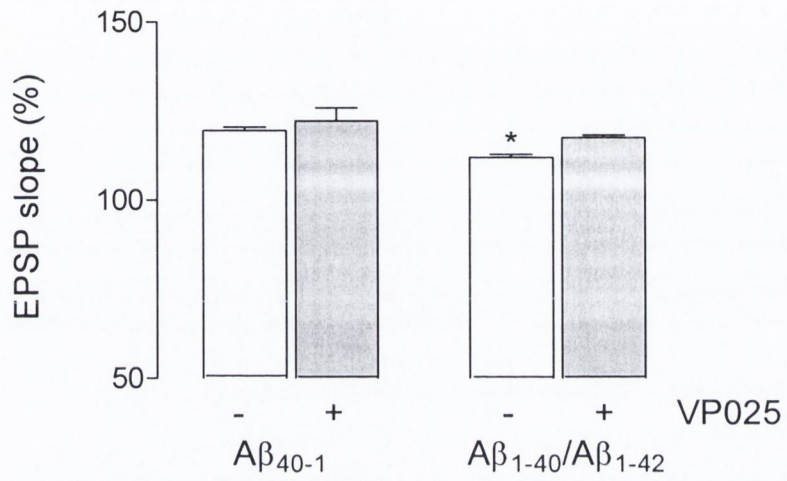
Saline + A $\beta_{40-1}$     VP025 + A $\beta_{40-1}$     Saline + A $\beta_{1-40}/A\beta_{1-42}$     VP025 + A $\beta_{1-40}/A\beta_{1-42}$

**Figure 3.12 VP025 intervention reverses the A $\beta$ -induced percentage change in EPSP slope**

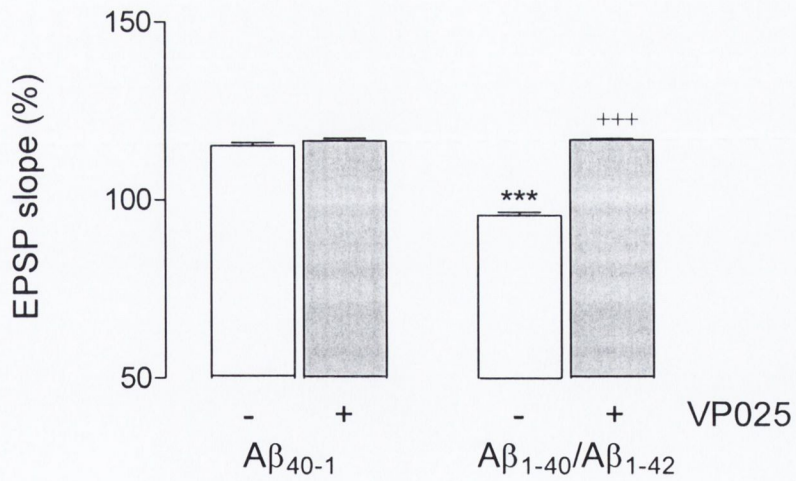
(a) Mean percentage increase in EPSP slope in the first 2min after stimulation was significantly decreased in A $\beta_{1-40}$ /A $\beta_{1-42}$ -treated rats (\* $p < 0.05$ ; ANOVA). There was no significant effect with VP025 treatment on the early change in EPSP slope.

(b) Mean percentage change in population EPSP slope in A $\beta_{1-40}$ /A $\beta_{1-42}$ -treated rats in the last 5min of the recording period was significantly decreased compared with A $\beta_{40-1}$ -treated rats (\*\* $p < 0.001$ ; ANOVA). Whereas treatment with VP025 had no significant effect on EPSP slope in A $\beta_{40-1}$ -treated animals it significantly attenuated the A $\beta_{1-40}$ /A $\beta_{1-42}$ -induced decrease. (++++ $p < 0.001$ ; ANOVA). Values are expressed as a percentage of the mean EPSP slope recorded in the 5 min immediately prior to tetanic stimulation and are the mean  $\pm$  SEM;  $n = 4 - 6$ .

a) 0-2 min post tetanus

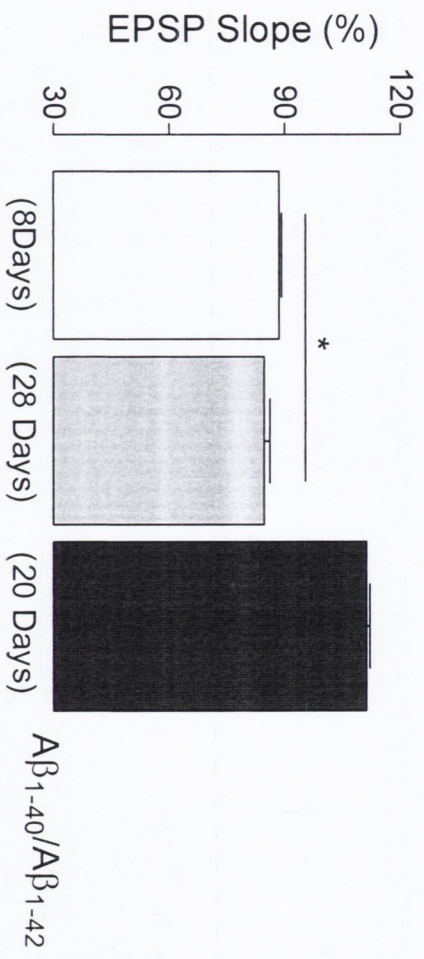


b) 40-45 min post tetanus



**Figure 3.13 Maintenance of LTP is significantly impaired with increasing exposure time to  $A\beta_{1-40}/A\beta_{1-42}$**

Treatment of rats with  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days exerted a significantly decrease on mean EPSP slope compared with treatment for 8 days (\* $p < 0.05$ ; t-test). Treatment of rats with  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days did not exert a significantly detrimental additive effect on mean EPSP slope compared with treatment for 8 days. Data are expressed at means  $\pm$  SEM;  $n = 3 - 5$ .







The objectives of these studies were to assess the effects of acute and prolonged A $\beta$  administration on LTP in perforant path-granule cell synapses *in vivo*, and to assess the effectiveness of VP025, a novel anti-inflammatory in abrogating any A $\beta$ -induced changes. The data show that acute A $\beta$  treatment with A $\beta_{1-40}$  (200 $\mu$ M) significantly inhibits LTP in perforant path granule cell synapses when compared with saline control. Acute administration of A $\beta_{1-40}$ /A $\beta_{1-42}$  (45 or 200 $\mu$ M) did not significantly impair LTP but did significantly lower maintenance in LTP over 45 minutes with respect to A $\beta_{40-1}$  control peptide. In addition, chronic infusion with A $\beta_{1-40}$ /A $\beta_{1-42}$  inhibited LTP in a time-dependent manner with the most profound change observed after 28 days of perfusion. Importantly, treatment of rats with VP025 attenuated the effect of both acute and 8 or 28 days A $\beta$  treatment on LTP.

The present findings that acute icv injection of A $\beta_{1-40}$  (200 $\mu$ M) inhibits LTP in dentate gyrus synapses parallel a similar effect seen in CA1 following acute icv A $\beta_{1-40}$  (200 $\mu$ M) administration (Minogue, PhD Thesis, 2005). Data from this study indicates, however, that rats treated acutely with A $\beta_{1-40}$ /A $\beta_{1-42}$  at two concentrations (45 and 200 $\mu$ M) maintained LTP when compared to A $\beta_{40-1}$ -treated rats albeit to a significantly lesser extent than their control counterparts.

Acute injections of A $\beta$  have previously been shown in this lab to block LTP. Minogue (2005) showed that acute icv administration of A $\beta_{1-40}$  at concentrations ranging from 20 to 200 $\mu$ M impaired LTP in dentate gyrus. Cullen and colleagues (1997) and Freir and colleagues (2001) have also reported LTP impairment in CA1 with A $\beta_{1-40}$  or A $\beta_{1-42}$  at doses ranging from 0.1 to 100nmol. Other evidence from this lab shows acute A $\beta_{1-42}$  (200 $\mu$ M) treatment increases microglial activation as evidenced by increased cytokine production in rat hippocampus (Lyons *et al.*, 2007; Clarke *et al.*, 2007). This augmentation of microglial activity was accompanied by impairment in LTP at perforant path granule cell synapses and was responsive to various anti-inflammatory strategies both *in vivo* and *in vitro*.

Experiments conducted by Walsh and colleagues (2002) reported that icv injection of conditioned medium gleaned from A $\beta$ O-secreting cultured cells subsequently inhibited LTP in anaesthetized rats. Immunodepletion of A $\beta$ O with R1282, a high titre

polyclonal A $\beta$  antibody, completely prevented the LTP blockade observed in these animals. Similarly, Klyubin and colleagues (2005) demonstrated that naturally secreted human A $\beta$ O inhibited LTP in rat hippocampus *in vivo* and that a monoclonal antibody to A $\beta$  prevented such inhibition.

Here a comparison was made between the effects of A $\beta$ <sub>1-40</sub> (200 $\mu$ M) and two concentrations of A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (45 and 200 $\mu$ M). The data indicate that A $\beta$ <sub>1-40</sub> (200 $\mu$ M) exerts a greater effect than A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> at either concentration. One might expect that rats treated with A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> would display a greater impairment in LTP compared with those which received A $\beta$ <sub>1-40</sub> alone and it might be anticipated that 200 $\mu$ M A $\beta$  would exert a greater effect on LTP than 45 $\mu$ M. This expectation stems from that fact A $\beta$ <sub>1-42</sub> is generally considered the more toxic of the two A $\beta$  species, toxicity being conferred by its greater number of  $\beta$ -sheet formations which increases its propensity to aggregate (Jarret and Lansbury, 1993; Jarret *et al.*, 1993; Klein *et al.*, 1999). Previous *in vitro* experiments show that both A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> peptides self-assemble into A $\beta$  fibrils (Fraser *et al.*, 1992; Ma *et al.*, 1994). Despite soluble A $\beta$ <sub>1-40</sub> being more abundantly secreted than soluble A $\beta$ <sub>1-42</sub> (Seubert *et al.*, 1992; Vigo-Pelfrey *et al.*, 1993; Suzuki *et al.*, 1994) post mortem examination shows that A $\beta$ <sub>1-42</sub> tends to be deposited in greater density in the parenchyma than its shorter counterpart in AD (Iwatsubo *et al.*, 1994, 1995). Thus, these distinct A $\beta$  species seem to be metabolised differently and may play different roles in the deposition of A $\beta$ .

Klein and colleagues (1999) assessed the neurotoxic properties of A $\beta$ <sub>1-42</sub> compared to A $\beta$ <sub>1-40</sub> by injecting both peptides into the frontal cortex of C57BL/6 mice. A $\beta$ <sub>1-42</sub> was associated with a significantly larger area of glial fibrillary acidic protein (GFAP) immunoreactivity and a greater density of reactive astrocytes than A $\beta$ <sub>1-40</sub>. Immunohistochemical staining for markers of oxidative stress were also significantly more intense around the area of A $\beta$ <sub>1-42</sub> administration compared to the A $\beta$ <sub>1-40</sub> injection sites. It was suggested that A $\beta$ <sub>1-42</sub> is more neurotoxic and may generate more free radical damage than A $\beta$ <sub>1-40</sub>. Further support for the greater toxicity reported for A $\beta$ <sub>1-42</sub> comes from recent results published by Parameshwaran and colleagues (2007). Using whole cell patch clamp techniques they reported that A $\beta$ <sub>1-42</sub>, and not A $\beta$ <sub>1-40</sub>, significantly affected

the functioning of AMPA receptors in rat hippocampus.  $A\beta_{1-42}$  reduced AMPA synaptic channel open times by 65% and increased close times by several fold. It was argued that  $A\beta_{1-42}$ , but not  $A\beta_{1-40}$ , interacts with and exhibits inhibitory effects on synaptic receptors and that this may contribute to the memory impairment observed in AD.

Based on this evidence, one could ask why an infusion of  $A\beta_{1-40}$  in combination with the more toxic  $A\beta_{1-42}$  does not exert more profound effects on LTP than  $A\beta_{1-40}$  alone. The answer may be in the interactions between  $A\beta$  peptides. In 2002, Zou and colleagues reported a novel function for  $A\beta_{1-40}$ . They found that freshly prepared  $A\beta_{1-40}$  and  $A\beta_{1-42}$  exhibited completely opposite actions on neurons *in vivo*. It was reported that the solution of  $A\beta_{1-40}$  tended to remain largely in monomeric form preventing neuronal death by curbing oxygen radical production, while  $A\beta_{1-42}$  aggregated immediately and exerted neurotoxic effects. Additional experiments (Zou *et al.*, 2003) indicated that  $A\beta_{1-40}$  protects neurons from  $A\beta_{1-42}$ -induced neurotoxicity in a ratio-dependant manner. These authors report that  $A\beta_{1-40}$  prevented neuronal death and tau phosphorylation in rat brain by inhibiting directly the amyloidogenic nature of  $A\beta_{1-42}$ .

Wild-Type human APP transgenic mice (Mucke *et al.*, 2000) have shown that the relative levels of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  are determinants of the aggregation state of the  $A\beta$  peptides *in vivo*, with relatively high concentrations of  $A\beta_{1-40}$  being potentially anti-amyloidogenic. This could account for the relatively reduced effect the higher (200 $\mu$ M)  $A\beta_{1-40}/A\beta_{1-42}$  dose had compared with the lower  $A\beta_{1-40}/A\beta_{1-42}$  concentration (45 $\mu$ M) on LTP. Using spectrophotometric analysis, Snyder and colleagues (1994) found that when  $A\beta_{1-40}$  and  $A\beta_{1-42}$  are mixed together  $A\beta_{1-40}$  retards the aggregation of  $A\beta_{1-42}$  in a concentration-dependent manner. This finding appears to be specific to  $A\beta_{1-40}$  as shorter  $A\beta$  peptides such as  $A\beta_{25-32}$  for example, displayed less ability to interfere with  $A\beta_{1-42}$  aggregation. Kim and colleagues (2007) reported that bi-transgenic mice (BRI-A40/BRI-A42A) over-expressing  $A\beta_{1-40}$  and  $A\beta_{1-42}$  displayed 60-90% less  $A\beta$  deposition than BRI-A42A mice which over-express  $A\beta_{1-42}$  alone. The authors demonstrated that  $A\beta_{1-42}$  and  $A\beta_{1-40}$  have opposing effects on amyloid deposition;  $A\beta_{1-42}$  promotes amyloid deposition but  $A\beta_{1-40}$  inhibits it. The authors argue that their data show protective properties of  $A\beta_{1-40}$  with respect to amyloid deposition and suggest that strategies that preferentially target

$A\beta_{1-40}$  may actually worsen the progression of AD. Controversially, Shin and colleagues (1997) found following intrahippocampal injection of solubilized  $A\beta_{1-40}$  and  $A\beta_{1-42}$  that it was  $A\beta_{1-40}$  alone which aggregated to form the fA $\beta$  associated with A $\beta$  plaques and that  $A\beta_{1-40}$  but not  $A\beta_{1-42}$  aggregates showed congo red birefringence which would indicate the presence of A $\beta$  fibrils in these deposits. These findings confound the commonly held belief that  $A\beta_{1-42}$  is the more aggressive aggregator and more toxic of the two peptide species *in vivo*.

Neurotoxicity requires polymeric A $\beta$  fibril formation (Pike *et al.*, 1993). The role of stressors, such as A $\beta$  accumulation, in mediating neuronal disruption has been investigated by studying their direct effects on synaptic mechanisms, especially LTP. As highlighted in the introduction, LTP is a neurophysiological model that describes a type of activity dependant synaptic modification important for certain forms of memory storage in the mammalian brain (Byrne *et al.*, 1993; Bartsch *et al.*, 1995; Lüscher *et al.*, 2000). Mesulam (1999) and Selkoe and colleagues (2002) have both suggested that it is the APP mis-processing associated with AD, which leads to an eventual failure in central neuroplasticity. Aredns and colleagues (2000) would argue that the plasticity of brains is region specific and that areas associated with greater plasticity are areas of the brain affected in early AD.

Other evidence linking the production and aggregation of toxic A $\beta$  species to cell loss with the pathogenesis of AD comes from analysis of the processing of its pre-cursor protein APP. Mutation of the genes encoding PSen1 and PSen2 are linked to the most common cause of fAD. This defect in the production of PSen1 and PSen2 alters  $\gamma$ -secretase activity.  $\gamma$ -secretase intra-membranous proteolysis of APP provides the final step in the generation of A $\beta$  from APP. Compromised  $\gamma$ -secretase means an increase in the production of A $\beta$ , in particular the highly amyloidogenic  $A\beta_{1-42}$  isoform. Deletion of PSen1 in mice has been show to greatly reduce  $\gamma$ -secretase activity (Wolfe *et al.*, 1999) indicating an important role for it in subsequent A $\beta$  plaque formation. Using mutant mouse models, Lazarov and colleagues (2006) demonstrate that mutations in PSen1 contribute to neuronal cell loss in regions of the brain associated with the induction and maintenance of LTP. Using PDAPP mice which over-express mutated human APP,

Giacchino and colleagues (2000), have shown complete inhibition of LTP in the CA1 area *in vivo*. Changes in hippocampal transmission and plasticity associated with early synaptic dysfunction have been observed in these animals prior to any evidence of A $\beta$  (Hsia *et al.*, 1999). Similarly, it has been shown that brief perfusion of hippocampal slices with non-neurotoxic concentrations of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> (200 $\mu$ M) is sufficient to significantly inhibit LTP induction (Chen *et al.*, 2000). Such an effect the authors suggest may underlie the memory deficits seen in AD even before neuronal loss has occurred.

Data from the chronic A $\beta$ -infusion studies show the attenuation of both early and later responses of perforant path-granule cell synapses to tetanic stimulation in rats treated with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) treatment over 8, 20 or 28 days. It was interesting to note that the impairment in LTP increased as the A $\beta$  infusion time increased suggesting that A $\beta$  neurotoxicity increases with prolonged administration to the brain of experimental animals.

In these studies maximal aggregation of A $\beta$  was confirmed using Thioflavin-T fluorescence. A significant reduction in oligomeric content was confirmed following the aggregation period using Coomassie blue assay technique but analysis by electrophoresis confirmed the presence of oligomers particularly the 13.5kDa species. Sahlin and colleagues (2007) have shown that oligomeric A $\beta$ , in protofibrillar form, is capable of being produced both *in vivo* and *in vitro*. Soluble oligomers are elevated in the AD brain (Kuo *et al.*, 1996; Funato *et al.*, 1999; Gong *et al.*, 2003; Georganopolou *et al.*, 2005) and the pathogenic importance of this form of A $\beta$  has been confirmed by the recent discovery of the Arctic fAD mutation, which has increased capacity for oligomerization (Nilsbeth *et al.*, 2001; Paivio *et al.*, 2004). Klyubin and colleagues (2004) examined the effects of icv injection of A $\beta$ <sub>1-40</sub> peptides created by the Arctic mutation in APP; the mutant peptide was 100 fold more potent in inhibiting LTP than wild-type A $\beta$ . Fibrillar material was detected in both preparations of the peptides but when soluble fractions were prepared using high speed centrifugation the supernatant obtained from the Arctic mutated peptide retained full activity in terms of its ability to inhibit LTP. This suggests that in AD sufferers expressing the Arctic fA $\beta$  mutation, soluble A $\beta$  may be the primary mediator of

their observed cognitive deficits. Concurring with the *in vivo* evidence, *in vitro* experiments have also demonstrated potent neurotoxicity upon exposure to soluble oligomers and protofibrils (Dahlgren *et al.*, 2002; Kim *et al.*, 2003). Further analysis of the relative oligomeric content of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> in the fibrillar A $\beta$  preparations used in the current work is needed and assessment of the effects of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> on soluble A $\beta$  content and their contribution to toxicity in both the acute and chronic experiments is also required.

In support of these findings, Nitta and colleagues (1994; 1997) show that chronic A $\beta$  infusion impairs learning behaviour in rats. Significantly these results are accompanied by a marked decrease in choline acetyltransferase activity in the hippocampus. These results suggest that under experimental conditions A $\beta$  deposition in the brain could lead to a cholinergic neuronal based impairment in learning similar to that found in AD patients. Subsequent studies (1999) performed by the same group also show that chronic A $\beta$  infusion inhibits LTP *ex vivo* and that this effect is nicotine sensitive also. Taken together their findings suggest that chronic A $\beta$  protein infusion impairs signal transduction mechanisms via nicotinic acetylcholine receptors and that this dysfunction could be responsible, in part at least, for their observed A $\beta$ -induced impairment in LTP.

In 2003, Kim and colleagues examined the role of oxidative stress in A $\beta$ -induced learning and memory impairment. Following 14 days of icv A $\beta$ <sub>1-42</sub> infusion they examined the immunoreactivity of endogenous antioxidant systems such as mitochondrial superoxide dismutase (SOD) and glutathione. Long-term A $\beta$  administration resulted in a significant reduction in anti-oxidant substances in several brain areas including hippocampus. The A $\beta$  induced changes were not uniform but were specific for various antioxidants and specific brain regions. Lecanu and colleagues (2006) found that chronic (4 week) icv infusion of Long-Evans rats with A $\beta$ <sub>1-42</sub> and buthionine-sulfoximine (FAB), but not A $\beta$ <sub>1-42</sub> alone, induced functional memory impairments accompanied by increased hyperphosphorylated Tau protein levels in the CSF. FAB depletes cells of the antioxidant glutathione resulting in free radical induced apoptosis. FAB-infused animals displayed thioflavin-S-positive amyloid deposits,

hyperphosphorylated Tau protein, neuronal loss, and gliosis. Animals treated with A $\beta$ <sub>1-42</sub> alone failed to show the histological modifications observed when A $\beta$ <sub>1-42</sub> was co-administered with FAB. These data suggest that chronic exposure to A $\beta$ <sub>1-42</sub> alone is not sufficient to induce an AD-like symptomology but supports a hypothesis whereby a decrease in the brain's antioxidant defence system may contribute to the A $\beta$ <sub>1-42</sub> oxidative stress necessary for the peptide to induce histopathological changes and memory loss.

Frautschy and colleagues (2001) have similarly found that chronic co-infusion of both A $\beta$  peptides induces spatial memory deficits in the Morris Water Maze and post-synaptic density loss in rat. They also find that chronic icv co-infusion of both A $\beta$  species induced oxidative damage, synaptophysin loss, a microglial response and widespread A $\beta$  deposits in the rat brain. Treatment of these rats with curcumin, which possesses anti-inflammatory properties, reversed many of these A $\beta$ -induced changes.

How A $\beta$  affects neurons to produce impairments in cognitive performance is unclear but persistent inflammation in the AD affected brain appears to play a role. Animal models have repeatedly shown that inflammation is associated with deficits in synaptic function (Vereker *et al.*, 2001) and consequently impairments in cognitive function (Braidia *et al.*, 2003). Significantly, administration of anti-inflammatory agents restores synaptic function in LPS-treated animals (Hausse-Wegrzyniak *et al.*, 1999; Nolan *et al.*, 2004; Lynch *et al.*, 2004). Data from this study reveal that pre-treatment of rats with VP025 significantly reverses the acute effects of A $\beta$  administration on LTP. Nolan and colleagues (2005) report that similar treatment of aged rats with VP015 (a phosphatidylserine (PS)-based liposome similar to VP025) significantly reversed impairment of LTP. Importantly, VP015 treatment significantly increased the concentration of the anti-inflammatory cytokine IL-4 in the hippocampus of these rats. In addition LTP-induced impairments in LTP were also accompanied by a significant decrease in hippocampal IL-1 $\beta$  levels by VP015 pre-treatment (Nolan *et al.*, 2004). PS expression on apoptotic cells promotes their phagocytosis and induces anti-inflammatory responses in phagocytes.

Earlier studies showed that treatment of rats with liposomes causes a reduction in LPS-induced increases in serum TNF- $\alpha$  (Monastra and Bruni, 1992) which indicates an



anti-inflammatory effect of liposomes. Subsequent findings showed that PS inhibits macrophage production of pro-inflammatory cytokines and nitric oxide and that it blocks macrophage killing of intracellular parasites (Aramaki *et al.*, 1997; 2000).

Further experiments carried out by Nolan and colleagues (2004) confirmed that VP015 reversed LPS-induced impairment in LTP. In addition they found that liposome treatment also prevented the LPS promotion of microglial activation while it also down-regulated p38 activation in hippocampal neurons. This was accompanied by an increase in the expression of anti-inflammatory cytokine IL-10, which is known to prevent antigen presentation to Th1 cells (Ozawa *et al.*, 1996). These results corroborate findings (Aramaki *et al.*, 2001), which show that PS liposome treatment inhibited LPS-induced phosphorylation of p38 in macrophage *in vitro*. Aramaki and colleagues (2001) suggested that the liposomes exert their anti-inflammatory influence by preventing the expression of iNOS and that this occurred upstream of NF $\kappa$ - $\beta$  activation. Ramos and colleagues (2007) also showed that PS liposomes reversed carrageenan-induced inflammation in mice *in vivo*. They found it lowered IL-1 $\beta$  concentration in the tissue collected and that it was effective as an anti-inflammatory agent even 48 hours following carrageenan injection. Hoffman and colleagues (2005) investigated the effects of PS-containing liposomes on adaptive immune responses in mice. They found that PS liposomes specifically inhibited responses to antigens as determined by decreased draining lymph node tissue mass, with reduced numbers of total leukocytes and antigen specific CD4<sup>+</sup> T cells. They also established that transforming growth factor- $\beta$  (TGF- $\beta$ ) plays a critical role in this inhibition, as the inhibitory effects of PS were reversed by *in vivo* administration of anti-TGF- $\beta$  antibodies.

As yet the molecular mechanisms governing the action of VP025 are unclear. Unlike VP015 it comprises liposomes containing anionic phosphatidylglycerol (PG), and neutral phosphatidylcholine (PC). Gololobov and colleagues (1998) report that PG prevents the breakdown of vasoactive intestinal peptide (VIP) and suggested that it was the charge on the lipid which interfered with enzymatic breakdown of the peptide. VIP is broadly distributed in the CNS and exerts diverse biological effects, including anti-inflammatory and immunomodulatory effects (Yanaihara, 1992; Paul and Ebadi, 1993). Similar to PS, PG also seems to govern NF $\kappa$ -B activation. Wu and colleagues (2003)

have shown that PG inhibits the synthesis of pro-inflammatory sPLA<sub>2</sub>-IIA by alveolar macrophages. These cells are the prime source of PLA<sub>2</sub> in acute lung injury. The transcription factor NFκ-B governs sPLA<sub>2</sub>-IIA expression in macrophage and PG completely suppressed its activation.

In addition to its ability to attenuate the acute effects of Aβ<sub>1-40</sub> on LTP, VP025 pre-treatment also attenuates the effects of chronic Aβ<sub>1-40</sub>/Aβ<sub>1-42</sub> administration. Of particular significance is the finding that VP025 effectively reversed the deficit in LTP when administered over 3 days on an intervention basis, starting midway through the 28 day infusion period. It is exciting to note that VP025 intervention rescues any Aβ-induced impairment in LTP as successfully as pre-treatment does. It is not yet clear how VP025 exerts its anti-inflammatory effect. Martin and colleagues (2004) have shown that, in parallel with its ability to prevent age-related impairments in LTP, VP025 also attenuated an age-induced increase in hippocampal IL-1β and JNK phosphorylation while it reversed the age-induced decrease in the pro-survival neuronal signaling molecule ERK. In the chronic Aβ administration studies, it is unlikely that VP025 administration directly affects Aβ fibrillogenesis or the ratio of soluble Aβ species to aggregates. It is more likely that it modulates synaptic function and controls the inflammatory state of the brain by governing the direct interaction of microglia and neurons or via signal transduction pathways. Evidence for this will be discussed in the next chapter.

## **Chapter 4**

The pathogenesis of idiopathic AD remains elusive, although evidence has suggested that neuroinflammation characterized by activation of resident microglia in the brain may contribute significantly to neurodegeneration in AD. Consistent with this is the observation that several neurodegenerative disorders such as AD are accompanied by an increase in the concentration of pro-inflammatory cytokines such as IL-1 $\beta$  in the CNS. IL-1 $\beta$  has a negative impact on synaptic plasticity and therefore an increase in IL-1 $\beta$ , such as that in the hippocampus of the A $\beta$ -treated rat, is associated with a deficit in LTP (Lynch and Lynch, 2000; Minogue *et al.*, 2003). This chapter investigates the effects of acute and long-term A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> administration (200 $\mu$ M; 63.8 $\mu$ M respectively) on expression of cell surface markers of inflammation on the resident immune competent cells of the brain, microglia, and subsequently investigates what contribution, if any, they make to our observed A $\beta$ -induced impairment in LTP. Expression of cell surface markers of microglial activation (CD86, ICAM-1 and MHCII) and changes in release of IL-1 $\beta$  in hippocampal tissue and RNA extract were examined by immunoblotting (CD86 and ICAM-1), RT-PCR and QPCR (MHCII) and ELISA (IL-1 $\beta$ ) respectively. The protocols are outlined in detail in chapter 2, sections 2.6, 2.7 and 2.8.

The data presented here indicate a significant increase in phenotypic markers of microglial activation in that CD86 and ICAM-1 expression were increased in hippocampal tissue prepared from rats treated with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 or 20 days, MHCII mRNA expression remained unaffected by A $\beta$  treatment. VP025 treatment did not alter CD86 expression but significantly reversed the A $\beta$ -induced increase in ICAM-1 expression. There was however, a lack of functional evidence for microglial activation in that there was no evidence of cytokine production in this tissue. Interestingly, there was a significant increase in IL-1 $\beta$  production in hippocampal tissue prepared from rats treated with A $\beta$  for 28 days, there was however no evidence of increased CD86 or ICAM-1 in this tissue. These data may underpin an alternative process, which involves stimulus dependent differentiation in roles for microglia, which changes with increasing exposure time to stress – i.e. from phagocytic entity to cytokine producer.

#### **4.1 Acute amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ administration increases cluster of differentiation 86 and intracellular adhesion molecule-1 expression in rat hippocampus**

The protein CD86 is expressed on activated microglia. It is a molecule which provides a costimulatory signal necessary for T cell activation and survival priming them against antigen. ICAM-1, a member of the IgG superfamily is up-regulated on microglia at sites of inflammation and promotes adhesion of LFA-1 expressing cells to those sites. An increase in CD86 and ICAM-1 expression was observed in the hippocampal tissue of rats which received an icv injection of  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) ( $0.90 \pm 0.02$  arbitrary units; \*\*  $p < 0.01$ ; ANOVA; Figure 4.1) and ( $0.66 \pm 0.01$  arbitrary units; \*  $p < 0.05$ ; ANOVA; Figure 4.2) when compared with hippocampal tissue prepared from rats that received the control peptide  $A\beta_{40-1}$  ( $0.68 \pm 0.06$  arbitrary units;  $0.35 \pm 0.07$  arbitrary units, respectively). Data are expressed as means  $\pm$  SEM;  $n = 4 - 6$ .

#### **4.2 Chronic amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ administration increases cluster of differentiation 86 and intracellular adhesion molecule-1 expression in rat hippocampus**

Having observed that acute  $A\beta$  administration increases CD86 and ICAM-1 expression in rat hippocampal tissue further experiments were conducted to establish whether chronic  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) administration regimes would induce similar results. On this occasion these experiments were carried out in the presence or absence of the novel anti-inflammatory VP025.

CD86 expression was significantly increased in hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) ( $0.66 \pm 0.07$  arbitrary units; \*  $p < 0.05$ ; ANOVA; Figure 4.3) over 8 days compared with tissue prepared from saline pre-treated rats that received  $A\beta_{40-1}$  ( $0.46 \pm 0.02$  arbitrary units). Similarly, ICAM-1 expression was also significantly increased in hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) ( $0.76 \pm 0.13$  arbitrary units; \*  $p < 0.05$ ; ANOVA; Figure 4.4) for 8 days compared with tissue prepared from saline pre-

treated rats that received  $A\beta_{40-1}$  ( $0.33 \pm 0.08$  arbitrary units). Although the effect was not statistically significant, VP025 slightly decreased the  $A\beta$ -induced change in both proteins so that the mean values for CD86 ( $0.62 \pm 0.07$  arbitrary units) and ICAM-1 ( $0.47 \pm 0.10$  arbitrary units) were not significantly greater than control.

CD86 expression was also significantly increased in hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 20 days ( $0.76 \pm 0.10$  arbitrary units; \* $p < 0.05$ ; ANOVA; Figure 4.5) compared with tissue prepared from saline pre-treated rats that received  $A\beta_{40-1}$  ( $0.39 \pm 0.04$  arbitrary units). ICAM-1 expression was also significantly increased in hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 20 days ( $0.65 \pm 0.24$  arbitrary units; \* $p < 0.05$ ; ANOVA; Figure 4.6) when compared with tissue prepared from saline pre-treated rats that received  $A\beta_{40-1}$  ( $0.15 \pm 0.001$  arbitrary units). Although the effect was not statistically significant, VP025 slightly decreased the  $A\beta$ -induced change in CD86 expression so that the mean value for CD86 ( $0.71 \pm 0.12$  arbitrary units) and was not significantly greater than saline pre-treated  $A\beta_{40-1}$ -treated rats. VP026 did however significantly decrease the  $A\beta$ -induced change in ICAM-1 expression ( $0.15 \pm 0.024$  arbitrary units; + $p < 0.05$ ; ANOVA; Figure 4.6).

Interestingly, neither CD86 nor ICAM-1 expression were significantly increased in hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 28 days ( $0.70 \pm 0.07$  arbitrary units;  $0.59 \pm 0.10$  arbitrary units respectively; Figures 4.7, 4.8) compared with hippocampal tissue prepared from saline pre-treated rats that received  $A\beta_{40-1}$  ( $0.73 \pm 0.13$  arbitrary units;  $0.51 \pm 0.06$  arbitrary units respectively; Figures 4.7, 4.8). No effect of VP025 pre-treatment was observed. Data are expressed as means  $\pm$  SEM;  $n = 4 - 6$ .

### **4.3 Amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ administration does not increase major histocompatibility complex II expression in the rat hippocampus**

Expression of MHC II mRNA was assessed in hippocampal tissue prepared from rats, which received  $A\beta_{1-40}/A\beta_{1-42}$  acutely for 4 hours ( $200\mu\text{M}$ ), or from saline pre-treated rats, which were infused with  $A\beta_{1-40}/A\beta_{1-42}$  for 8 or 28 days ( $63.8\mu\text{M}$ ).  $A\beta$ -treatment

failed to significantly alter the expression of MHCII mRNA in tissue prepared from any of the groups (Figures 4.9, 4.10, 4.11 respectively). Data are expressed as means  $\pm$  SEM; n = 5 - 6.

#### **4.4 Chronic amyloid- $\beta_{1-40}$ /amyloid- $\beta_{1-42}$ infusion increases the concentration of interleukin-1 $\beta$ in rat hippocampus**

Analysis of IL-1 $\beta$  in hippocampal tissue prepared from rats treated A $\beta_{1-40}$ /A $\beta_{1-42}$  (200 $\mu$ M) acutely ( $169 \pm 3.1$  pg IL-1 $\beta$ /mg) or, saline pre-treated rats, which were infused with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 ( $408.2 \pm 35.2$  IL-1 $\beta$  pg/mg) or 20 days ( $641.8 \pm 29.9$  IL-1 $\beta$  pg/mg), revealed no significant A $\beta$ -induced change when compared with saline pre-treated rats infused with A $\beta_{40-1}$  ( $171 \pm 5.1$ ;  $383.3 \pm 38.1$ ;  $688.4 \pm 45.3$  IL-1 $\beta$  pg/mg respectively; Figures 4.12, 4.13, 4.14). However, IL-1 $\beta$  concentration was significantly increased in hippocampal homogenate prepared from saline pre-treated rats which received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 28 days ( $164.5 \pm 12.5$  IL-1 $\beta$  pg/mg) when compared with saline pre-treated rats which were treated with A $\beta_{40-1}$  ( $138.4 \pm 3.45$  IL-1 $\beta$  pg/mg; \* $p < 0.05$ ; ANOVA; Figure 4.15). The effect of VP025 pre-treatment was only assessed in rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 and 20 days; it was shown not to exert any effect on IL-1 $\beta$  production in hippocampal tissue prepared from these animals ( $420.0 \pm 50.90$ ;  $569.0 \pm 43.05$  IL-1 $\beta$  pg/mg respectively; Figures 4.13 and 4.14). Data are expressed as means  $\pm$  SEM; n = 4 - 6. The results pertaining to hippocampal tissue prepared from rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  for 28 days were obtained by collaboration with Alessia Piazza, the tissue was kindly donated by Dr. Anthony Lyons.

## **Chapter 4**

### **Figures**

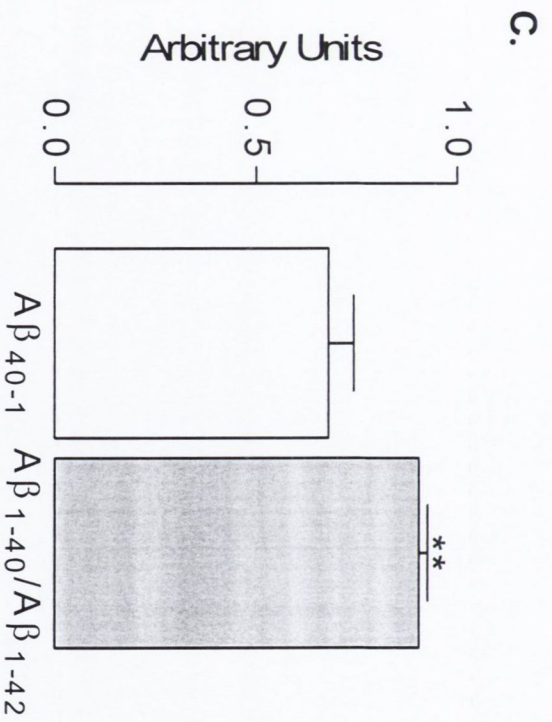


**Figure 4.1 Acute  $A\beta_{1-40}/A\beta_{1-42}$  administration increases CD86 expression in rat hippocampus**

A sample immunoblot shows that CD86 expression was increased after 4 hours in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (lane 2) compared with  $A\beta_{40-1}$  treated rats (lane 1).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean CD86 expression was increased after 4 hours in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (200 $\mu$ M) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats (\*\* $p < 0.01$ ; ANOVA). Values are expressed as arbitrary units of CD86 expression and are the mean  $\pm$  SEM;  $n = 4 - 6$ .



**Figure 4.2 Acute  $A\beta_{1-40}/A\beta_{1-42}$  administration increases ICAM-1 expression in rat hippocampus**

A. A sample immunoblot shows that ICAM-1 expression was increased after 4 hours in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (lane 2) compared with  $A\beta_{40-1}$  treated rats (lane 1).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean ICAM-1 expression was increased in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (200 $\mu$ M) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats (\* $p < 0.05$ ; ANOVA).

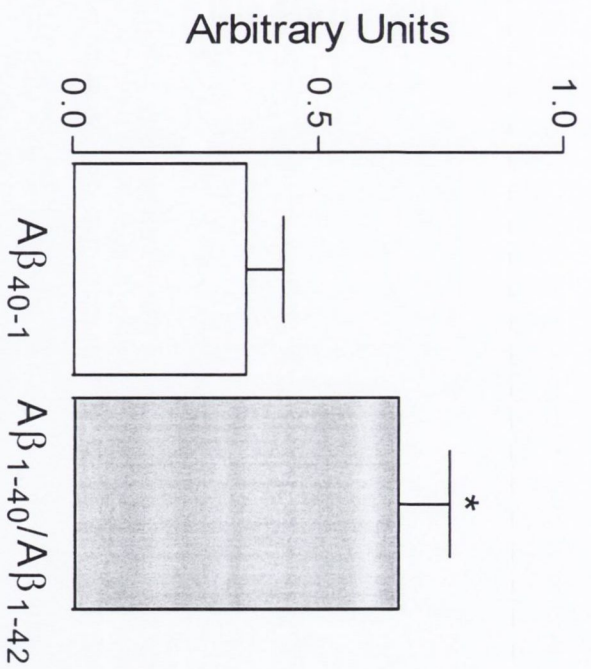
Values are expressed as arbitrary units of ICAM-1 expression and are the mean  $\pm$  SEM;  $n = 4 - 6$ .

1 2

A. ICAM-1(85kDa)

B. Actin (43kDa)

C.



**Figure 4.3 CD86 expression is significantly increased by 8 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

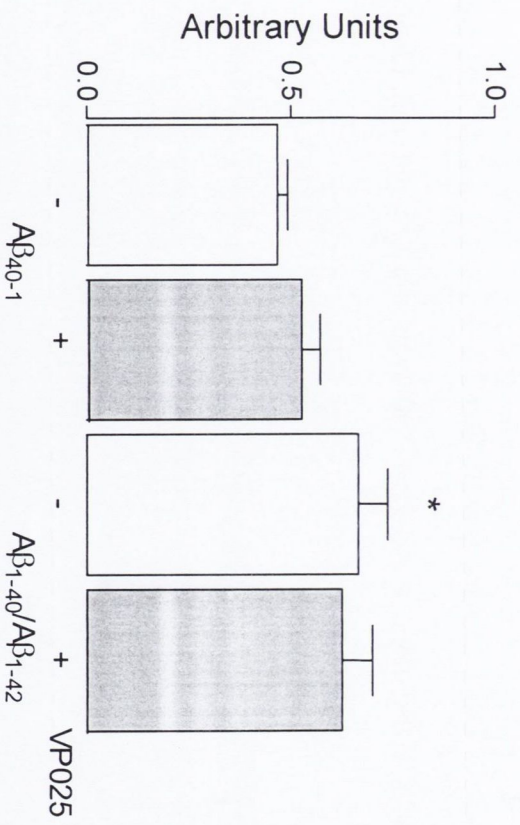
A. A sample immunoblot shows that CD86 expression was significantly increased in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days (lane 3) (\* $p < 0.05$ ; ANOVA) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 treatment exerted no significant effect in this experiment (lanes 2 and 4).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean CD86 expression was increased in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (63.8 $\mu$ M) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats (\* $p < 0.05$ ; ANOVA). Values are expressed as arbitrary units of CD86 expression and are the mean  $\pm$  SEM;  $n = 5 - 6$ .



**C.**



**Figure 4.4 ICAM-1 expression is significantly increased by 8 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

A. A sample immunoblot shows that ICAM-1 expression was significantly increased in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days (lane 3) (\* $p < 0.05$ ; ANOVA) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 treatment exerted no significant effect in this experiment (lanes 2 and 4).

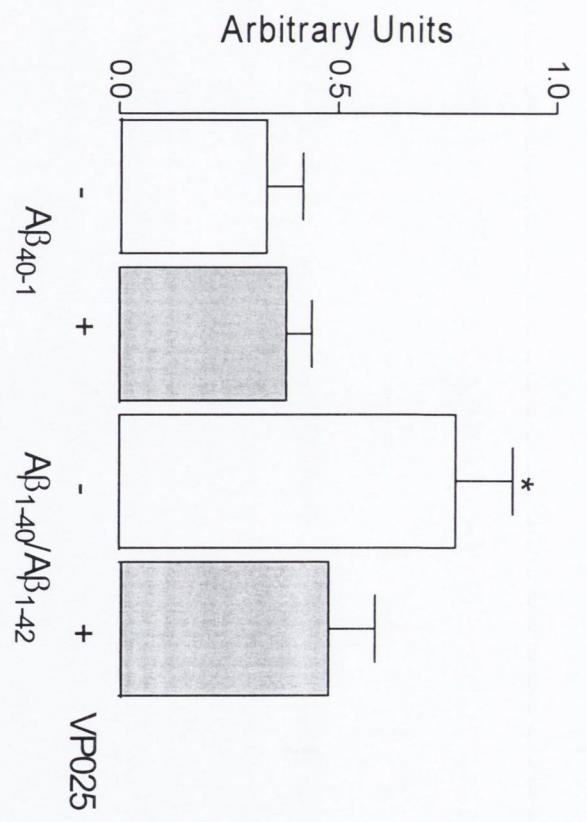
B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean ICAM-1 expression was increased hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $63.8\mu\text{M}$ ) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats (\* $p < 0.05$ ; ANOVA). Values are expressed as arbitrary units of ICAM-1 expression and are the mean  $\pm$  SEM;  $n = 4 - 5$ .

1 2 3 4



**C.**





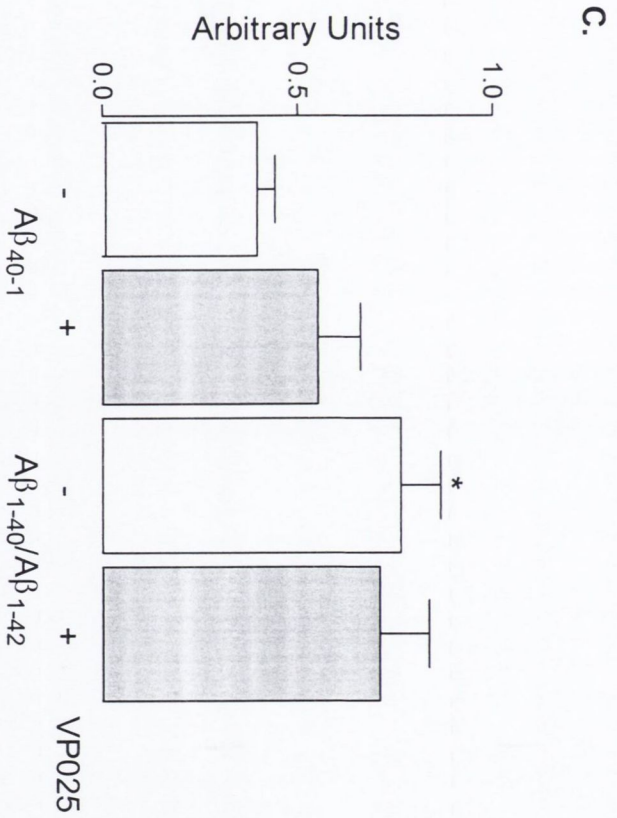
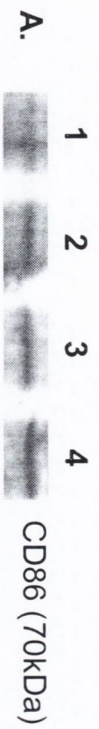
**Figure 4.5 CD86 expression is significantly increased by 20 days**

**$A\beta_{1-40}/A\beta_{1-42}$  treatment**

A. A sample immunoblot shows that CD86 expression was significantly increased in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days (lane 3) (\* $p < 0.05$ ; ANOVA) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 treatment exerted no significant effect in this experiment (lanes 2 and 4).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean CD86 expression was increased in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (63.8 $\mu$ M) compared with tissue prepared from control-treated rats (\* $p < 0.05$ ; ANOVA). Values are expressed as arbitrary units of CD86 expression and are the mean  $\pm$  SEM;  $n = 4 - 6$ .

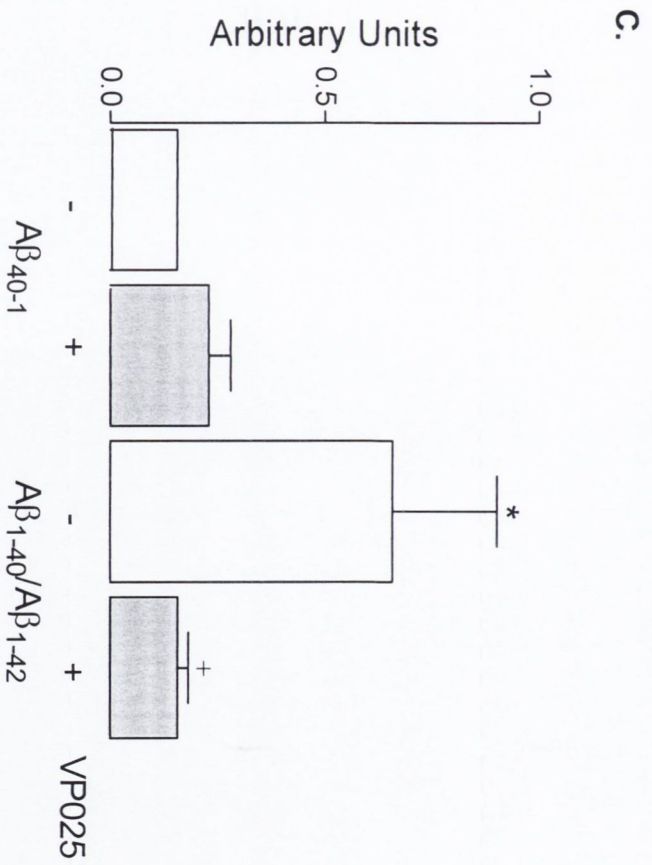


**Figure 4.6 VP025 reverses the increase in ICAM-1 expression induced by 20 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

A. A sample immunoblot shows that ICAM-1 expression was significantly increased in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days (lane 3) (\* $p < 0.05$ ; ANOVA) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 exerted a significant effect in this experiment (lane 4).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean ICAM-1 expression was increased in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (63.8 $\mu$ M) compared with tissue prepared from control-treated rats (\* $p < 0.05$ ; ANOVA). VP025 pre-treatment exerted a significant effect in this experiment ( $^{\dagger}p < 0.05$ ; ANOVA). Values are expressed as arbitrary units of ICAM-1 expression and are the mean  $\pm$  SEM;  $n = 4 - 5$ .



**Figure 4.7 CD86 expression is unaffected by 28 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

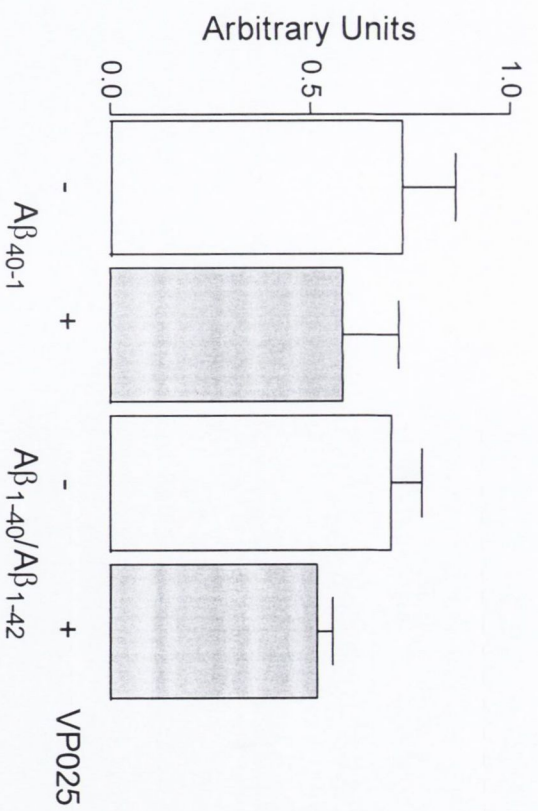
A. A sample immunoblot shows that CD86 expression was unchanged in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days (lane 3) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 treatment exerted no significant effect in this experiment (lanes 2 and 4).

B. No change in actin was observed.

C. Data from densitometric analysis revealed that mean CD86 expression was unchanged in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats ( $63.8\mu\text{M}$ ) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats. Values are expressed as arbitrary units of CD86 expression and are the mean  $\pm$  SEM; n = 5 - 6.



**C.**



**Figure 4.8 ICAM-1 expression is unaffected by 28 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

A. A sample immunoblot shows that ICAM-1 expression was unchanged in hippocampal tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days (lane 3) when compared with hippocampal tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (lane 1). VP025 treatment exerted no significant effect in this experiment (lanes 2 and 4).

B. No change in actin was observed.

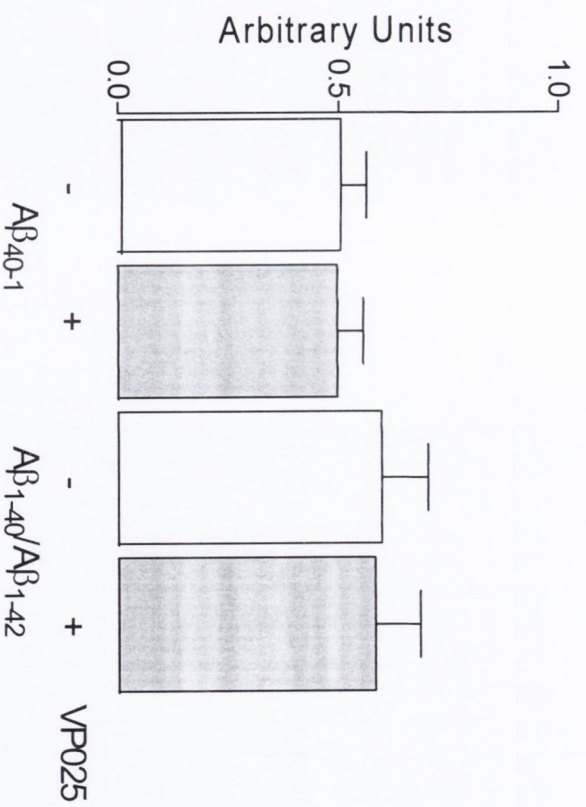
C. Data from densitometric analysis revealed that mean ICAM-1 expression was unchanged in hippocampal tissue prepared from  $A\beta_{1-40}/A\beta_{1-42}$ -treated rats (63.8 $\mu$ M) compared with tissue prepared from  $A\beta_{40-1}$ -treated rats. Values are expressed as arbitrary units of ICAM-1 expression and are the mean  $\pm$  SEM; n = 5 - 6.

1 2 3 4

A.  85kDa ICAM-1

B.  43kDa Actin

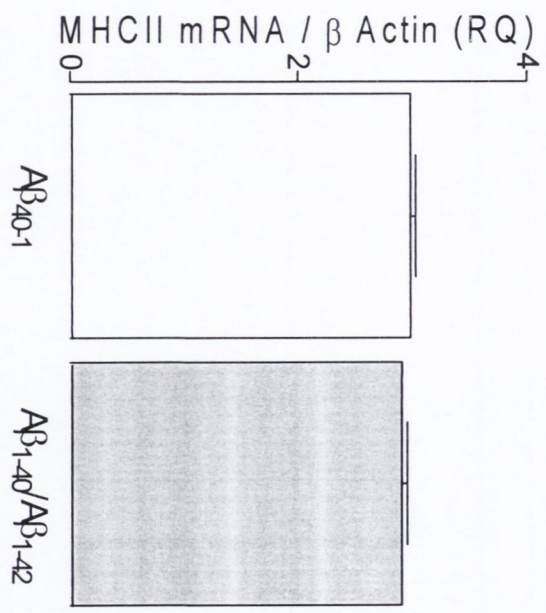
C.





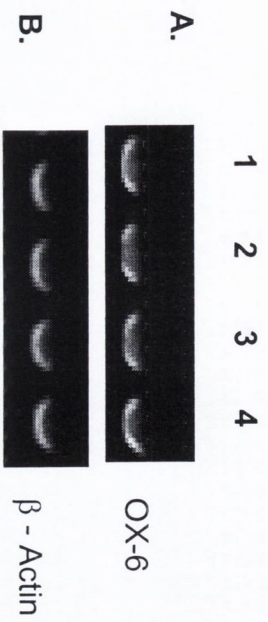
**Figure 4.9 Acute  $A\beta_{1-40}/A\beta_{1-42}$  administration does not increase MHCII mRNA expression**

MHCII mRNA expression was not significantly affected in hippocampal tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) for four hours when compared with hippocampal tissue prepared from rats that received  $A\beta_{40-1}$ . Data expressed as the mean  $\pm$  SEM; n = 6. **Note:** MHCII mRNA expression was assessed in this tissue by QPCR all other MHCII mRNA results were assessed by RT-PCR.

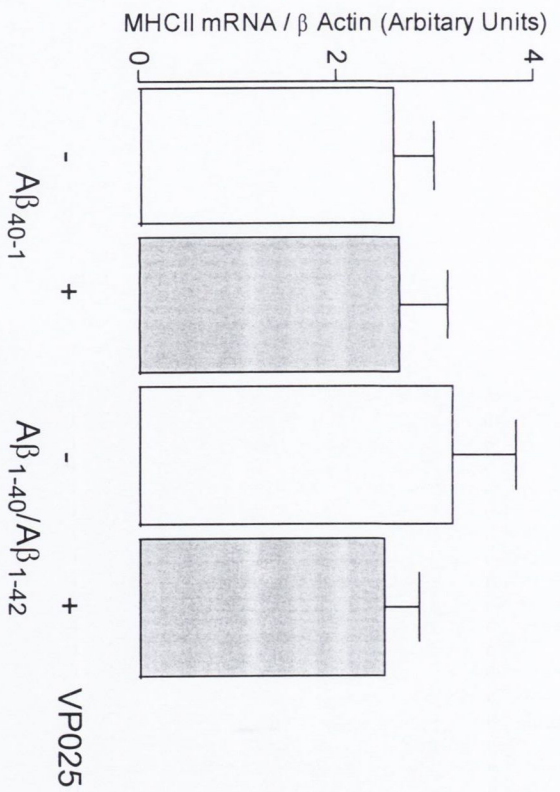


**Figure 4.10 MHCII mRNA expression in not altered by 8 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

MHCII mRNA expression was not significantly affected in hippocampal tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8 days when compared with hippocampal tissue prepared from rats that received  $A\beta_{40-1}$ . VP025 exerted no effect in this experiment. Data expressed as the mean  $\pm$  SEM; n = 6.



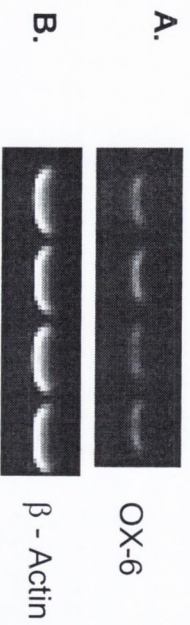
**C.**



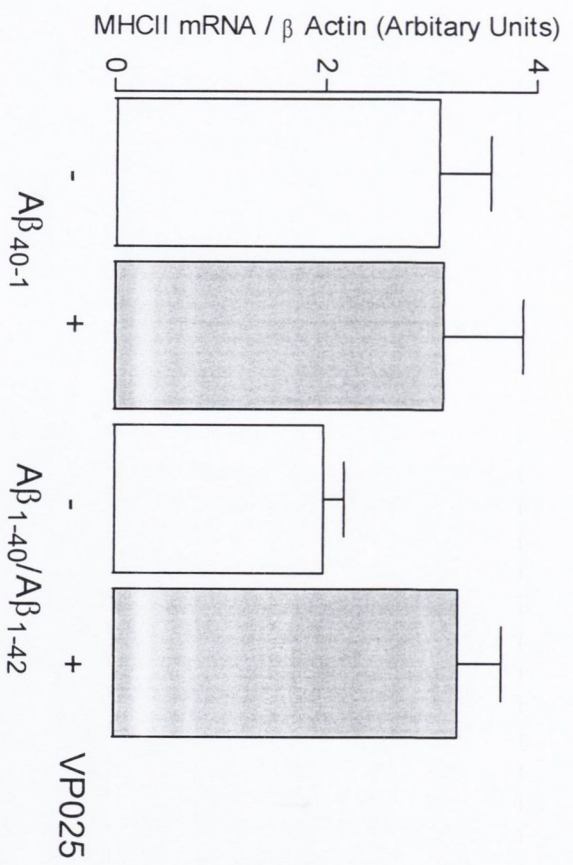
**Figure 4.11 MHCII mRNA expression is not altered by 28 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

MHCII mRNA expression was not significantly affected in hippocampal tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 28 days when compared with hippocampal tissue prepared from rats that received  $A\beta_{40-1}$ . VP025 exerted no effect in this experiment. Data expressed as the mean  $\pm$  SEM; n = 6.

1 2 3 4



**C.**



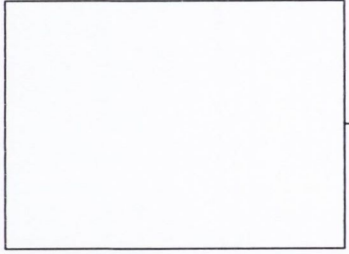
**Figure 4.12 Acute  $A\beta_{1-40}/A\beta_{1-42}$  injection did not alter hippocampal IL-1 $\beta$  concentration**

Interleukin-1 $\beta$  (IL-1 $\beta$ ) concentration was similar in hippocampal homogenate prepared from rats which received  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) and rats which were treated with  $A\beta_{40-1}$ . Values are expressed as pg IL-1 $\beta$  / mg tissue corrected for protein and are the mean  $\pm$  SEM; n = 6. The effect of VP025 pre-treatment was not assessed in this experiment.

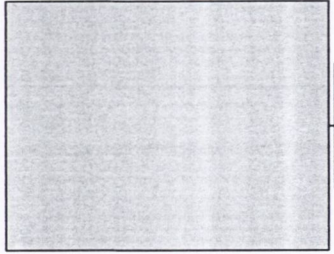
Hippocampal IL-1 $\beta$  (pg/mg)

200  
100  
0

AB<sub>40-1</sub>



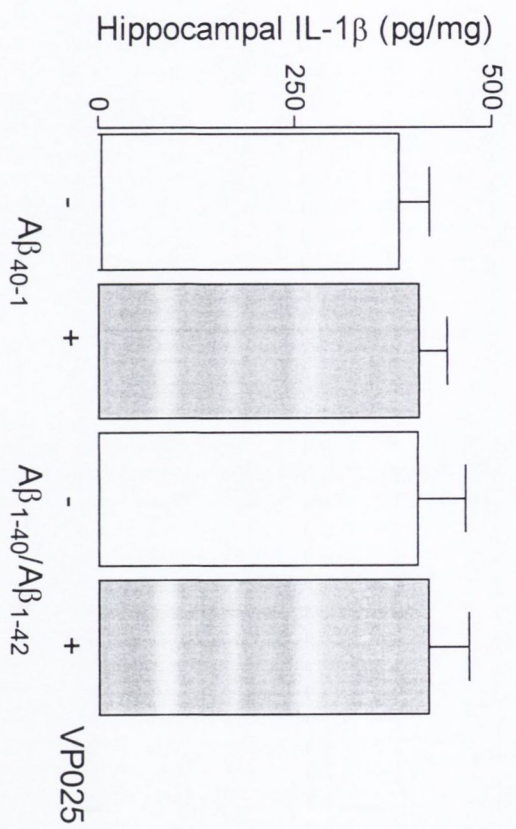
AB<sub>1-40</sub>/AB<sub>1-42</sub>





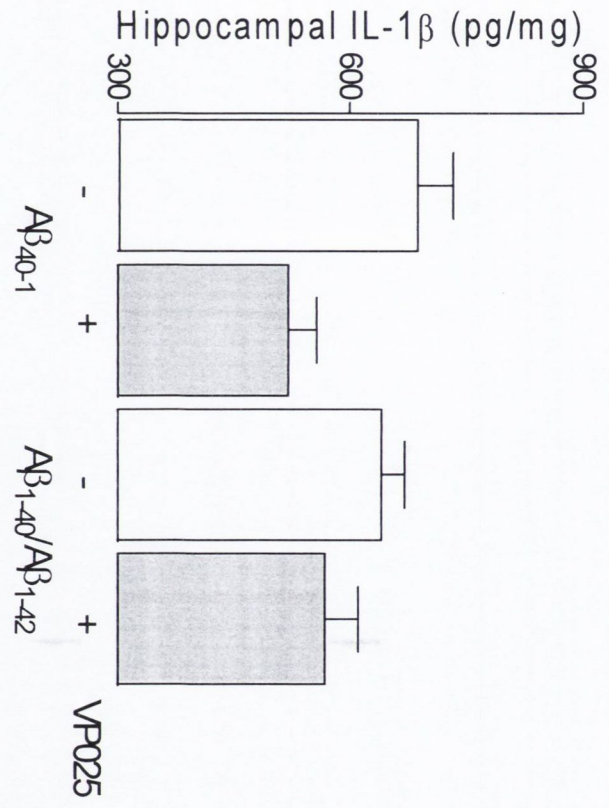
**Figure 4.13 Infusion of  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days did not alter hippocampal IL-1 $\beta$  concentration**

Interleukin-1 $\beta$  (IL-1 $\beta$ ) concentration was similar in hippocampal homogenate prepared from rats which received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8 days and rats which were treated with  $A\beta_{40-1}$ . VP025 did not exert an effect in this experiment. Values are expressed as pg IL-1 $\beta$  / mg tissue corrected for protein and are the mean  $\pm$  SEM; n = 6.



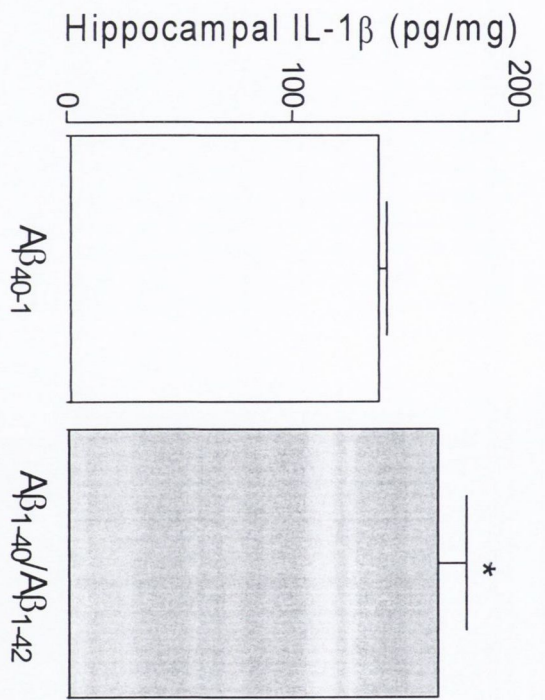
**Figure 4.14 Infusion of  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days did not alter hippocampal IL-1 $\beta$  concentration**

Interleukin-1 $\beta$  (IL-1 $\beta$ ) concentration was similar in hippocampal homogenate prepared from rats which received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 20 days and rats which were treated with  $A\beta_{40-1}$ . VP025 did not exert an effect in this experiment. Values are expressed as pg IL-1 $\beta$  / mg tissue corrected for protein and are the mean  $\pm$  SEM; n = 6.



**Figure 4.15 Infusion of  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days significantly increases hippocampal IL-1 $\beta$  concentration**

Interleukin-1 $\beta$  (IL-1 $\beta$ ) concentration was significantly increased in hippocampal homogenate prepared from rats which received  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 28 days when compared with rats which were treated with  $A\beta_{40-1}$  (\* $p$ <0.05; ANOVA). Values are expressed as pg IL-1 $\beta$  / mg tissue corrected for protein and are the mean  $\pm$  SEM; n = 6. The efficacy of VP025 pre-treatment was not assessed in this experiment.





The main objective of this study was to establish the underlying cause of the A $\beta$ -induced impairment in LTP, focusing on a possible role for microglial activation. Therefore the expression of a number of phenotypic markers of microglial activation was examined to establish whether persistent A $\beta$ -induced microglial activation could contribute to the observed neuronal dysfunction. To this end CD86 and ICAM-1 protein and MHCII mRNA expression were assessed in hippocampal tissue from the various treatment groups. CD86 is expressed on microglia and provides the co-stimulatory signal necessary for T cell activation, its principle mode of action is to bind CD28 on T cells. Along with CD80, CD86 molecules provide the required stimulus to prime T cells against antigens such as A $\beta$  peptides engulfed by activated microglia. ICAM-1, along with its receptor LFA-1, is known to play a key role in inflammatory processes. Combined, these cell surface proteins facilitate the recruitment of macrophage to sites of inflammation. MHCII molecules are found only on a few specialised APC types including microglia. APCs are expected to present 'foreign or deleterious' material via the MHCII complex to T cells thereby driving an immune response. The peptides presented by class II molecules are derived from endocytosed extracellular proteins such as A $\beta$  peptide, as well as cytosolic self proteins. These proteins are digested and bound by the APC to the MHCII molecule prior to the molecules migration to the plasma membrane, making the pathway of peptide loading onto MHCII of critical relevance in the regulation of immunological responses and immune self-tolerance (Dani *et al.*, 2004).

The data indicate that acute administration of A $\beta_{1-40}$ /A $\beta_{1-42}$  (200 $\mu$ M) increases CD86 and ICAM-1 protein expression but not MHCII mRNA in rat hippocampus. The expression of CD86 and ICAM-1 was also examined in hippocampal tissue prepared from rats chronically infused with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) in the absence or presence of VP025. Both CD86 and ICAM-1 expression were significantly increased in hippocampus of rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 8 and 20 but not 28 days. VP025 pre-treatment seemed to exert little effect in modulating the A $\beta$ -induced increase in expression of CD86. ICAM-1 expression differs from that of CD86 in that it appears to be modulated by VP025 pre-treatment; it was reduced greatly, albeit not significantly, in VP025 pre-treated hippocampal tissue prepared from rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 days and was significantly decreased in hippocampal tissue prepared from



rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) for 20 days. Findings by Apelt and colleagues (2002) provide some support for these results. These authors examined the relationship between A $\beta$ -mediated microgliosis and ICAM-1. Using immunocytochemistry in a Tg2576 mouse model they demonstrated a diffuse immunostaining of ICAM-1 in the corona around fA $\beta$  plaques and an up-regulation of ICAM-1 in activated microglial cells located in close proximity to the plaques.

It is interesting to note that MHCII mRNA in rat hippocampus showed no response to either acute or chronic A $\beta$ -treatment. It is generally accepted that up-regulation of MHCII by microglia is needed for them to rescue neural tissue from a potentially harmful situation; including the toxicity they themselves can cause (Schwartz *et al.*, 2003). However, Butovsky and colleagues (2005) found that, in their hands at least, a pro-inflammatory environment in the CNS could confer 'protective autoimmunity' by dampening MHCII expression. They reported that T cells in the human AD affected brain rendered microglia neutral in response to aggregated A $\beta$ , and signal transduction pathways activated by A $\beta$  suppressed genes associated with MHCII expression in microglia. They authors argued that microglia in this state are potentially harmful to neural tissue in that they fail to engulf 'self compounds' such as A $\beta$  peptides. Not only this, these non-activated microglia exacerbate the process long-term by evading local interaction with T cells because of their failure to express MHCII.

Despite the phenotypic evidence for microglial activation following 8 or 20 days A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> infusion, i.e. the increases in CD86 and ICAM-1 protein expression presented here, there is no evidence of an increase in IL-1 $\beta$  protein at these time points. This raises the question of whether an inflammatory effect is generated by A $\beta$  administration over this time period. It has consistently been shown that microglia are the most likely source of inflammatory mediators like IL-1 $\beta$  and that IL-1 $\beta$  has a negative impact on LTP (Murray & Lynch, 1998; Meda *et al.*, 2001). Data from this laboratory has consistently shown that IL-1 $\beta$  can also be released from neurons, albeit to a lesser extent. However, other pro-inflammatory cytokines like IL-6, TNF $\alpha$  and IFN $\gamma$  also inhibit LTP (D'Arcangelo *et al.*, 1991; Li *et al.*, 1997; Wang *et al.*, 2005) and the evidence, at least in the case of IL-6 and TNF $\alpha$ , is that they are released from activated

microglia (Hwang *et al.*, 2006; Li *et al.*, 2007). The lack of concordance between the A $\beta$ -induced changes in ICAM-1 and CD86 protein and MHCII mRNA, following 8 and 20 days infusion, was unexpected. One possible explanation for the MHCII result is that changes in MHC mRNA may not parallel changes in its protein expression; recent unpublished data from this lab supports this hypothesis. Immunohistochemical staining for MHCII showed a greater intensity in MHCII expression in the hippocampus of rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 28 days compared with control-treated animals (Belinda Grehan, personal communication).

In addition, the lack of a parallel change in these markers of microglial activation and IL-1 $\beta$  requires investigation. Up-regulation of CD86, ICAM-1 and MHCII, typically thought to reflect microglial activation, may not be associated with cytokine release in this model. Again, unpublished data from this lab supports this hypothesis. Immunohistochemistry has shown positive staining for CD68 in the hippocampus of rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 28 days compared with control-treated animals. CD68 is a macrophage marker related to lysosomal glycoproteins whose expression is up-regulated during phagocytosis. It is also recognized as having low constitutive cell surface expression which is significantly up-regulated on cell stimulation (Ramprasad *et al.*, 1996). Significantly, immunohistochemical staining for CD68 showed co-localization with MHCII in the hippocampus of rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 28 days compared with control-treated animals, linking microglial activation (CD86/ICAM-1) and phagocytosis (CD68) (Belinda Grehan, personal communication) to chronic A $\beta_{1-40}$ /A $\beta_{1-42}$  treatment. This question is currently being further investigated in the laboratory using FACS analysis to triple label cells for surface markers and intracellular cytokines. In addition, recent findings from this lab have also shown that an imbalance between pro- and anti-inflammatory cytokines in hippocampus, rather than absolute concentration of IL-1 $\beta$ , is more robustly correlated with LTP (Nolan *et al.*, 2005; Maher *et al.*, 2006), in this light, tissue prepared from animals treated acutely with A $\beta$  or chronically for 8 or 20 days need to be further examined.

Interestingly, in contrast to hippocampal tissue prepared from rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 or 20 days there is a significant increase in IL-1 $\beta$  protein in the hippocampus of rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  at a similar concentration for 28 days.

Historically the etiology of neurodegenerative disorders varies greatly but increased IL-1 $\beta$  appears to be a common link in processes leading to neuronal death (Rothwell *et al.*, 1997; Loddick *et al.*, 1997). It is postulated that IL-1 $\beta$  and other inflammation-related molecules contribute to the complex of destructive processes that result in a progressive loss of neuronal structures (Hanisch *et al.*, 2001; McGeer and McGeer, 2001). The contribution of inflammation to the etiology of AD is based on evidence that the prevalence of the disease is markedly reduced in patients under NSAID-treatment (McGeer and McGeer, 2007).

It has only recently been appreciated that microglia exhibit an array of phenotypes and that these may be controlled by the immediate local environment (Goerdts and Orfanos; 1999, Gordon; 2003). Evidence from this lab has suggested that acute A $\beta$  treatment increases microglial activation as revealed by increased cytokine production in rat hippocampus (Minogue *et al.*, 2003; Lyons *et al.*, 2007; Clarke *et al.*, 2007). This augmentation in microglial activity was accompanied by impairment in LTP and was responsive to various anti-inflammatory strategies. Cytokine levels have also been shown to correlate with amyloid load in transgenic mouse models of AD (Patel *et al.*, 2005). Whereas previous studies have linked a decrease in LTP with an increase in IL-1 $\beta$ , the present data fail to provide evidence for this association.

Examination of the results from this study indicates that there may well be a temporal element governing the expression of phenotypic markers of microglial activation such as CD86 and ICAM-1 as well as IL-1 $\beta$  production. Correlation analysis of the data, fails however, to establish a link between increased CD86 and ICAM-1 expression in hippocampus and impairment in LTP in dentate gyrus following chronic A $\beta$  administration. Similar correlative analysis fails to establish a link between alteration of ICAM-1 expression by VP025 pre-treatment and the observed VP025 reversal of A $\beta$ -induced impairment in LTP maintenance. This finding is surprising as VP025 pre-treatment has been shown previously to successfully decrease CD40 expression in hippocampal preparations from aged rats, as well as restore age associated attenuation in LTP in these rats. In addition, VP025 pre-treatment decreases JNK expression and IL-1 $\beta$  and IFN- $\gamma$  production in hippocampus of aged and LPS-treated rats (Martin *et al.*, 2005, unpublished data and by personal communication), indicating that VP025 pre-treatment

can manipulate neuronal-microglial interaction and decrease both pro-inflammatory microglial activation and output in rat brain. As VP025 does not alter CD86 and ICAM-1 expression in parallel in these experiments, this may indicate that VP025 pre-treatment may alter ICAM-1 expression on cells other than microglia in the hippocampus, such as blood vessel epithelial cells.

What has been ignored to date is the fact that the major function of ICAM-1 is one of adhesion molecule, anchoring, not just microglia and T cells but many cell types, thereby facilitating intracellular or indeed trans BBB signalling. It is possible that VP025 treatment alters cell adhesion and connectivity between cells of the CNS in times of inflammation, thereby facilitating control of the inflammatory response. In the case of ICAM-1, by diminishing its expression, VP025 dampens the pro-inflammatory response in the CNS either by preventing migration of pro-inflammatory cell from the periphery, or proliferation of pro-inflammatory signal between endogenous cells of the CNS. Unpublished data from this laboratory supports the idea that VP025 may play an immunomodulatory role in the CNS in that it has been shown to modulate connectivity between cells with respect to inflammatory signalling. Martin and colleagues (unpublished) have shown that VP025 pre-treatment increased CD200 ligand expression in the hippocampus of aged, LPS-treated and A $\beta_{1-40}$  (200 $\mu$ M)-treated rats. The role of CD200 ligand, which is expressed on neurons, is to bind its receptor on microglia, on receptor binding a series of intracellular signalling cascades and gene expression is initiated to down-regulate the inflammatory response in microglia (Lyons *et al.*, 2007). The data most certainly indicates however, that in these experiments, VP025 treatment affects neuronal viability and synaptic function in hippocampus, by means other than direct manipulation of antigen presentation by microglia. A putative role for VP025 in preservation of neuronal functioning will be more extensively discussed in the following chapters.

There is plenty of evidence in the literature to support temporal governance of changes in expression of markers of microglial activation. Examining the temporal activation of microglia in response to A $\beta$ , Sasaki and colleagues (1997) investigated the relationship between MHCII activation and A $\beta$  protein deposition in post mortem tissue in AD brains. Using single and double staining techniques against A $\beta$  and various

markers of microglial activation they found that middle-aged non-demented subjects had small amounts of cerebral A $\beta$  deposits in diffuse plaques. Despite 70% of these plaques containing microglia, no evidence for microglial activation was found in this tissue. In age-matched AD affected brains double staining for A $\beta$  and MHCII showed that only 20% of diffuse plaques contained minimal activated microglia but that this increased with severity of the disease. It was argued that microglial activation is absent in the early stages of the disease despite the presence of A $\beta$ , and that activation only occurs as plaques mature from diffuse to primitive entities i.e. when amounts of A $\beta$  deposits and the degree of neuritic change increases.

Baker and colleagues (1999) also found that expression of markers of microglial activation, in their case; cathepsin S and CCR5, are differentially regulated in various *in vivo* models of Creutzfeldt-Jakob disease (CJD). Similar to AD, CJD is also thought to have its origins in the mis-processing and accumulation of protein, in this instance prion protein. The authors report that the onset and peak magnitude of up-regulation in cathepsin S and CCR5 to be protocol dependent. Depending on the CJD experimental paradigm used increases in cathepsin S and CCR5 either occurs before or after major accumulation of prion protein. These findings may suggest a link between the aggregation state of peptides and the temporal reaction of microglia to a foreign peptide.

Chen and colleagues (2004), using multivariate analysis of G93A SOD1 mice, examined gene expression in 21 different genes governing many areas associated with the progressive neurodegenerative disorder ALS. Mutant animals of this kind are symptomatic at approximately 12 weeks and die at 18 – 20 weeks of age. Activation of genes governing CD86 expression was observed in pre-symptomatic mice as early as 6 weeks whereas gene activity governing IL-1 production was not altered until week 15 when the mice were fully symptomatic. Overall these data suggest that experimental evidence of microglial activation is dependent on the marker chosen and on the model under investigation, with different phenotypic markers of activation being expressed at different times or not at all depending on the experimental protocol used and extent of CNS injury.

Differential expression in cell surface markers, such as CD86 or ICAM-1, on microglia reflects the changes in their activity with time and could account for some of

the results seen in the current experiments. Hanisch and colleagues (2001) argue that it is possible to inhibit cytokine release from microglia by blocking signaling pathways without affecting other activation parameters in these cells. In the present study it is possible that immediate or early exposure of microglia to A $\beta$  leads to reactivity in these cells as they attempt to eliminate the foreign A $\beta$  peptide from the CNS by phagocytosis. As the A $\beta$  infusion continues it is possible that the clearance of A $\beta$  cannot be sustained with the microglia switching phenotype to that of cytokine producer. The work of Hanisch and colleagues (2002) lend credence to this theory. They speculate that the phagocytic machinery of microglia is paralyzed by augmented cytokine production and that increasing toxicity in the CNS condemns microglia to an inflammatory response exacerbating the severity in pathology of chronic neurodegenerative diseases such as AD. Koenigsnecht-Talboo and Landreth (2004) report that pro-inflammatory cytokines, such as IL-1 $\beta$ , attenuate microglial phagocytosis stimulated by fibrillar A $\beta$  and argue that this may, in part at least, contribute to the accumulation of fibrillar A $\beta$  containing plaques in the AD brain. The inhibition of phagocytic activity by A $\beta$  was relieved, in their hands at least, by co-incubation with anti-inflammatory cytokines, findings that support anti-inflammatory strategies as therapies for AD. In addition, Koenigsnecht-Talboo and Landreth (2005) reported that inhibition of microglial-phagocytosis by pro-inflammatory cytokines was elicited by engagement of the A $\beta$  receptor complex and complement receptor 3 (CR3). Interestingly, Perry and colleagues (2006) also report a lack of *in vivo* evidence for cytokine production (IL-1 $\beta$ , IL-6 or TNF $\alpha$ ) in their mouse model of prion disease. Prion disease is another neurodegenerative disorder whose etiology is based in the misfolding of amyloid proteins.

There is much evidence in the literature showing cells of the macrophage lineage (which include microglia) capable of compartmentalizing their functionality in an environment-specific manner. Indeed results from this lab show microglial responses to stressors to be stimulus specific. Microglia appear to respond to A $\beta$ -treatment *in vitro* by increasing phagocytosis and suppressing cytokine production but their response to H<sub>2</sub>O<sub>2</sub> (which induces oxidative stress) appears to be the reverse (O'Reilly, personal communication). The increase in ICAM-1 and CD86 expression observed in this study indicate up-regulation in microglial activation with respect to antigen presentation.

Adhesion molecules functionally represent cell surface receptors that enable cell–cell and cell–extracellular matrix interactions. The phagocytosis of damaged neurons, foreign material, and neuronal regeneration, requires the interaction of microglial cells with degenerating axons and neuronal somata, this is known to be mediated by the expression of cell adhesion molecules such as ICAM-1 (Brown and Perry, 1998).

Fadok and colleagues (1998) suggest that the phagocytosis of damaged cells or debris by macrophage is a ‘quiet process’ that does not lead to production of inflammatory mediators. Indeed phagocytosis of apoptotic neutrophils actively inhibited the production of IL-1 $\beta$ , IL-8 and TNF $\alpha$  by human monocyte-derived macrophages while production of TGF- $\beta$  and PGE<sub>2</sub> was increased. The latter appeared to be involved in the inhibition of pro-inflammatory cytokine production because addition of exogenous TGF $\beta$  and PGE<sub>2</sub> resulted in the inhibition of LPS-stimulated cytokine production. It was suggested that binding and/or phagocytosis of detrimental material induces active anti-inflammatory or suppressive properties in human macrophages and the authors concluded that resolution of inflammation depends, not only on the removal of foreign material, but also on active suppression of inflammatory mediator production. These findings may reflect some of the processes occurring in the brains of rats chronically administered A $\beta$  over 8 or 20 days.

Townsend and colleagues (2005) also reported a time dependent phagocytic response in murine microglia challenged with A $\beta$ . This response was not associated with production of IL-1 $\beta$  or TNF $\alpha$ . Similar findings were reported by Minghetti and colleagues (2005) which saw microglia challenged not with A $\beta$  but with apoptotic cells. Depino and colleagues (2003) report a lack of cytokine production in the sub-acute intrastriatal 6-hydroxydopamine-rat model of PD. Immunohistochemistry showed that microglial cells still show signs of activation 6 – 30 days post-lesion in the substantia nigra pars compacta (SNpc). This microglial activation was accompanied by atypical pro-inflammatory cytokine production. IL-1 $\beta$  mRNA was found to be elevated (16 fold) 30 days post-injection but no concomitant induction of IL-1 $\beta$  at the protein level was observed. These data suggest that IL-1 $\beta$  expression is subjected to a tight control in the SNpc during the course of neurodegeneration. In the periphery, this observation has been documented repeatedly for other cell types such as monocytes or keratinocytes under a

variety of stimuli, such as calcium ionophores, adhesion, or exposure to C5a. (Dinarello, 1996). Cells which increase IL-1 $\beta$  mRNA expression, but not to translate it to IL-1 $\beta$  protein are considered to be 'primed' and small amounts of other stimuli rapidly trigger translation, usually resulting in higher than normal IL-1 $\beta$  production (Dinarello, 1996) In addition, Depino and colleagues argue that, IL-1 $\beta$  mRNA induction even if it were translated to protein, should have no dramatic functional consequences in the development of neuronal degeneration in their particular model in that IL-1 $\beta$  was shown to be expressed differentially only at 30 days, when 60% of neurons are already lost in the SNpc (Sauer and Oertel, 1994). It would be interesting to examine IL-1 $\beta$  mRNA expression in a similar light in the current A $\beta$  experiments.

The data show that chronic A $\beta$  administration causes an impairment in LTP in perforant path granules cells of the dentate gyrus which is relieved by VP025 anti-inflammatory therapy. However, analyses of hippocampal tissue from this study have produced results which show an 'atypical' response by microglia to prolonged infusion of A $\beta$  peptides into the rat brain. Except for hippocampal tissue prepared from rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 28 days there is a lack of evidence for any functional inflammatory output from microglia which would link their persistent activation to the A $\beta$ -induced impairment in LTP. These results support the notion that neuronal compromise *per se* does not induce secretion of pro-inflammatory cytokines but that an additional stimulus is necessary to stimulate pro-inflammatory cytokine production. The lack of functional pro-inflammatory output in our A $\beta$ -treated hippocampal tissue could be explained by *in vitro* findings reported by Kurosaka and colleagues (2003). The authors observed a lack of cytokine production by macrophage whilst they were actively phagocytosing apoptotic cells. The authors argue that a lack of cytokine production by macrophage during phagocytosis could be interpreted as an attempt by the host to maintain a natural immune homeostatic balance whilst dealing with a discrete issue. This process only becomes a problematic one if the existence of apoptotic cells or toxic entities persists due to defective clearance, in general terms this occurs mainly as a result of the host's system becoming chronically stressed and overwhelmed (Roos *et al.*, 2004).



The main objective of this study was to establish the underlying cause of the A $\beta$ -induced impairment in LTP, focusing on a possible role for microglial activation and persistent inflammation. The results highlighted in this discussion do not explain what exactly is occurring in the hippocampus of rats treated chronically with A $\beta$  but may go some way towards contextualizing the up-regulation of microglial activity in the absence of a definitive pro-inflammatory environment. In the absence of a typical inflammatory response in the rat hippocampus following A $\beta$  treatment we must look to other factors, such as A $\beta$ -associated increases in caspase activity, that could be affecting neuronal functioning in the rat brain, evidence for this will be discussed in the following chapter.

## **Chapter 5**

In the absence of a typical inflammatory response in the hippocampus we must look to other factors that may contribute to our observed A $\beta$ -stimulated impairment in LTP. Evidence suggests a link between widespread dystrophy in neuronal processes in contact with A $\beta$  deposits and the observed major alterations in cortical synaptic responses seen in dementia affected brain. Studies have shown that dying cells display the characteristics of apoptosis in AD brains and A $\beta$  peptides have also been shown to increase initiators and effectors of programmed cell death *in vivo* and *in vitro*. Not only this, A $\beta$  is thought to contribute to neuronal dysfunction and death by causing perturbations in neuronal membrane functioning by increasing the activation of a number of enzymes whose role it is to cleave integral membrane components.

In light of the lack of direct evidence implicating microglial driven inflammation in the compromise of LTP, this chapter investigates the effect of A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) in the rat cortex, with particular emphasis on its role played in caspase activation, as well as its role in the compromise of neuronal membrane integrity. The effects of A $\beta$  administration were examined in these experiments initially in cortex to avoid tissue wastage as hippocampal tissue was limited and many of the experimental assays were being used for the first time. In addition, as the effects of acute and chronic A $\beta$  administration on hippocampus had been limited with respect to the original experimental hypothesis it was then of interest to examine whether these particular experimental paradigms affected cortex rather than hippocampus or whether it was A $\beta$ -induced changes in cortex which was affecting hippocampal function.

The data shows both *in vivo* and *in vitro* an A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) or A $\beta_{1-42}$  related increase in caspase activation with a related decrease in cell viability and increase in cell death. These findings were accompanied by A $\beta_{1-40}$ /A $\beta_{1-42}$ -induced increases in sphingomyelinase and sPLA<sub>2</sub> activity in cortical tissue prepared from the cortex of rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 days, indicating that chronic A $\beta_{1-40}$ /A $\beta_{1-42}$  administration disturbs the integrity of neuronal membranes *in vivo*. *In vitro* experiments strengthen these findings, showing that A $\beta$  (10 $\mu$ M) or ceramide (100 $\mu$ M) (a bioactive lipid product of sphingomyelin cleavage) treatment directly impairs cell viability and increases the activation of the executioner caspase, caspase-3. Use of a neutral SMase

inhibitor GW4869, showed these results to be sphingomyelinase dependent. These data are consistent with the idea that A $\beta$  peptides have negative effects on neuronal function and could provide evidence supporting the notion that A $\beta$  can contribute to inhibition of LTP from outside the hippocampus. Significantly, VP025 exerted a neuroprotective role in these experiments in that it reversed the A $\beta_{1-40}$ /A $\beta_{1-42}$  -associated increases in caspase-8, caspase-3 and sphingomyelinase activation observed in cortical tissue.

## 5.1 The amyloid- $\beta$ -induced increase in caspase-8 in cortex is reversed by VP025 pre-treatment

Caspases are cysteine proteases, specific for aspartic residues, and are thought to be key players in the initiation and execution of cell death.  $A\beta$  is thought to participate in the neurodegeneration associated with AD contributing to the morphological and biochemical changes characteristic of apoptosis including the induction of caspase activation. Caspase-8 is known as an apoptotic initiator caspase. Figure 5.1 shows that caspase-8 activation was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 days ( $1360 \pm 11.58\text{pmol pNA produced/min/mg}$ ; \*\*\*  $p < 0.001$ ; ANOVA; Figure 5.1) compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $845.5 \pm 13.04\text{pmol pNA produced/min/mg}$ ). Caspase-8 activation was also significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) ( $1348 \pm 73.53\text{pmol pNA produced/min/mg}$ ; \*\*\*  $p < 0.001$ ; ANOVA; Figure 5.2) for 20 days compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $912 \pm 59.11\text{pmol pNA produced/min/mg}$ ). Similarly, caspase-8 activation was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days ( $63.8\mu\text{M}$ ) ( $3127 \pm 56.47\text{pmol pNA produced/min/mg}$ ; \*\*\*  $p < 0.001$ ; ANOVA; Figure 5.3) compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $2574 \pm 29.23\text{pmol pNA produced/min/mg}$ ). VP025 treatment significantly reduced caspase-8 activation in cortical tissue, so that activity in cortical tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 ( $911.9 \pm 16.28\text{pmol pNA produced/min/mg}$ ; ANOVA; Figure 5.1), 20 ( $731.9 \pm 53.58\text{pmol pNA produced/min/mg}$ ; ANOVA; Figure 5.2) and 28 days ( $2833 \pm 52.3\text{pmol pNA produced/min/mg}$ ; ANOVA; Figure 5.3) was significantly decreased compared with tissue obtained from animals treated with  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) alone ( $^{+++}p < 0.001$ ; ANOVA; Figure 5.1, 5.2, 5.3). Data are expressed as means  $\pm$  SEM;  $n = 5 - 6$ .

## 5.2 The amyloid- $\beta$ -induced increase in caspase-3 activation is reversed by VP025 pre-treatment

Activation of caspase-3 which is known as an apoptotic effector caspase was also assessed in tissue prepared from these rats and also in rats treated acutely with A $\beta$ . Figure 5.4 shows that caspase-3 activation was significantly increased in cortical tissue prepared from rats that received an acute injection of A $\beta_{1-40}$ /A $\beta_{1-42}$  (200 $\mu$ M) ( $809.7 \pm 5.1$  pmol pNA produced/min/mg; \*\*\*  $p < 0.001$ ; t-test; Figure 5.4) compared with tissue prepared from rats that received A $\beta_{40-1}$  ( $771.0 \pm 6.26$  pmol pNA produced/min/mg). Caspase-3 activation was also significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 days ( $799.5 \pm 11.68$  pmol pNA produced/min/mg; \*\*\*  $p < 0.0001$ ; ANOVA; Figure 5.5) compared with tissue prepared from saline-treated rats that received A $\beta_{40-1}$  ( $604 \pm 21.7$  pmol pNA produced/min/mg). Similarly, caspase-3 activation was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 20 days ( $1936 \pm 161.4$  pmol pNA produced/min/mg; \*\*  $p < 0.01$ ; ANOVA; Figure 5.6) and 28 days ( $1863 \pm 54$  pmol pNA produced/min/mg; \*\*\*  $p < 0.001$ ; ANOVA; Figure 5.7) compared with tissue prepared from saline-treated rats that received A $\beta_{40-1}$  ( $1049 \pm 127$ ;  $1370 \pm 30.08$  pmol pNA produced/min/mg respectively). VP025 treatment significantly reduced caspase-3 activation, so that activity in cortical tissue prepared from rats that received A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 ( $678 \pm 24.99$  pmol pNA produced/min/mg; ANOVA; Figure 5.5), and 28 days ( $1548 \pm 22.64$  pmol pNA produced/min/mg; ANOVA; Figure 5.7) was significantly decreased compared with tissue prepared from control rats that received A $\beta_{40-1}$  ( $^{+++}p < 0.001$ ;  $^+p < 0.05$ ). VP025 did not significantly reduce caspase-3 activation in rats treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 20 days ( $1836 \pm 162.2$  pmol pNA produced/min/mg; ANOVA; Figure 5.7). Data are expressed as means  $\pm$  SEM;  $n = 5 - 6$ .

### **5.3 VP025 reverses the amyloid- $\beta$ -induced increase in cortical sphingomyelinase activity**

Ceramide is a second messenger involved in many biochemical events, which occur during cell senescence, and its concentration is increased in the AD brain. Endogenous ceramide is generated by hydrolysis of sphingomyelin by the action of sphingomyelinase and the evidence indicates that neutral sphingomyelinase generates the

signaling-active ceramide implicated in cell death. Sphingomyelinase activation was measured in cortical homogenate prepared from adult rats and figure 5.8 indicates that activity was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 days ( $558.5 \pm 13.29\text{mU/ml}$ ; \*  $p < 0.05$ ; ANOVA) compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $481.7 \pm 11.27\text{mU/ml}$ ). However enzyme activity was not significantly increased in cortical tissue prepared from control rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 20 days ( $1069 \pm 132.2\text{mU/ml}$ ; Figure 5.9) compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $828.2 \pm 62.8\text{mU/ml}$ ) but it was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 28 days ( $834.8 \pm 34.2\text{mU/ml}$ ; \*  $p < 0.05$ ; ANOVA; Figure 5.10) when compared with tissue prepared from control rats which received  $A\beta_{40-1}$  ( $735.6 \pm 43.6\text{mU/ml}$ ). VP025 treatment significantly reduced sphingomyelinase activation, so that activity in cortical tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 days was significantly decreased compared with tissue prepared from control rats that received  $A\beta_{40-1}$  ( $467.9 \pm 7.56\text{mU/ml}$ ;  $^{++}p < 0.001$ ; ANOVA; Figure 5.8). VP025 pre-treatment failed to exert any significance in cortical tissue prepared from rats that received  $A\beta$ -treatment for 20 or 28 days. Data are expressed as means  $\pm$  SEM;  $n = 4 - 6$ .

#### **5.4 Amyloid- $\beta_{1-40}/\text{amyloid}\beta_{1-42}$ induces an increase in cortical secretory phospholipase $A_2$ activity**

$PLA_2$  belongs to a family of enzymes that catalyze the cleavage of fatty acids in phospholipids. The phospholipases are involved in complex signaling that link receptor agonists, oxidative agents and pro-inflammatory cytokines to the release of arachidonic acid and the synthesis of eicosanoids and have been implicated in apoptotic processes. Figure 5.11 shows that  $PLA_2$  activation was significantly increased in cortical preparations obtained from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8 days ( $55.68 \pm 4.62\text{U/ml}$ ) but not 20 or 28 days compared with tissue prepared from saline treated rats that received  $A\beta_{40-1}$  ( $42.55 \pm 3.87\text{U/ml}$ ;  $^{**}p < 0.005$ ; ANOVA; Figures 5.11,

5.12, 5.13). VP025 pre-treatment failed to exert any effect on enzyme activation. Data are expressed as means  $\pm$  SEM; n = 4 - 6.

### **5.5 Amyloid- $\beta_{1-42}$ and ceramide treatment decrease cell viability *in vitro***

To determine whether A $\beta$  peptides, and ceramide, are directly capable of inducing cell death, preparations enriched for cortical neurons were pre-treated with VP025 for 1h and then treated for 24 hours with A $\beta_{1-42}$  (10 $\mu$ M) or ceramide (100 $\mu$ M). Cell viability was assessed using the MTS assay, which measures the efficiency of mitochondrial functioning in a cell. Figure 5.12 shows that neuronal viability was significantly decreased in cortical cells treated with A $\beta_{1-42}$  ( $0.15 \pm 0.004$ Abs490nm) or ceramide ( $0.10 \pm 0.005$ Abs490nm) compared with (DMSO) control-treated cortical neurons ( $0.23 \pm 0.012$ Abs490nm) (\*\*\*/§§§p < 0.001; ANOVA; Figure 5.14). VP025 pre-treatment exerted no significant effect in this experiment. Data are expressed as means  $\pm$  SEM; n = 7 - 12.

### **5.6 Ceramide increases interleukin-1 $\beta$ release *in vitro***

To determine whether this impairment in cellular function was accompanied by cytokine release, IL-1 $\beta$  expression was measured in supernatants taken from A $\beta$ - or ceramide-treated cortical neurons. Figure 5.15 shows that release of IL-1 $\beta$  was only significantly increased in cortical neurons treated with ceramide ( $122.0 \pm 46.49$ pg/ml) compared with (DMSO) control-treated neurons ( $18.61 \pm 7.9$ pg/ml; \*p < 0.05; ANOVA). Although elevated, the increase in IL-1 $\beta$  release induced with A $\beta$  treatment, did not reach statistical significance ( $56.58 \pm 11.08$ pg/ml). VP025 pre-treatment exerted no significant effect in this experiment. Data are expressed as means  $\pm$  SEM; n = 3 - 5.

### **5.7 Viability of cortical neurons is maintained with a sphingomyelinase inhibitor *in vitro***

A $\beta$ - and ceramide- treatment has been shown to decrease the viability of cortical neurons. Ceramide also causes a significant increase in their production of the pro-



inflammatory cytokine IL-1 $\beta$ . To determine whether sphingomyelinase activation might play a role in A $\beta$ -induced toxicity, a neutral sphingomyelinase inhibitor (GW4869) was co-incubated with A $\beta$ <sub>1-42</sub> for 24 hours. Analysis of the mean data shows that cell viability was significantly decreased in cortical neurons treated with A $\beta$ <sub>1-42</sub> (0.18  $\pm$  0.007Abs490nm) when compared to control-treated (DMSO) cortical neurons (0.24  $\pm$  0.02Abs490nm; \*p < 0.05; ANOVA; Figure 5.16). There was a significant increase in cell viability in cortical neurons co-treated with A $\beta$ <sub>1-42</sub> and the GW4869 when compared to those treated with A $\beta$ <sub>1-42</sub> alone (0.23  $\pm$  0.01Abs490nm; †p < 0.05; ANOVA; Figure 5.16). Data are expressed as means  $\pm$  SEM; n = 6.

### **5.8 Interleukin-1 $\beta$ release is unaffected by sphingomyelinase inhibition *in vitro***

Analysis of supernatant from these cells revealed that IL-1 $\beta$  was not significantly increased in cortical neurons treated with A $\beta$ <sub>1-42</sub> (60.16  $\pm$  21.15 pg/ml) compared with (DMSO) control-treated neurons (24.57  $\pm$  5.20 pg/ml; Figure 5.17). There was no significant change in IL-1 $\beta$  release in cells, which were incubated in the presence of GW4869 co-treatment (30.62  $\pm$  11.69 pg/ml). Data are expressed as means  $\pm$  SEM; n = 5 – 6.

### **5.9 Amyloid- $\beta$ <sub>1-42</sub> and ceramide treatment are associated with an increase in caspase-3 activity *in vitro***

Caspase-3 activity was assessed in cultured cortical neurons to investigate whether A $\beta$ <sub>1-42</sub> or ceramide exerted a similar effect on caspase-3 as on cell viability. Figure 5.18 shows that caspase-3 activity was significantly increased in cortical neurons treated with A $\beta$ <sub>1-42</sub> (785  $\pm$  30.86 pmol pNA produced/min/mg) or ceramide (659.6  $\pm$  31.93 pmol pNA produced/min/mg) compared with control-treated neurons (586.6  $\pm$  7.88 pmol pNA produced/min/mg; \*\*p < 0.001; ANOVA; §p < 0.05; t-test). Co-treatment with GW4869 significantly decreased the A $\beta$ <sub>1-42</sub>-induced increase in caspase-3 activation (628.4  $\pm$  18.3 pmol pNA produced/min/mg; ††p < 0.001; ANOVA). These findings

indicate that A $\beta$  and ceramide significantly affect the viability of cultured cortical neurons – an effect possibly mediated by sphingomyelinase activation since inhibition of this enzyme prevents an A $\beta$ -induced decrease in cell viability. Data are expressed as means  $\pm$  SEM; n = 5 – 6.

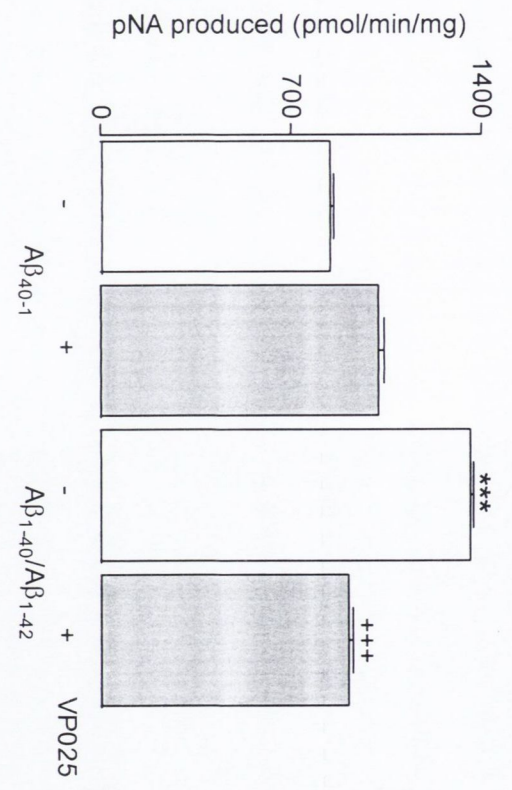


# **Chapter 5**

## **Figures**

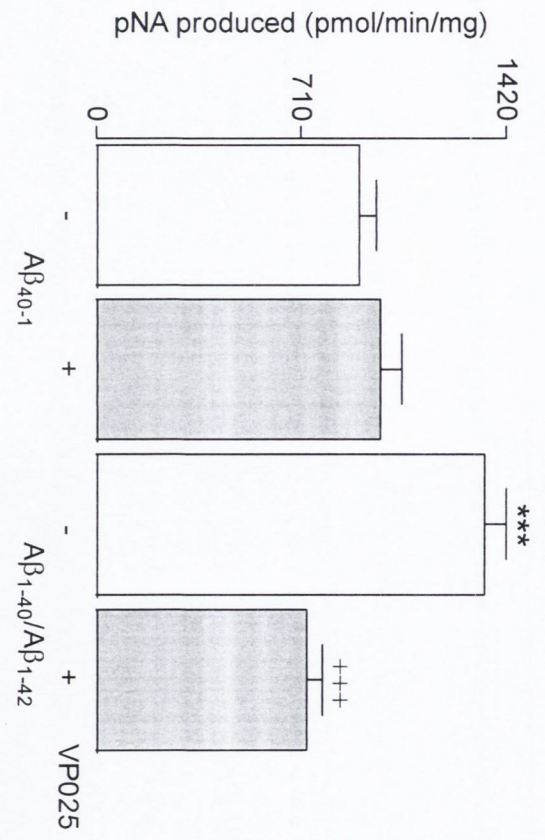
**Figure 5.1 VP025 reverses the increase in caspase-8 activity induced by 8 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

Caspase-8 activity was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days compared with tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (\*\* $p < 0.0001$ ; ANOVA). VP025 significantly attenuated the  $A\beta$ -induced increase in caspase-8 activity (+++ $p < 0.0001$ ; ANOVA). Data are expressed as the mean  $\pm$  SEM;  $n = 5 - 6$ .



**Figure 5.2 VP025 reverses the increase in caspase-8 activity induced by 20 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

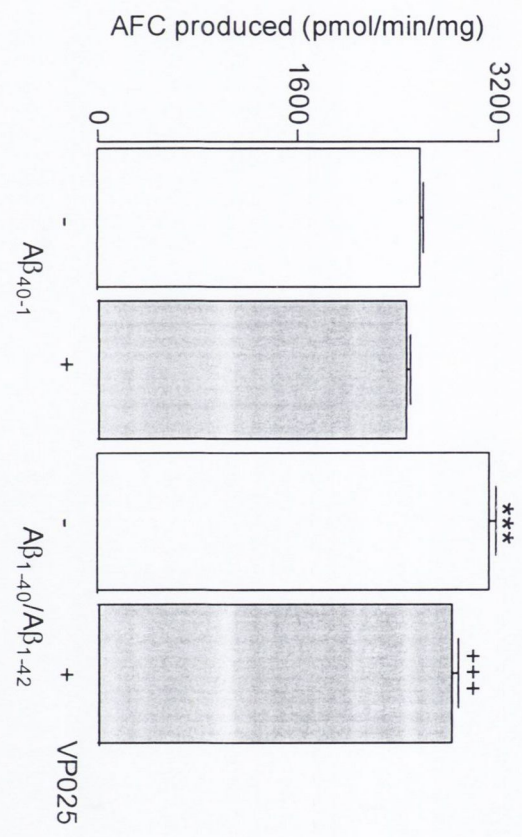
Caspase-8 activity was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub> (\*\*p < 0.001; ANOVA). VP025 significantly attenuated the A $\beta$ -induced increase in caspase-8 activity (+++p < 0.0001; ANOVA). Data are expressed as the mean  $\pm$  SEM; n = 5 – 6.





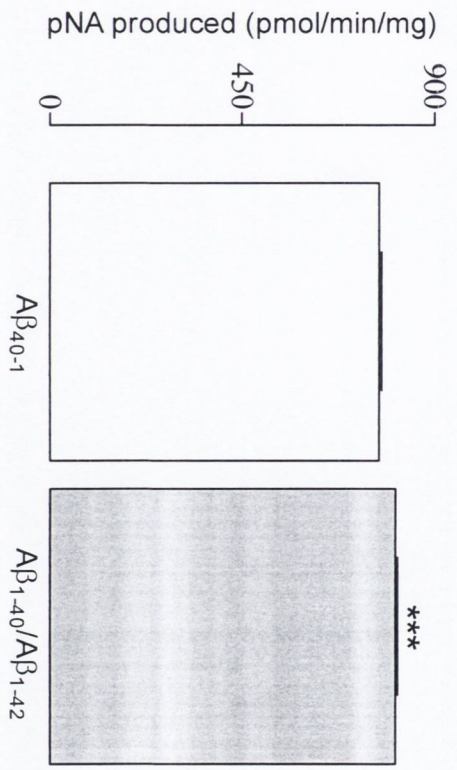
**Figure 5.3 VP025 reverses the increase in caspase-8 activity induced by 28 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

Caspase-8 activity was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days compared with tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (\*\* $p < 0.0001$ ; ANOVA). VP025 significantly attenuated the  $A\beta$ -induced increase in caspase-8 activity ( $^{+++}p < 0.0001$ ; ANOVA). Data are expressed as the mean  $\pm$  SEM;  $n = 5 - 6$ .



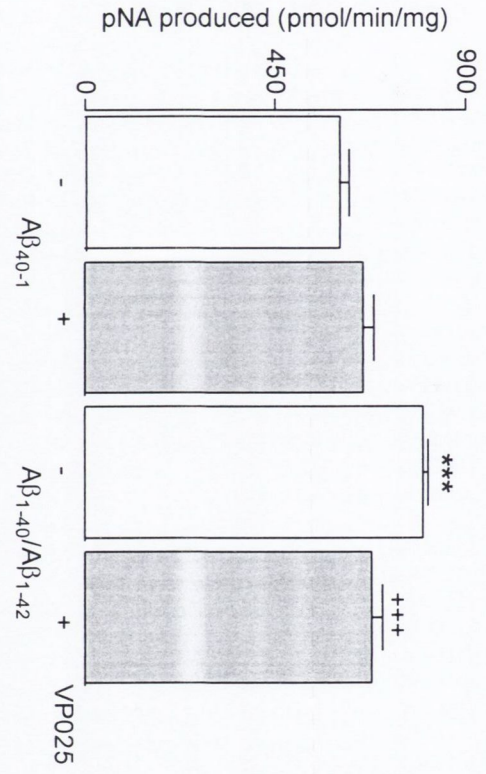
**Figure 5.4 Acute  $A\beta_{1-40}/A\beta_{1-42}$  infusion induces significant increases in cortical caspase-3 activation**

Caspase-3 activation was significantly increased in cortical tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 4 hours (\*\* $p < 0.001$ ; ANOVA) when compared with tissue prepared from rats that received  $A\beta_{40-1}$ . Data expressed as means  $\pm$  SEM;  $n = 6$ .



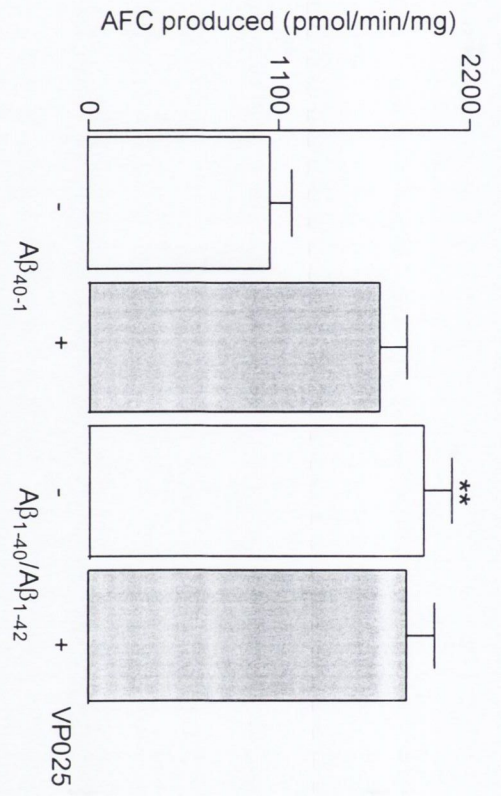
**Figure 5.5 VP025 reverses the increase in caspase-3 activity induced by 8 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

Caspase-3 activity was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days compared with tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (\*\* $p < 0.0001$ ; ANOVA). VP025 significantly attenuated the  $A\beta$ -induced increase in caspase-3 activity ( $+++p < 0.001$ ; ANOVA). Data are expressed as the mean  $\pm$  SEM;  $n = 5 - 6$ .



**Figure 5.6 VP025 does not reverse the increase in caspase-3 activity induced by 20 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

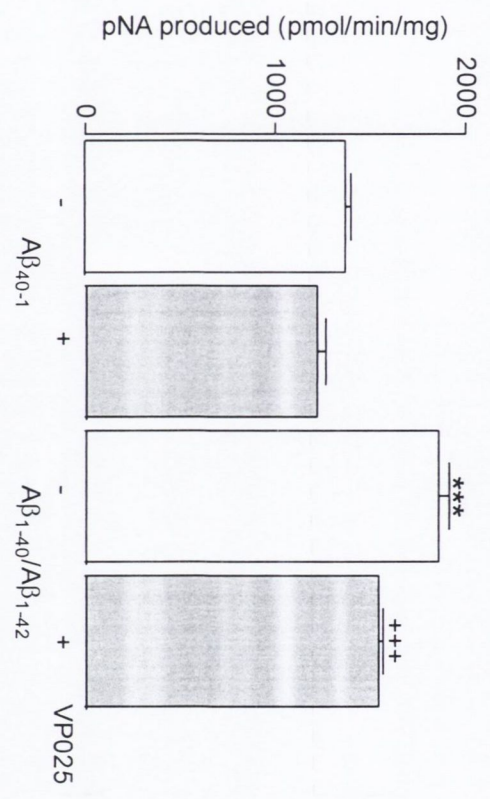
Caspase-3 activity was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 20 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub> (\*\*p < 0.01; ANOVA). VP025 pre-treatment did not attenuate the A $\beta$ -induced increase in caspase-3 activity. Data are expressed as the mean  $\pm$  SEM; n = 5 – 6.





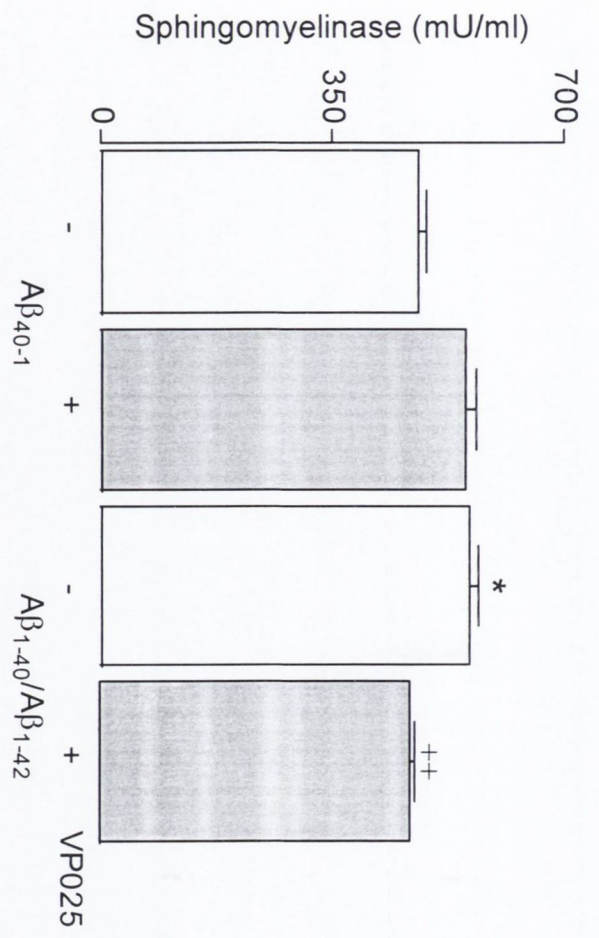
**Figure 5.7 VP025 reverses the increase in caspase-3 activity induced by 28 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

Caspase-3 activity was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 28 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub> (\*\**p* < 0.0001; ANOVA). VP025 significantly attenuated the A $\beta$ -induced increase in caspase-3 activity (+++*p* < 0.001; ANOVA). Data are expressed as the mean  $\pm$  SEM; n = 5.



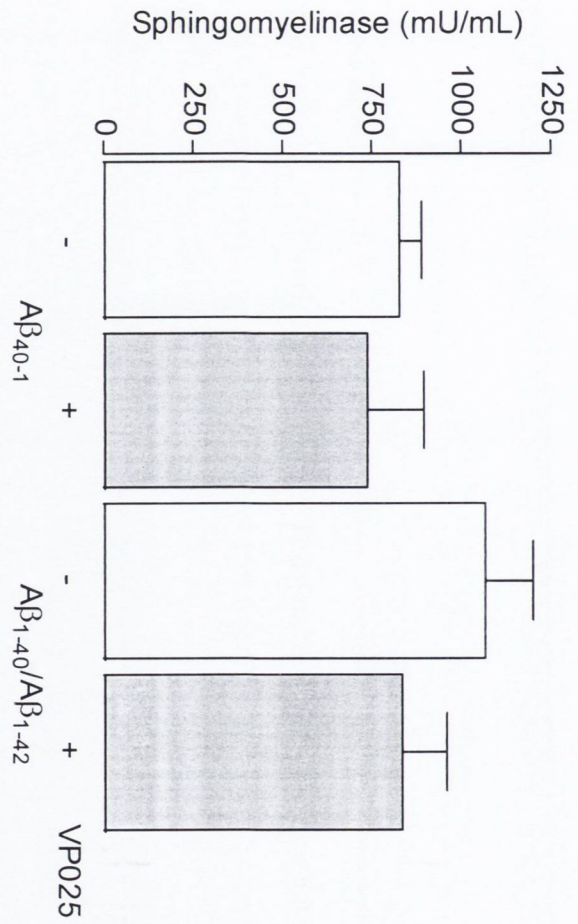
**Figure 5.8 VP025 reverses the increase in sphingomyelinase activity induced by 8 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

Sphingomyelinase activity was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub> (\*p < 0.05; ANOVA). VP025 significantly attenuated the A $\beta$ -induced increase in sphingomyelinase activity (††p < 0.001; ANOVA). Data are expressed as the mean  $\pm$  SEM; n = 4.



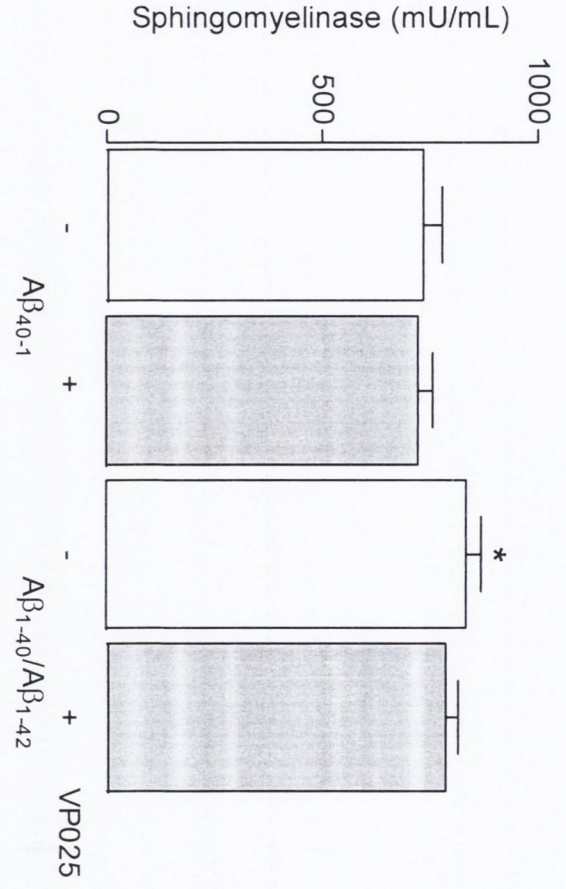
**Figure 5.9 Sphingomyelinase activity is unaffected by 20 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

Sphingomyelinase activity was increased but not significantly in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days compared with tissue prepared from saline-treated rats that received  $A\beta_{40-1}$ . VP025 exerted no effect in this experiment. Data are expressed as the mean  $\pm$  SEM; n = 5 - 6.



**Figure 5.10 Sphingomyelinase activity is significantly increased by 28 days treatment with  $A\beta_{1-40}/A\beta_{1-42}$**

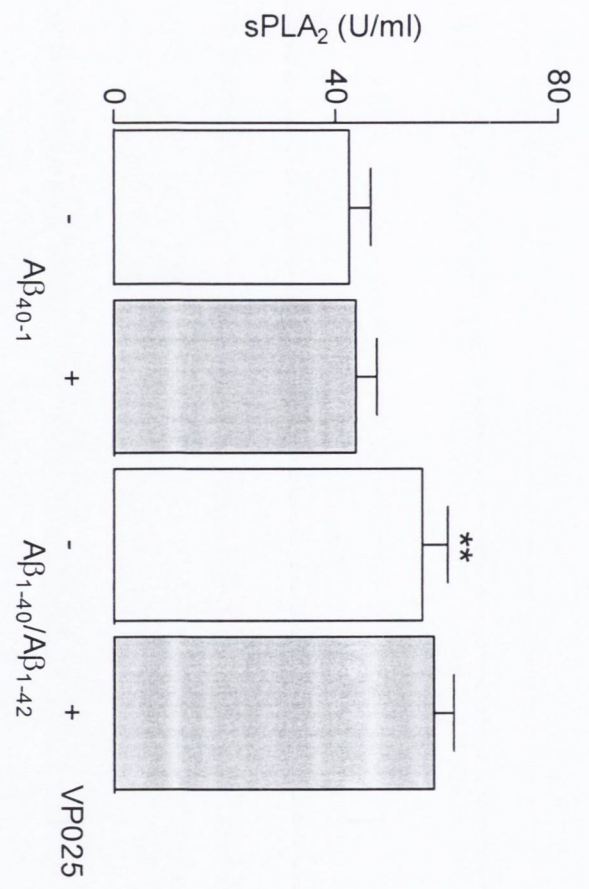
Sphingomyelinase activity was significantly increased in cortical tissue prepared from saline-treated rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days compared with tissue prepared from saline-treated rats that received  $A\beta_{40-1}$  (\* $p < 0.05$ ; ANOVA). VP025 exerted no significant effect in this experiment. Data are expressed as the mean  $\pm$  SEM;  $n = 4 - 6$ .





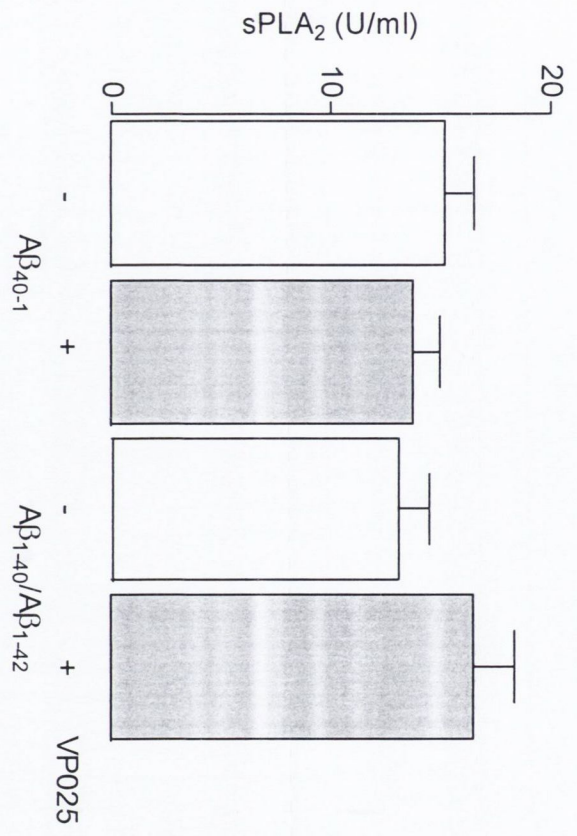
**Figure 5.11 sPLA<sub>2</sub> activity is significantly increased by 8 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

sPLA<sub>2</sub> activity was significantly increased in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub> (\*\*p < 0.005; ANOVA). VP025 exerted no significant effect in this experiment. Data are expressed as the mean  $\pm$  SEM; n = 4.



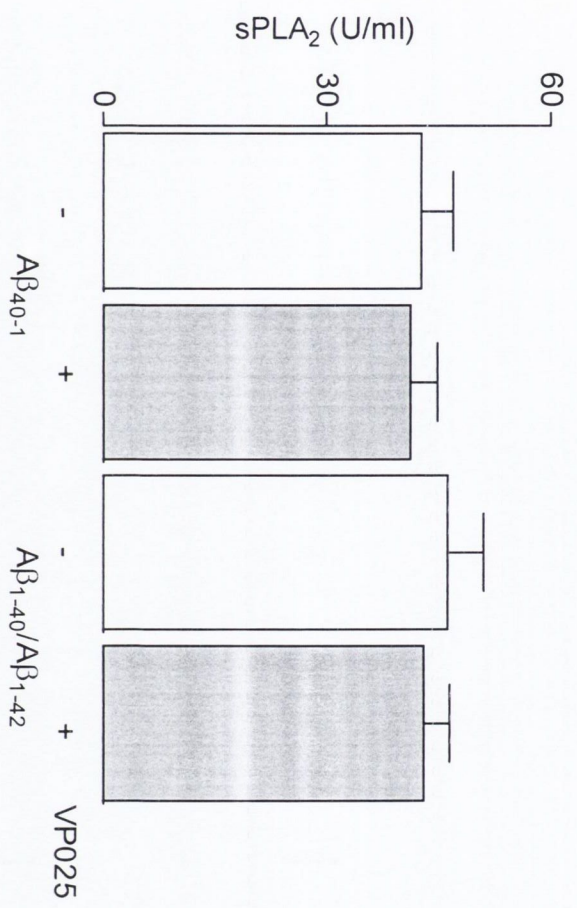
**Figure 5.12 sPLA<sub>2</sub> activity is unaffected by 20 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

sPLA<sub>2</sub> activity was unchanged in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 20 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub>. VP025 exerted no significant effect in this experiment. Data are expressed as the mean  $\pm$  SEM; n = 5 - 6.



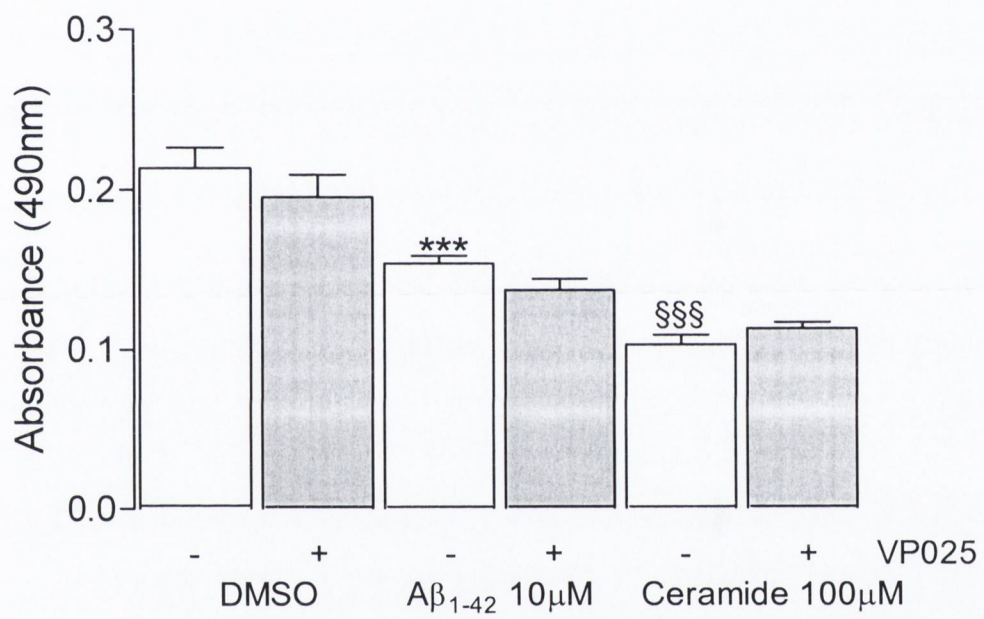
**Figure 5.13 sPLA<sub>2</sub> activity is unaffected by 20 days treatment with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>**

sPLA<sub>2</sub> activity was unchanged in cortical tissue prepared from saline-treated rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 28 days compared with tissue prepared from saline-treated rats that received A $\beta$ <sub>40-1</sub>. VP025 exerted no significant effect in this experiment. Data are expressed as the mean  $\pm$  SEM; n = 5 - 6.



**Figure 5.14  $A\beta_{1-42}$  and ceramide decrease neuronal viability *in vitro***

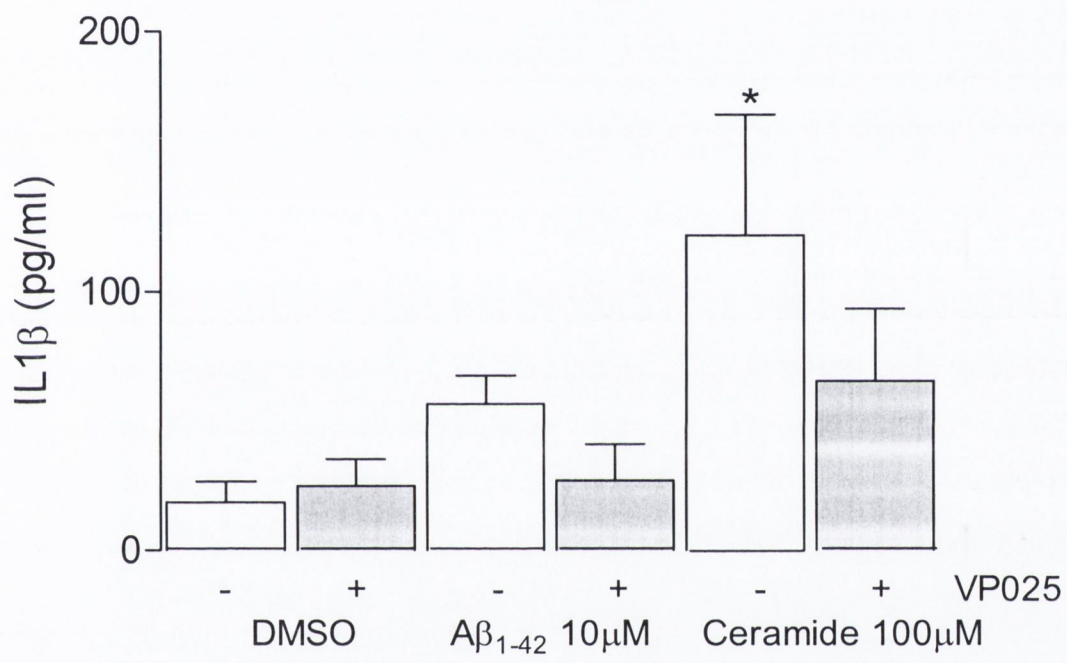
Cell viability was significantly decreased in cortical neurons treated with  $A\beta_{1-42}$  or ceramide (\*\*\*/§§§p < 0.001; ANOVA). VP025 exerted no effect in this experiment. Values are expressed as the mean  $\pm$  SEM; n = 7 – 12.





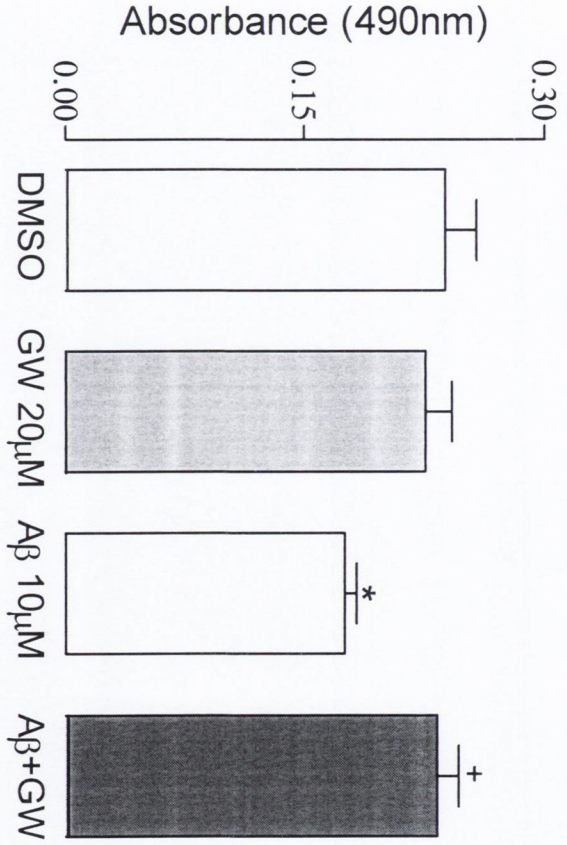
**Figure 5.15 Ceramide increases IL-1 $\beta$  release *in vitro***

Release of IL-1 $\beta$  was significantly increased from cortical neurons treated with ceramide compared with control-treated neurons (\* $p < 0.05$ ; ANOVA). VP025 exerted no effect in this experiment. Values are expressed as pg IL-1 $\beta$ /ml and are the means  $\pm$  SEM;  $n = 3 - 5$ .



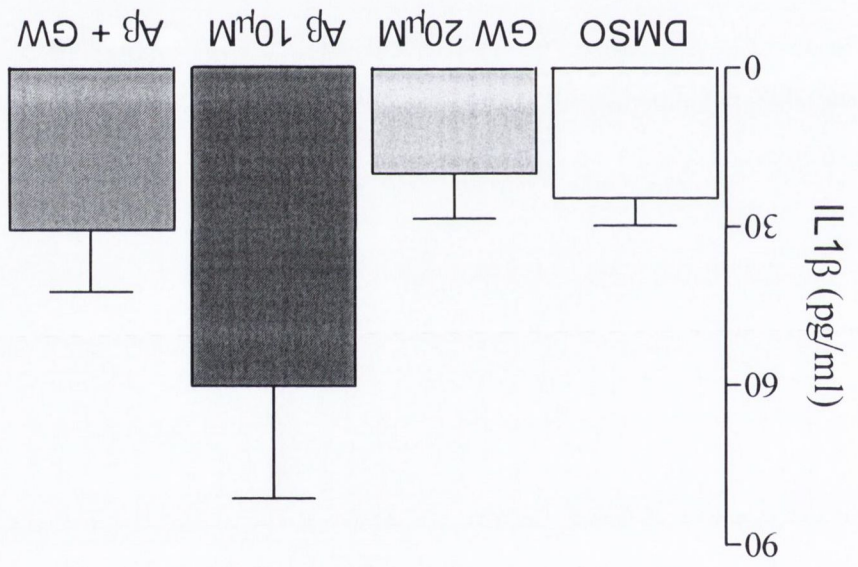
**Figure 5.16 Inhibition of sphingomyelinase attenuates the A $\beta$ -induced decrease in cell viability in vitro**

Cell viability was significantly decreased in cortical neurons treated with A $\beta_{1-42}$  (\*  $p < 0.05$ ; ANOVA). This was significantly attenuated by the sphingomyelinase inhibitor (GW4869) (+ $p < 0.05$ ; ANOVA). Values are expressed as mean absorbance  $\pm$  SEM;  $n = 6$ .



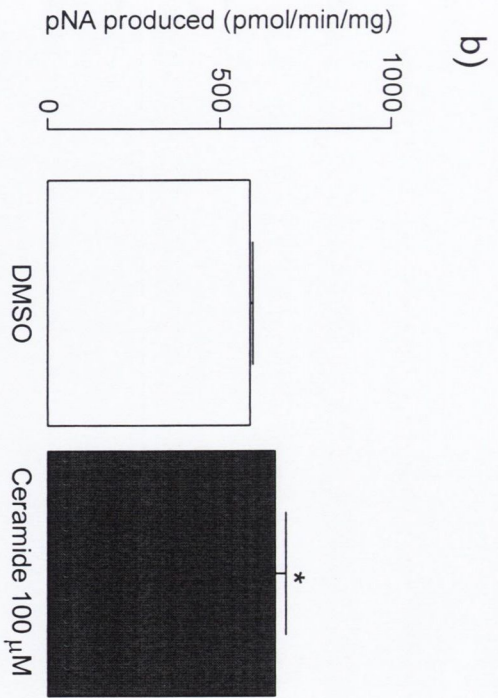
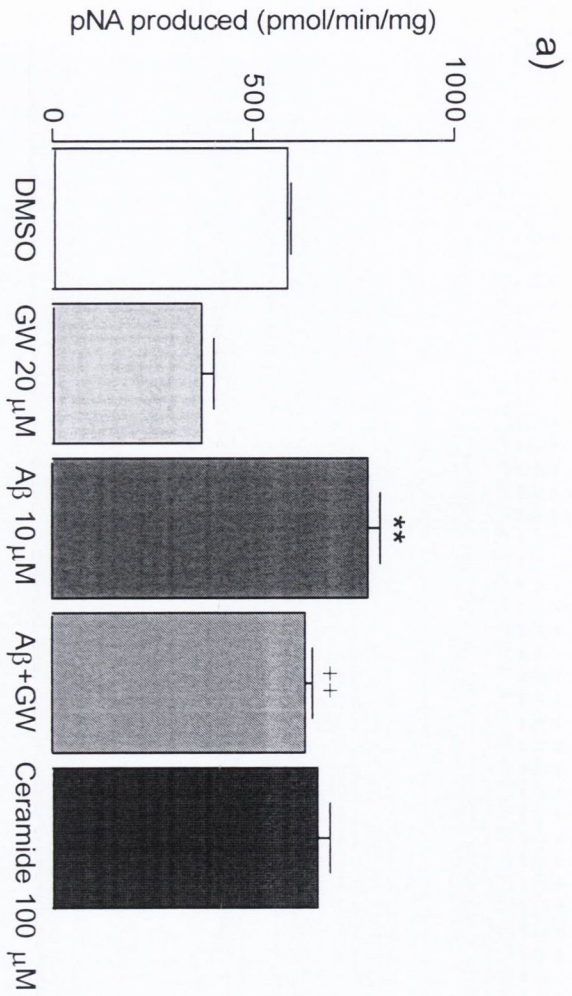
**Figure 5.17 Sphingomyelinase inhibition does not affect IL-1 $\beta$  *in vitro***

Neither A $\beta_{1-42}$  alone nor in the presence of the neutral sphingomyelinase inhibitor (GW4869) exerted any effect on IL-1 $\beta$ . Values are expressed as pg IL-1 $\beta$ /ml and are means  $\pm$  SEM; n = 5 – 6.



**Figure 5.18  $A\beta_{1-42}$  and ceramide increase in caspase-3 activity *in vitro***

Caspase-3 activation was significantly increased in cortical neurons treated with  $A\beta_{1-42}$  (a) (\*\* $p < 0.001$ ; ANOVA). GW4869 significantly attenuated the  $A\beta_{1-42}$ -induced increase in caspase-3 activation (a) (++ $p < 0.001$ ; ANOVA). Ceramide treatment however, did not affect caspase-3 activation when analysed by ANOVA, but significantly increased Caspase-3 activation in cortical neurons when analysed by t t-test versus DMSO control (b) (\* $p < 0.05$ ; t-test). Data expressed as the means  $\pm$  SEM; n = 5 – 6.





The objective of this study was to establish whether chronic infusion of  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) might trigger changes in rat brain which are indicative of cell death and that may affect neuronal membrane integrity, and subsequently account for our observed  $A\beta$ -induced impairment in LTP. The data indicate that long-term administration of  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) significantly increased cortical caspase-8 activity *in vivo*, which was paralleled by activation of caspase-3. The data also show that  $A\beta$ -induced changes in caspase activation were paralleled by increases in SMase and evidence from the *in vitro* data suggest that SMase activity contributes to the increase in caspase activity. One of the most robust findings presented is that administration of  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) for 8, 20 or 28 days increases activation of caspase-8 and -3 in cortical tissue. Significantly almost all these changes are attenuated by VP025 pre-treatment indicating the neuroprotective effects of this treatment. The *in vivo* data demonstrate the neurotoxic effect of  $A\beta_{1-40}/A\beta_{1-42}$  ( $63.8\mu\text{M}$ ) and suggest that chronic administration induces cell death. Several studies have reported that  $A\beta$  induces changes indicative of cell death. Minogue and colleagues (2003) reported activation of JNK in rat hippocampus following acute  $A\beta_{1-40}$  ( $200\mu\text{M}$ ) administration this was coupled with increased expression of pro-apoptotic proteins such as caspase-3, Bax, cytosolic cytochrome c and Fas ligand expression, as well as a significant impairment in LTP in CA1. Combined, these data link  $A\beta$  to an associated increase in cell death which accompanies inhibited expression of LTP *in vivo*.

$A\beta$ -induced neuronal cell death seems to be differentially regulated by the various  $A\beta$  species. Using homogeneous preparations of  $A\beta\text{O}$ , ADDLs and f $A\beta$ , Deshpande and colleagues (2006) performed a side-by-side comparison of the effect of  $A\beta$  species on human cortical neurons (HCNs) *in vitro*.  $A\beta\text{Os}$  exert a faster effect, killing most neurons in 24 hours. Five to seven days of incubation were required to generate similar levels of cell death with ADDLs. Chronic incubation for 10 days, and higher concentrations of f $A\beta$  were required to produce generalized dystrophic changes and only accounted for modest amounts of cell death in these experiments. Structural differences between  $A\beta\text{Os}$ , ADDLs and f $A\beta$  influence their pore-forming or receptor-binding activities and are likely to account for this disparity. An alternative possibility is that, the

toxic effect of ADDLs or fA $\beta$  could be receptor-mediated, leading to mitochondrial changes via downstream signals over a longer time course. A $\beta$ O and ADDLS show remarkable co-localization with synaptic sites in rat brain and the mechanism by which they are targeted to synaptic sites may be a crucial step in the pathological cascade (Lacor *et al.*, 2004).

Recent results suggest that ADDLs bind to and reduce the number of NMDA receptors, providing an alternative pathway by which soluble forms of A $\beta$  may cause synaptic failure (Lacor *et al.*, 2004). The fast and highly toxic effect of A $\beta$ O<sub>s</sub> *in vitro* also correlates with a sequence of cellular alterations, consistent with the activation of a mitochondrial death pathway, including early changes in adenosine tri-phosphate (ATP) production, cytoplasmic translocation of cytochrome *c*, caspase activation, and nuclear condensation (Desphande *et al.*, 2006). Experiment involving chronic exposure of rat cortical neurons in culture to fibrillar A $\beta$ <sub>1-40</sub> (20 $\mu$ M) and immunohistochemical analysis of post mortem examination of AD affected human brain tissue, have shown that apart from interfering with hippocampal dependent memory, that A $\beta$ <sub>1-40</sub> also induces abnormal activation of signaling pathways leading to neuritic dystrophy and synaptic loss throughout the brain (Grace *et al.*, 2002; Grace and Busciglio, 2003). Chronic and subtle impairment of mitochondrial function by low concentrations of A $\beta$ -soluble species may underlie defective synaptic activity and cognitive impairment in AD patients and may play a role in the present chronic A $\beta$  administration studies, a hypothesis which awaits future examination.

Jellinger and Stadelmann (1999) using immunohistochemical techniques, reported that in the post mortem AD brain, a considerable number of hippocampal neurons and glia which lay close to amyloid deposits displayed a 3- to 6- fold increase in DNA fragmentation. Experiments conducted by Matsui and colleagues (2006) showed elevated levels of mRNA for caspases-7 and -8 in AD affected temporal neocortex when compared to control brains but interestingly no differences were seen in caspase-3 or caspase-9 activation. Multiple regression analysis indicated, however, a positive correlation between caspase-8 and caspase-3 activation and furthermore a positive correlation between caspase-8 activation and the amount of A $\beta$ <sub>1-42</sub> protein concentration extracted from the same post mortem tissue. These findings suggest that, at a

transcriptional level at least, activation of key elements of the apoptotic cascade correlate with accumulation of A $\beta$ <sub>1-42</sub>.

The *in vitro* data in this work shows that neuronal cortical cell cultures exposed to A $\beta$ <sub>1-42</sub> (10 $\mu$ M) also demonstrate signs of apoptosis with significantly lower levels of cell viability and elevated caspase-3 activity following treatment (24hr). VP025 pre-treatment completely reverses the pro-apoptotic effects of A $\beta$  on neurons *in vivo*. What is unknown at present is at what stage in the apoptotic cascade VP025 acts to manipulate caspase activation. These data suggest a contributory role for caspase activation in the A $\beta$ -induced impairment in LTP seen in chronically A $\beta$ -treated rat hippocampus.

Marques and colleagues (2003) and Keil and colleagues (2004) have shown using APP transgenic mice and *in vitro* APP transfection techniques that A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> are neurotoxic via apoptotic pathways (Marques *et al.*, 2003). Galvan and colleagues (2006) reported that curtailing caspase activity on aspartate residues associated with AD (Asp 664) blocks the development of symptoms of AD in transgenic mice by preventing APP-C-31 peptide production, which is thought to have pro-apoptotic properties in its own right. Circular dichroism spectroscopy has shown A $\beta$ <sub>1-40</sub> capable of promoting apoptosis by interacting with cell membranes (Terzi *et al.*, 1997), leading to an increase in A $\beta$  fibrillogenesis and modifications in lipid bilayer properties. The lipid philicity displayed by A $\beta$  and the membrane perturbations it elicits could render neurons vulnerable to increases in levels of oxidative stress and impairments in cellular energy metabolism as seen in fibroblasts prepared from AD patients and in neurons prepared from PS1 mutant mice leading to increases in caspase driven apoptosis (Gibson *et al.*, 2002; Huang *et al.*, 2005).

The *in vivo* data presented indicate that chronic A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) administration does indeed cause disturbances in the integrity of cortical neuronal membranes; marked increases in SMase activation in cortical tissue prepared from rats treated with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) for 8 and 28 days were observed. Significantly, pre-treatment of rats with VP025 attenuates this A $\beta$ -induced effect in cortical tissue prepared from rats treated with A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 days. The findings of Alessenko and colleagues (2004) support this, they found that a single intra-cerebral injection of A $\beta$ <sub>25-35</sub> (3nmol)

significantly increased SMase activity in rat cerebral cortex and hippocampus, these effects were still seen 7 days post injection. In this work, *in vitro* results indicate that ceramide (100 $\mu$ M), significantly lowered cell viability, and increased caspase-3 activation in cortical neurons. SMase acts on sphingomyelin to yield ceramide, a lipid molecule and second messenger. *In vitro* experiments involving rat glomerular mesangial cells stimulated with IL-1 $\beta$ , have previously shown, that ceramide increases the cellular oxidative state and is implicated in apoptotic paradigms (Coroneos *et al.*, 1995). Ceramide has been shown to be capable of inducing cell senescence and to cause apoptosis in most cancer cells (Mathias *et al.*, 1998). Soderberg and colleagues (1992) reported lower sphingomyelin levels and higher ceramide levels in AD affected brains, implying that increased sphingomyelin degradation and ceramide accumulation may contribute to AD pathogenesis. Consistent with its ability to induce apoptosis Willaime-Morowek and colleagues (2003) demonstrated that ceramide levels increased upon survival-factor withdrawal in primary cultured cortical neurons. Interestingly, survival-factor withdrawal or addition of exogenous c(2)-ceramide (25mM) induces JNK pathway activation in these cells. Previous findings from this lab also implicate p-JNK as playing a pivotal role in A $\beta$ <sub>1-40</sub>-induced deterioration of neuronal function (Minogue, 2005) and, specifically, inhibition of JNK attenuated the A $\beta$ -induced increase in caspase-3 activation.

A $\beta$  and ceramide share cell death signaling characteristics, A $\beta$ -induced apoptosis involves TNFR1 and p75 cell surface receptors that relay death signals through the sphingomyelin-ceramide pathway (Hannun and Luberto, 2000). Campbell and Lynch (2000) showed that ceramide elevated Ca<sup>2+</sup> in cortical synaptosomes in a p42 MAP kinase-dependent manner. Their finding that ceramide is involved in the p42 MAP kinase-dependent regulation of neuronal Ca<sup>2+</sup> homeostasis by IL-1 $\beta$  suggests a role for sphingolipid metabolites in the pro-inflammatory-mediated regulation of critical Ca<sup>2+</sup>-dependent neuronal events such as neurotransmitter release and synaptic plasticity. Data from *in vitro* experiments described here show that ceramide-treatment (100 $\mu$ M), of cultured cortical neurons, significantly increases IL-1 $\beta$  production. A $\beta$ <sub>1-42</sub>-treatment (10 $\mu$ M) failed to mimic this effect however, this may be due the relatively low A $\beta$ <sub>1-42</sub> treatment concentration used compared to that used for ceramide or may reflect the 27%

decrease in neuronal viability caused by A $\beta$  which may interfere with their ability to produce IL-1 $\beta$ .

The findings of this present study suggest that instigators of neuronal membrane instability such as SMase could be contributing to neuronal cell loss because inhibition of SMase by GW4869 (20 $\mu$ M) blocked the A $\beta$ -induced increase in caspase-3 and prevented the A $\beta$ -induced decrease in cell viability *in vitro*. However, these analyses have not been assessed *in vivo* to date and it will be important to assess, in future experiments, whether the restorative action of VP025 is dependent on its ability to modulate SMase. Interestingly VP025 treatment prevented SMase activation in rat cortex prepared from animals treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 8 days indicating that it may be facilitating the restoration of LTP by suppressing its activity; changes in hippocampus, in response to A $\beta_{1-40}$ /A $\beta_{1-42}$  or VP025 remain to be investigated. Further investigation is also required to establish that the effect of VP025 on caspase activity is mediated by a change in SMase.

Not only did 8 days A $\beta_{1-40}$ /A $\beta_{1-42}$  infusion increase SMase enzyme activity in rat cortex, it also enhanced sPLA $_2$  activity although VP025 failed to abrogate this particular A $\beta$ -induced change. sPLA $_2$  isoforms are expressed in several cells including macrophages (Triggiani *et al.*, 2005) and PLA $_2$  is activated during systemic inflammation or allergy. sPLA $_2$  acts on neuronal membranes hydrolysing fatty acids at the sn-2 position of AA containing phospholipids which results in the generation of free AA which is the pre-cursor of eicosanoids and lysophospholipids. AA and its metabolites have a variety of physiological effects and AA is the substrate for the synthesis of more potent lipid mediators such as the eicosanoids and 4-hydroxynonenal (4-HNE), which at low concentrations act as second messengers. They affect and modulate several cell functions, including signal transduction, gene expression, and cell proliferation, but at high concentrations, these lipid mediators cause neurotoxicity. sPLA $_2$  also interacts with other entities namely heparin sulphate proteoglycans in membrane microdomains called lipid rafts. sPLA $_2$  in these pockets of AA enriched phospholipids allows the fatty acid to come in contact with other enzymes such as COX and LOX, creating conditions for efficient eicosanoids synthesis (Kudo *et al.*, 2002). sPLA $_2$  serves therefore to provide the

substrate for the biosynthesis of pro-inflammatory mediators other than cytokines and may play a role in A $\beta$ -induced neuronal dysfunction seen in these experiments.

Sphingolipids and their metabolites link specific cell surface receptors and environmental stresses to apoptosis (Pettus *et al.*, 2002). Kriem and colleagues 2005 found that A $\beta$  peptides induced rapid calcium-dependent release of AA from cortical neurons and that this release was specifically inhibited by PLA<sub>2</sub> antisense oligonucleotides. Inhibition also served to increase cell viability and decrease the apoptotic events induced by A $\beta$ . Malaplate-Armand and colleagues (2006) reported that soluble A $\beta$  oligomers induced activation of neutral SMase resulting in apoptotic cell death and that this process was governed by PLA<sub>2</sub> activation and AA production. The authors reported that neuronal viability was compromised in a dose-dependant manner with AA treatment and changes in AA were paralleled by an increase in SMase. Significantly, it was also found that inhibitors of neutral SMase strongly inhibited AA-induced apoptosis; it was noted that other polyunsaturated fatty acids such as DHA or EPA did not display toxic effects similar to AA. In addition, Jayadev and colleagues (1997) reported that L929 cells deficient in PLA<sub>2</sub> were incapable of hydrolyzing SMase in response to TNF- $\alpha$  *in vitro*. Based on these findings and the results of the present work, it might be suggested that activation of PLA<sub>2</sub> could be a necessary step in the initiation of SMase activation in rat brain and might account for its transitory activation by A $\beta$  in this work.

The initial hypothesis that an increase in microglial activation, and by extrapolation pro-inflammatory-induced signaling, mediate A $\beta$ -induced neurotoxicity and enhance susceptibility of hippocampus of rats no longer seems reasonable since A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>, despite having a significant effect on LTP, failed in most circumstances to elicit a typical pro-inflammatory response, with the exception, of course, of increased IL-1 $\beta$  production in the hippocampus following 28 days A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) infusion.

Data concerning A $\beta$ -induced damage in the CNS focuses for the most part on A $\beta$ -elicited microglial-mediated inflammatory responses in the hippocampus. Microglia have been shown to associate with fA $\beta$  and invest plaques of the peptide with their processes, such interaction is thought to drive a change in their phenotypic activation (Stalder *et al.*,

2001). Typically, microglial activation is accompanied by the elaboration of a wide range of pro-inflammatory molecules that mediate the auto-activation of these cells. Murine models of AD that over express APP and develop A $\beta$  plaques have also been shown to exhibit pro-inflammatory activation of microglial cells (Frautschy *et al.*, 1998; Benzing *et al.*, 1999; Stalder *et al.*, 1999; Mehlhorn *et al.*, 2000; Bornemann *et al.*, 2001) and the inflammatory response has been associated with the demise of neurons adjacent to the plaques (Kalaria, 1999).

What is now apparent is that A $\beta$ 's effect on hippocampal and cortical functioning is much more complex and interdependent. The hippocampus is not the sole seat of memory formation in the brain and it would be disingenuous to believe that it functions in isolation. The hippocampus plays a time-limited role in long-term memory storage of certain types of information, such that extra-hippocampal structures, namely cortical regions, eventually become capable of supporting the retrieval of remote memories independently (Bontempi and Durkin, 2007).

Nakamura and colleagues (2001) and Olariu and colleagues (2002) have both independently prolonged A $\beta$ <sub>1-42</sub> or A $\beta$ <sub>1-40</sub> administration (14 days) impairs cognitive performance in the rat. The authors report a dose- and time-dependent impairment in the spontaneous alternation performance in the Y-maze (spatial working memory), place navigation task in a water maze (spatial reference memory) and passive avoidance retention (non-spatial long-term memory). The learning impairments reported by Nakamura and colleagues (2001) were observed to be more severe 80 days after infusion of A $\beta$ <sub>1-42</sub> when compared to their level of competency 20 days after A $\beta$ -treatment. This demonstrates that prolonged A $\beta$  administration establishes a chain of deleterious events which adversely affects neuronal functioning.

*In vivo* experiments in transgenic models with pan-cortical amyloid load show the presence of widespread dystrophy in neuronal processes in contact with fA $\beta$  deposits, which contribute to major alterations in neocortical synaptic responses (Tsai *et al.*, 2004; Spiers *et al.*, 2005; Stern *et al.*, 2004). MRI based ante mortem studies of AD affected brains have shown extensive tissue loss with the majority of the dystrophy observable around the temporal and pre-frontal lobes spreading to primary sensorimotor areas as the disease progresses in severity (Silbert *et al.*, 2003; Thompson *et al.*, 2007). Despite this

very tangible global effect of the disease on the whole brain little is known about the underlying mechanisms responsible for the neuronal dystrophy and degeneration associated with it.

The data described here provides compelling evidence that chronic A $\beta$  administration induces SMase, caspase-8 and caspase-3 in rat cortex, this implicates A $\beta$  in the ultimate destruction of neuronal membrane integrity and invocation of cell death. Due to the interdependent nature of cortex and hippocampus, A $\beta$ -induced cortical cell loss could contribute to impairment in synaptic function in hippocampus, specifically the impairment identified here in LTP. *In vitro* analysis failed to show an effect of VP025 treatment on ceramide or A $\beta$ -induced neurotoxicity but one must question the value of *in vitro* analysis in this instance given that VP025 is normally delivered im, and that the treatment concentration is so low. VP025 treatment successfully reversed A $\beta$ -induced changes associated with impairment in LTP, as well reversing SMase and caspase activation *in vivo*, thus highlighting the importance of this novel anti-inflammatory in maintaining neuronal function.



**Chapter 6**  
**Summary and Discussion**

In addition to a noticeable decline in cognitive ability in the sufferer, the major neuropathological features, which underlie AD, are, synaptic loss, neuronal loss, neurofibrillary tangles and the deposition of A $\beta$  in plaques. A great deal of evidence suggests that the neuronal loss associated with the disease is a consequence of the actions of A $\beta$  (Resende *et al.*, 2007). Much of the pathology associated with the disease has been linked to persistent A $\beta$ -induced microglial activation allied to an increase in their production of inflammatory cytokines such as IL-1 $\beta$  (Arends *et al.*, 2000; Walker *et al.*, 2001; Stephan and Phillips, 2005; Ranario *et al.*, 2006). Microglial activation has been shown to localize to pathologically vulnerable regions of AD brain such as the medial temporal lobes, and in particular, the hippocampus (Saskai *et al.*, 1997). Clinical and animal experimental models have established that the formation of new memories requires an intact and functioning hippocampus so it is perhaps not surprising that cognitive impairment is one of the main features of AD.

There is a significant body of evidence indicating that synaptic function is disrupted in animal studies in which there is evidence of increased microglial activation (Lynch *et al.*, 2007) and increased concentration of pro-inflammatory cytokines (Lynch *et al.*, 2004). It has emerged in several experimental models that A $\beta$  is also associated with deficits in hippocampal synaptic function. In each of these experimental conditions, LTP, a model of synaptic plasticity, was markedly impaired, and this impairment was coupled with an increased hippocampal concentration of IL-1 $\beta$  (Minogue *et al.*, 2003; Townsend *et al.*, 2006; Gengler *et al.*, 2007; Lyons *et al.*, 2007). The importance of the negative impact of microglial activation on synaptic plasticity has been highlighted by the finding that a number of anti-inflammatory strategies, which prevent activation of microglia, attenuate LPS- or A $\beta$ -associated inhibition of LTP (Nolan *et al.*, 2004; Wang *et al.*, 2005).

This thesis aims to investigate A $\beta$ -induced events that might explain its impact on LTP in the hippocampus as well as its contribution to compromised neuronal function and cell death. The effect of a novel anti-inflammatory agent, VP025 (Vasogen Inc.), on changes induced in the rat hippocampus and cortex by acute and chronic administration of A $\beta$  is also assessed. VP025 is an investigational drug based on synthetic lipid

technology and has been shown in the past to abrogate LPS- and age-induced up-regulation of IL-1 $\beta$  and IL-1 $\beta$ -induced signaling (Martin *et al.*, 2003, 2004).

## **6.1 Summary of results**

### **6.1.1 Chapter III: VP025 treatment abrogates the amyloid- $\beta$ -induced deficit in long-term potentiation**

- VP025 pre-treatment abrogates the A $\beta$ -induced deficit in LTP in rat dentate gyrus following acute icv A $\beta_{1-40}$  (200 $\mu$ M) administration.
- Acute A $\beta_{1-40}$ /A $\beta_{1-42}$  (45 and 200 $\mu$ M) impairs LTP maintenance in rat dentate gyrus with respect to A $\beta_{40-1}$ -treated controls.
- VP025 pre-treatment abrogates the deficit in LTP in rat dentate gyrus, following icv treatment with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 8 days.
- VP025 pre-treatment abrogates the deficit in LTP in rat dentate gyrus, following treatment icv with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 20 days.
- VP025 pre-treatment abrogates the deficit in LTP in rat dentate gyrus, following treatment with icv A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 28 days.
- VP025 intervention abrogates the deficit in LTP in rat dentate gyrus, following icv treatment with A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) for 28 days.
- The impairment in LTP in dentate gyrus was associated with increased exposure time to A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M).

### **6.1.2 Chapter IV: Amyloid- $\beta$ induces increases in microglial activation in rat hippocampus**

- Acute administration of A $\beta_{1-40}$ /A $\beta_{1-42}$  (200 $\mu$ M) increases CD86 and ICAM-1 expression in rat hippocampus. The effect of VP025 pre-treatment was not examined in this experiment.

- Acute administration of  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) does not increase IL-1 $\beta$  concentration in rat hippocampus. The effect of VP025 pre-treatment was not examined in this experiment.
- Chronic administration of  $A\beta$  increases CD86 and ICAM-1 protein expression but not MHCII mRNA expression in rat hippocampus following icv infusion of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8 days. VP025 pre-treatment does not alter CD86 or ICAM-1 expression in these experiments.
- Chronic administration of  $A\beta$  increases CD86 and ICAM-1 protein expression but not MHCII mRNA expression in rat hippocampus following icv infusion of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 20 days. VP025 pre-treatment does not alter CD86 expression but does significantly reduce ICAM-1 expression in these experiments.
- Chronic administration of  $A\beta$  does not increase CD86, ICAM-1 protein expression or MHCII mRNA expression in rat hippocampus following icv infusion of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 28 days.
- Chronic icv administration of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8 or 20 days does not increase IL-1 $\beta$  concentration in rat hippocampus. VP025 pre-treatment has no effect on IL-1 $\beta$  concentration in these experiments.
- Chronic  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) infusion increases the concentration of IL-1 $\beta$  in rat hippocampus. The effect of VP025 pre-treatment was not examined in this experiment.

### **6.1.3 Chapter V: VP025 treatment alleviates amyloid- $\beta$ -induced cortical neuronal cell death**

- The  $A\beta$ -induced increase in caspase-8 activation in rat cortex, following 8, 20 or 28 days icv infusion of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M), is significantly reduced by VP025 pre-treatment.
- The  $A\beta$ -induced increase in caspase-3 activation in rat cortex, following 8, 20 or 28 days icv infusion of  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M), is significantly reduced by VP025 pre-treatment.

- Chronic  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) infusion for 8 or 20 days significantly increases sphingomyelinase activation in rat cortex. A significant increase was not seen in the cortex of rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 20 days. VP025 pre-treatment significantly reduced sphingomyelinase activation in the cortex of rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days but not in the cortex of those treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days.
- Chronic  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) infusion for 8 days significantly increases sPLA<sub>2</sub> activation in rat cortex. A significant increase was not seen in the cortex of rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 20 or 28 days. VP025 pre-treatment had no effect on sPLA<sub>2</sub> activation in the cortex of rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 8 days.
- $A\beta_{1-42}$  (10 $\mu$ M) and ceramide (100 $\mu$ M) treatment decrease cell viability in neuronal enriched cultures *in vitro*.
- Ceramide (100 $\mu$ M) significantly increases IL-1 $\beta$  release in neuronal enriched cultures *in vitro*.
- Viability of cells in cultures enriched for cortical neurons is maintained with a sphingomyelinase inhibitor (GW4869) *in vitro*.
- IL-1 $\beta$  release by cells in cultures enriched for cortical neurons is unaffected by GW4869 sphingomyelinase inhibition *in vitro*.
- *In vitro*  $A\beta_{1-42}$  (10 $\mu$ M) and ceramide (100 $\mu$ M) treatment are associated with an increase in caspase-3 activity in cell cultures enriched for cortical neurons.

## 6.2 Discussion

The aim of this work was to assess the effect of acute,  $A\beta_{1-40}$  (200 $\mu$ M),  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M), and long-term  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) icv administration on LTP in rat hippocampus, and to examine the modulatory role played by VP025, a novel anti-inflammatory agent, in modulating any  $A\beta$ -induced changes. The results demonstrate that synaptic plasticity in the dentate gyrus of rat, assessed by analysis of LTP, is inhibited by acute ( $A\beta_{1-40}$ ; 200 $\mu$ M), impaired by acute ( $A\beta_{1-40}/A\beta_{1-42}$ ; 45 and 200 $\mu$ M) and significantly attenuated by chronic ( $A\beta_{1-40}/A\beta_{1-42}$ ; 63.8 $\mu$ M)  $A\beta$  administration, and this

inhibition of LTP was reversed by VP025 treatment ( $A\beta_{1-40}$ : 200 $\mu$ M and  $A\beta_{1-40}/A\beta_{1-42}$ ; 63.8 $\mu$ M only). The initial hypothesis was that  $A\beta$  icv administration would increase microglial activation and trigger release of pro-inflammatory cytokines and their downstream signaling. With the exception of evidence of an  $A\beta$ -induced increase in IL-1 $\beta$  concentration in the rat hippocampus following 28 days  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) infusion, the data do not support this hypothesis since the data show that  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) or  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) failed to elicit a 'typical' pro-inflammatory response in these experiments.

Western blot analysis of hippocampal tissue prepared from rats that received acute administration of  $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M) and  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8, 20 and 28 days showed a time dependent pattern of increased expression of CD86 and ICAM-1, with their expression significantly increased following acute, 8 or 20 days  $A\beta$  administration and absent following 28 days  $A\beta$  treatment; this pattern was not observed in MHCII mRNA expression. VP025 pre-treatment did not affect  $A\beta$ -induced changes in CD86 expression but substantially reduced ICAM-1 expression in hippocampal tissue prepared from rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M) for 8 days and significantly reduced its expression in hippocampal tissue prepared from rats treated with  $A\beta_{1-40}/A\beta_{1-42}$  for 20 days.

Analysis of hippocampal tissue by ELISA only provided evidence for IL-1 $\beta$  production in hippocampal tissue prepared from rats that received  $A\beta_{1-40}/A\beta_{1-42}$  for 28 days, despite the earlier increase in CD86 and ICAM-1, which is thought to parallel changes in IL-1 $\beta$ . The absence of IL-1 $\beta$  in the brains of rats, following acute or chronic (8 or 20 days)  $A\beta$  administration, does not correlate with what was found in post-mortem samples from AD patients (Cacabelos *et al.*, 1994). However, whereas post-mortem samples reflect the final stage of AD, this study analyses the progression of  $A\beta$ -induced neuroinflammatory changes in rat brain tissue over 28 days. In addition, these animals are kept in a pathogen-free environment. Nevertheless, the data do not preclude the fact that proinflammatory cytokines such as IL-1 $\beta$  could be functionally involved in the last stages of the human disease. VP025 played no modulatory role in governing IL-1 $\beta$  concentration in hippocampus in these experiments. Similar to the present data, Depino

and colleagues (2003) also found atypical microglial responses in their *in vivo* PD model in that they observed a 16 fold increase in IL-1 $\beta$  mRNA expression but no IL-1 $\beta$  production 30 days post lesion, IL-1 $\beta$  mRNA was not examined in these experiments however.

The ambiguous role played by VP025, in these experiments, in relation to microglial activation is an interesting one. It appears to modulate ICAM-1 expression in hippocampus but not CD86 expression or IL-1 $\beta$  production, this may indicate that under these experimental conditions VP025 modulation of ICAM-1 expression may not be microglial associated but may stem from modulation of other cell types such as brain microvessel endothelia, neurons or astrocytes. The lack of evidence for VP025 action on microglia is surprising as it has been shown in a number of other models of neuroinflammation and disease (ALS, PD, Age, LPS) to have both neuroimmunomodulatory and neuroprotective effects, thought to stem from modulation of microglial activation and apoptotic signalling.

In the case of ALS, *in vitro* experiments where co-cultures of microglia and motoneurons were treated with LPS or ALS IgG, showed that in both instances VP025 prevented motoneuron cell death when compared with PBS-treated controls. *In vivo* experiments which utilised a similar VP025 pre-treatment protocol to that used in these experiments, VP025 treatment of SOD1 mice delayed disease onset by ~8 days and death by ~18 days in ALS affected animals, and immunohistochemical analysis of spinal chord tissue from these animals showed suppression of microglial activation when compared with PBS-treated controls (Beers *et al.*, 2004 a, b).

VP025 pre-treatment also had a positive effect in a rat model of Parkinson's disease, the 6-hydroxydopamine (6-OHDA) lesion of the medial forebrain bundle (Crotty *et al.*, 2008). The authors found that the functional integrity of nigrostriatal dopaminergic neurons which was assessed 7 and 21 days post-lesion by amphetamine-induced rotational testing, showed that rotational counts were significantly less in rats pre-treated with VP025 compared with PBS pre-treated 6-OHDA-lesioned rats. Neurochemical analysis 10 and 28 days following lesion induction showed that VP025 prevented the 6-OHDA-induced reduction in concentrations of striatal dopamine and its metabolites. Immunocytochemical analysis of the ipsilateral substantia nigra showed that VP025

significantly inhibited 6-OHDA-induced loss of dopaminergic neurons; in addition, immunostaining showed that VP025 prevented activation of microglia (MHCII) and p38 activation in dopaminergic neurons of 6-OHDA-lesioned rats.

Finally, evidence from this lab shows that VP025 pre-treatment significantly influences microglial activation in the hippocampus of aged and LPS-treated rats. Not only does VP025 successfully reverse the age- and LPS-associated attenuation of LTP in dentate gyrus, it also reverses the age related increase in CD40 expression, IL-1 $\beta$  production, decreases in p-Erk expression, and JNK activation in aged animals. VP025 also displays immunomodulatory effects in that it reverses the A $\beta$ <sub>1-40</sub> (200 $\mu$ M), Age or LPS-induced reduction in CD200 expression in hippocampus thus ensuring continued neuronal interaction with microglia thereby maintaining them in a quiescent state (Martin *et al.*, unpublished).

The finding that VP025 modulates ICAM-1 expression may prove to be an important one in that it may give some indication as to how peripherally administered VP025 exerts central effects. ICAM-1 plays an important role in immune-mediated cell-cell adhesive interactions (Springer *et al.*, 1994) and intracellular signal transduction pathways through 'outside-in' signaling events (Lub *et al.*, 1997; Kim *et al.*, 2003). Under basal conditions constitutive expression of ICAM-1 is low (Wertheimer *et al.*, 1992), however its expression is markedly increased on endothelial cells in the presence of pro-inflammatory mediators such as TNF $\alpha$  and IL-1 $\beta$  (Harcourt *et al.*, 1999). Following stimulation endothelial cells increase expression of adhesion molecules (Couraud, 1998). There is strong correlative evidence for increased ICAM-1 expression on endothelia and increased BBB permeability (Pu *et al.*, 2003; Corti *et al.*, 2004; Isogai *et al.*, 2004). In addition, an association between increased ICAM-1 expression on BBB endothelia and activated microglia has been shown during central-mediated cerebral inflammation (Danton *et al.*, 2003; Kyrkanides *et al.*, 2001). Microglial cells are located throughout the brain and in great numbers near blood vessels, with their processes having direct contact with the basal lamina of cerebro microvessels (Lassmann *et al.*, 1991).

Unpublished confocal microscopy data has shown that VP025 is phagocytosed by human monocytic U937 cells showing that VP025 could readily be taken up by macrophage at the site of injection (Helen Skerrit, RCSI, by personal communication).



Further research has shown that VP025 is capable of modulating T cell function in the periphery in that it manipulates IL-2 and IFN- $\gamma$  production in PMA stimulated T cells prepared from human donors (Helen Skeritt, RCSI, by personal communication). It is possible that peripheral administration of VP025 changes the function and hence cell surface morphology of either or both cell types. This in turn may alter macrophage or T cell interaction with cells (endothelia) of the blood brain barrier which up-regulate ICAM-1 expression in times of stress and thus transmit VP025's immunoregulatory effects by cell-cell contact across the BBB or by transmigration of peripheral cells to the brain. As yet this hypothesis is purely speculative and needs extensive work.

There is evidence in the literature that it is possible to have microglial activation in the absence of cytokine output. Examination of activated macrophages in the periphery shows them to be incapable of expressing pro-inflammatory cytokines during phagocytosis of cells that have undergone apoptosis (Fadok *et al.*, 1998). In addition, microglial activation in the absence of cytokine production has also been seen in a model of prion disease (Perry *et al.*, 2002). Following middle cerebral artery occlusion (MCAO) in mice, Andersson and colleagues (1991) and Gregersen and colleagues (2000) report that pro-inflammatory cytokine production in the MCAO-induced lesion is observable for a short period of 48 hours in contrast to microglial activation that lasted for many weeks. Bowen and colleagues (2006) report similar findings, they found that IL-1 $\beta$ , TNF- $\alpha$  and IL-6 expression peaked significantly 24-48 hours following lesion induction but that this effect was no longer observable after this period. It was argued that brief expression of cytokines following MCAO is neuroprotective, as animals subjected to subsequent transient MCAO displayed significantly reduced post-ischemic expression of inflammatory genes including cytokines, as well as a significantly decreased infarct volume, and neurological dysfunction. Perry and colleagues (2002) argue that these observations serve to warn us not to assume that IL-1 $\beta$  production always relates to microglial activation.

Combrinck and colleagues (2002) argue that, activated microglial such as those observed in the present experiments become 'primed', in that they have not yet secreted proinflammatory cytokines. When stimulated at a later date (in these experiments somewhere between 20 and 28 days of A $\beta$  treatment) they produce IL-1 $\beta$ . The authors

suggest that it is this priming and delayed response that could potentially exaggerate microglial responses to systemic inflammation leading to enhanced sickness behaviour. These observations and conclusions may account for the production of IL-1 $\beta$  observed in rat hippocampus following 28 days A $\beta_{1-40}$ /A $\beta_{1-42}$  treatment and may also contribute to the enhanced impairment of LTP observed in these rats (Figure 6.1).

I considered that the A $\beta$ -induced changes observed in these experiments might also be associated with deterioration in neuronal function or even cell death. Brain weight loss in AD is around 20% (Ankarcrona and Winblad, 2005). This loss is due to substantial neuronal degeneration in regions involved in memory and learning processes. The data described here provides evidence that chronic A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration induces SMase and sPLA<sub>2</sub> following 8 days A $\beta$  administration and that the activation of pro-apoptotic mediators; caspase-8 and caspase-3, is up-regulated in rat cortex following 8, 20 and 28 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) infusion. These results implicate A $\beta_{1-40}$ /A $\beta_{1-42}$  in the ultimate destruction of neuronal membrane integrity and invocation of cell death. Importantly, VP025 treatment reversed A $\beta$ -induced changes in SMase and caspase activation *in vivo*, highlighting the importance of this novel anti-inflammatory in maintaining neuronal function. These data support earlier findings in the 6-OHDA-induced model of PD where VP025 pre-treatment prevented increases in pro-apoptotic p-p38 up to 10 day after 6-OHDA-induced lesion (Crotty *et al.*, 2008).

The induction of apoptosis by A $\beta$  correlates well with the absence of evidence for cytokine production in the early phases of these experiments, as apoptosis by definition does not cause inflammation. The data correlate well with the biology of AD, in that neuronal cell death in the AD affected brain is thought to be apoptotic. Postmortem analysis of AD brain showed, for example, transferase-mediated dUTP nick end labeling (TUNEL) positive neurons and glia in hippocampus and cortex indicating DNA fragmentation (Su *et al.*, 1994; Dragunow *et al.*, 1995; Lassmann *et al.*, 1995; Smale *et al.*, 1995; Lucassen *et al.*, 1997; Sugaya *et al.*, 1997). Increased expression of Bcl-2 family members (Kitamura *et al.*, 1998; Giannakopoulos *et al.*, 1999), increased caspase activities as well as cleavage of caspase substrates have also been detected in AD brain (Rohn *et al.*, 2001; Pompl *et al.*, 2003). These results correlate well with previous data from this lab; Minogue and colleagues (2003) reported an A $\beta_{1-40}$  (200 $\mu$ M)-induced

increase in hippocampal JNK expression *in vivo* which correlated with the observed impairment in LTP in CA1. In parallel an A $\beta$ -stimulated increase in pro-apoptotic associated proteins such as Bax, Fas ligand and cytochrome c, as well as significant increases in TUNEL staining and caspase-1 activation and a concomitant decrease in PARP expression, were also observed.

sPLA<sub>2</sub> is an enzyme activated during systemic inflammation. The data show that A $\beta$  significantly enhanced its activity in these experiments although VP025 failed to abrogate this A $\beta$ -induced change. The increase in sPLA<sub>2</sub> activation was only observed in cortical tissue prepared from rats that received A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> for 8 days. Although its expression appears to be discretely increased by A $\beta$ , sPLA<sub>2</sub> may nonetheless play an important role in this experimental model in that it has been shown to be necessary for sphingomyelinase activation (Malaplate-Armand *et al.*, 2006). In addition AA, a by-product of sPLA<sub>2</sub> hydrolysis of neuronal membrane fatty acids, has been shown to promote apoptosis in neurons (Malaplate-Armand *et al.*, 2006).

The *in vitro* data presented here links A $\beta$  treatment with sphingomyelinase activation, caspase activation and cell death in cortical neurons. They do indicate that ceramide, the bioactive and neuro-toxic product of sphingomyelinase activation, significantly decreases neuronal viability and increases IL-1 $\beta$  production and caspase-3 activation in cortical neurons. Significantly, GW4869 reverses these A $\beta$ -induced changes implying that SMase may play a pivotal role in A $\beta$ -induced cell death. These results are particularly exciting in light of the fact that VP025 has been shown to modulate SMase activation *in vivo*.

On the basis of the data presented here a working hypothesis describing the mechanism by which A $\beta$  induces cell death can be suggested which is largely supported by the previous data; this is outlined in figure 6.2. *In vitro* analysis provides support for several steps in this cascade although further work is necessary (e.g. analysis of PLA<sub>2</sub> activation, AA concentration, ceramide concentration) to provide confirmation. In addition several studies are necessary to confirm that this cascade plays a role in A $\beta$ -induced cell death *in vivo*. Importantly the action of VP025 remains to be clarified.

While the data indicating its ability to prevent A $\beta$ -induced SMase and caspase activation is robust, the mechanism by which this is achieved requires considerable work.

### 6.3 Future Studies

- Analysis of A $\beta$  immunoreactivity in tissue from all time points would be interesting to determine whether A $\beta$  deposition occurred in the brain during these experiments.
- Considering that the effects of VP025 treatment after 14, 15 and 27 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63. $\mu$ M) administration were comparable to VP025 pre-treatment analysis, the effect of VP025 intervention treatment on all other parameters examined in this thesis would be vital to establish whether VP025 as an intervention treatment could be a viable prospect.
- The issue of whether A $\beta$  was phagocytosed by microglia warrants investigation, immunohistochemical analysis of colocalisation between phagocytic microglia and A $\beta$  would be required to assess this. Further analysis on the links between CD86/ICAM-1 and CD68 by FACS would be beneficial for this point.
- The data suggest that the A $\beta$ -induced impairment in LTP impairment was associated with activated sPLA2, SMase, caspase-8 and caspase-3. It would be of interest to establish whether this effect was restricted to neurons, or whether astrocytes or microglia were similarly affected. *In vitro* experiments show that cultures enriched for neurons are susceptible to A $\beta_{1-42}$  (10 $\mu$ M) and ceramide (100 $\mu$ M) treatment *in vitro* and that caspase-3 activity is significantly increased in their presence. Further *in vitro* analysis is required to show whether glial cells (hippocampal and/or cortical) under similar conditions could contribute to the observed A $\beta$ -induced impairment in LTP.
- It was mentioned that A $\beta_{1-40}$  can retard the aggregation of A $\beta_{1-42}$  and that this could be contributing to the attenuated effect of acute administration of A $\beta_{1-40}$ /A $\beta_{1-42}$  on LTP. Analysis of the effect of A $\beta_{1-40}$  on A $\beta_{1-42}$  in the context of these experiments would address this issue.

- The majority of A $\beta$  treatment protocols in this study involved acute and chronic administration of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> in combination only, it would be interesting to compare the effects of A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> administration versus the same administration protocols with either A $\beta$  type administered alone. Similarly it would be interesting to assess the effect of VP025 treatment on either of the A $\beta$  species alone.
- Further analysis of the relative oligomeric content of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> in the fibrillar A $\beta$  preparations used in the current work is also needed, especially with respect to the pumps contents at the end of the 3 infusion periods. This would establish whether the nature of the A $\beta$  species changes with time and if there were changes, this could account for the differing results seen for the same parameter (e.g. CD86 / ICAM-1) seen across the various time points.
- Mentioned in chapter 4 is that VP025 attenuates the A $\beta$ -induced increase in ICAM-1 expression in rat hippocampus prepared following 8 and 20 days A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) administration. Further analysis of the effect of VP025 on other cell types in the hippocampus following chronic A $\beta$  administration is required to establish whether VP025's effect on ICAM-1 expression is restricted to glia alone or whether VP025 could be affecting other cell types which could contribute to the significant effect VP025 pre-treatment has on LTP maintenance in the dentate gyrus.
- As yet the molecular mechanisms governing the action of VP025 following chronic A $\beta$  administration are unclear. Work carried out previously in the lab (Martin *et al.*, unpublished) has shown that VP025 abrogates the attenuation in hippocampal LTP seen in aged rats. This was linked to VP025 reversing age-associated increases in IL-1 $\beta$  production, JNK activation and decreased levels of p-Erk and synaptophysin expression. Similar analysis on both acute (A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub>; 200 $\mu$ M) and chronic A $\beta$ <sub>1-40</sub>/A $\beta$ <sub>1-42</sub> (63.8 $\mu$ M) treated tissue should be carried out to see if VP025 has similar effects.
- The data suggests that instigators of neuronal membrane instability such as SMase could be contributing to neuronal cell loss as inhibition of SMase by GW4869

blocked the A $\beta$ -induced increase in caspase-3 and prevented the A $\beta$ -induced decrease in cell viability *in vitro*. However, these analyses have not been assessed *in vivo* to date and it will be important to assess, in future experiments, whether the restorative action of VP025 is dependent on its ability to modulate SMase and hence neuronal viability. Interestingly VP025 treatment prevented SMase activation in rat cortex prepared from animals treated with A $\beta_{1-40}$ /A $\beta_{1-42}$  for 8 days indicating that it may be facilitating the restoration of LTP by suppressing its activity; changes in hippocampus, in response to A $\beta_{1-40}$ /A $\beta_{1-42}$  or VP025 remain to be investigated.

- sPLA<sub>2</sub> activation was significantly increased in rat cortex by 8 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) infusion. As evidence for A $\beta$ -induced increases in cytokine production is scarce in this work, examination of the role of sPLA<sub>2</sub> warrants attention. sPLA<sub>2</sub> serves to provide the substrate for the biosynthesis of pro-inflammatory mediators other than cytokines and may play a role in A $\beta$ -induced neuronal dysfunction seen in these experiments.
- *In vitro* analysis provides support for several steps of the cascade described in figure 6.2. Further work is necessary to confirm that this cascade plays a role in A $\beta$ -induced cell death *in vivo* eg. AA and ceramide concentrations in hippocampal and cortical tissue from these experiments need to be examined. Extensive work is needed to establish the mode of action of VP025 within this framework, to include its fate from peripheral administration to its having long lasting central effects on neuronal viability and functioning.

High CD86 / ICAM-1 expression

Low CD86 / ICAM-1 expression

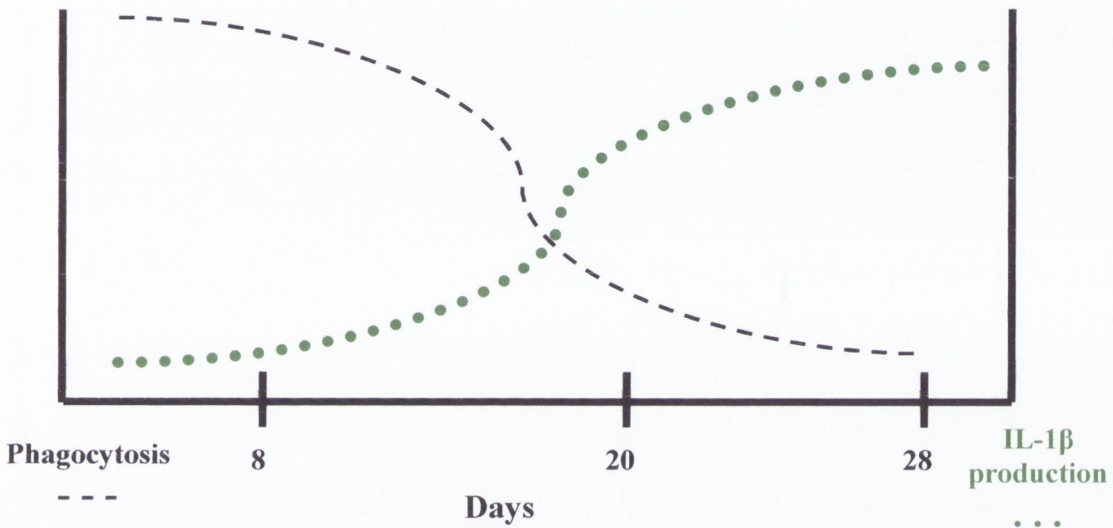
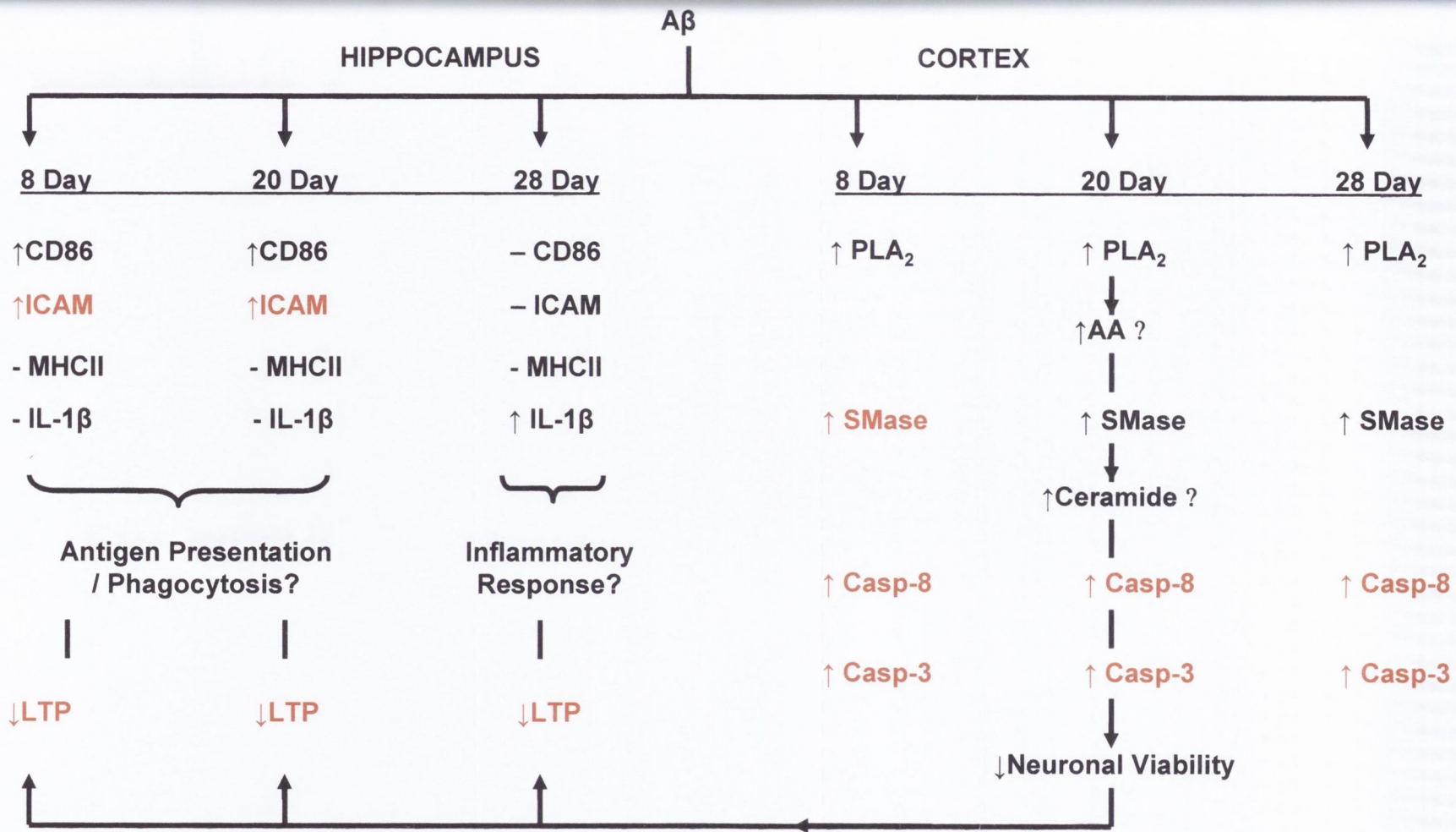


Figure 6.1 Hypothetical scheme illustrating microglial responses to prolonged A $\beta$  administration

Chronic administration of A $\beta_{1-40}$ /A $\beta_{1-42}$  over 8 or 20 days induces increases in CD86 and ICAM-1 hippocampal expression. This occurs in the absence of production of the pro-inflammatory cytokine IL-1 $\beta$ . The absence of cytokine production is thought to reflect increased phagocytic activity in microglia, which precludes them from producing cytokines. It is suggested that as the phagocytic activity of microglia becomes overwhelmed with time, the cells revert to a phenotype more closely associated with neuroinflammation and that they begin to produce IL-1 $\beta$ . This scheme may well reflect some aspects of disease progression in AD over time. CD86 = cluster of differentiation 86, ICAM-1 = intracellular adhesion molecule-1, AD = Alzheimer's disease, A $\beta$  = amyloid- $\beta$ , IL-1 $\beta$  = interleukin-1 $\beta$ .







**Figure 6.2 Summary Schematic and suggested scheme of events leading to an A $\beta$ -associated decline in neuronal viability via activation of sPLA<sub>2</sub>, sphingomyelinase and caspases-8 and -3.**

Chronic A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration did not induce microglial activation and cytokine output in the hypothesized manner and the data do not support A $\beta$ -induced microglial activation as the source of the observed impairment in LTP. However, chronic icv administration of A $\beta$  increases PLA<sub>2</sub> activity, which causes an increase in AA concentration in the cortex, this in turn leads to activation of SMase which in turn increases cortical ceramide concentration. Activation of caspase-8 and -3 by ceramide leads to apoptotic cell death. Chronic A $\beta$  administration leads to neuronal compromise and could account for the A $\beta$ -induced impairment in LTP seen in these experiments. Text in red denotes where VP025 treatment had a significant abrogative effect over the A $\beta$ -induced changes in hippocampus or cortex.



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### VIII Appendix I. Mean Data

Units  EPSP slope %	Control Saline		Control A $\beta_{1-40}$ (200 $\mu$ M)		VP025 Saline		VP025 A $\beta_{1-40}$ (200 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
	151.87 $\pm$ 2.08	115.4 $\pm$ 0.49	129.54 $\pm$ 2.05	102.5 $\pm$ 0.53	143.68 $\pm$ 1.39	114.59 $\pm$ 0.61	129.54 $\pm$ 0.84	123.15 $\pm$ 0.64

**Table 1.** The effect of A $\beta_{1-40}$  (200 $\mu$ M) administration and VP025 pre-treatment on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 8.

Units  EPSP slope %	A $\beta_{40-1}$		A $\beta_{1-40}$ /A $\beta_{1-42}$ (45 $\mu$ M)		A $\beta_{1-40}$ /A $\beta_{1-42}$ (200 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
	148.7 $\pm$ 0.94	129.01 $\pm$ 0.91	141.9 $\pm$ 4.91	118.7 $\pm$ 0.46	152.1 $\pm$ 2.66	118.6 $\pm$ 0.89

**Table 2.** The effect of A $\beta_{1-40}$ /A $\beta_{1-42}$  (45  $\mu$ M or 200 $\mu$ M) administration on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 2 - 4.

Units	Control A $\beta_{40-1}$ (63.8 $\mu$ M)		Control A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)		VP025 A $\beta_{40-1}$ (63.8 $\mu$ M)		VP025 A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
EPSP slope %	135.4 $\pm$ 1.11	112.8 $\pm$ 1.11	108.4 $\pm$ 1.56	88.66 $\pm$ 0.61	131.6 $\pm$ 1.49	114.4 $\pm$ 0.79	128.3 $\pm$ 1.35	109.58 $\pm$ 0.59

**Table 3** The effect of 8 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration  $\pm$  VP025 pre-treatment on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 4-6.

Units	Control A $\beta_{40-1}$ (63.8 $\mu$ M)		Control A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)		VP025 A $\beta_{40-1}$ (63.8 $\mu$ M)		VP025 A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
EPSP slope %	142.5 $\pm$ 1.03	123.61 $\pm$ 2.98	112.96 $\pm$ 2.93	111.70 $\pm$ 0.85	158.54 $\pm$ 3.65	127.29 $\pm$ 2.41	135.78 $\pm$ 1.02	114.46 $\pm$ 1.44

**Table 4** The effect of 20 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration  $\pm$  VP025 pre-treatment on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 5-6.

Units	Control A $\beta_{40-1}$ (63.8 $\mu$ M)		Control A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)		VP025 A $\beta_{40-1}$ (63.8 $\mu$ M)		VP025 A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
EPSP slope %	131.86 $\pm$ 2.26	113.63 $\pm$ 0.77	144.66 $\pm$ 3.58	85.36 $\pm$ 1.70	152.99 $\pm$ 3.23	123.16 $\pm$ 1.01	136.35 $\pm$ 1.99	127.0 $\pm$ 0.57

**Table 5** The effect of 28 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration  $\pm$  VP025 pre-treatment on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 5-6.

Units	Control A $\beta_{40-1}$ (63.8 $\mu$ M)		Control A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)		VP025 A $\beta_{40-1}$ (63.8 $\mu$ M)		VP025 A $\beta_{1-40}$ /A $\beta_{1-42}$ (63.8 $\mu$ M)	
	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min	0-2 min	40-45 min
EPSP slope %	120.18 $\pm$ 1.36	115.23 $\pm$ 0.85	113.32 $\pm$ 1.53	95.64 $\pm$ 0.99	127.06 $\pm$ 0.85	116.53 $\pm$ 0.92	118.14 $\pm$ 0.96	116 $\pm$ 0.35

**Table 6** The effect of 28 days A $\beta_{1-40}$ /A $\beta_{1-42}$  (63.8 $\mu$ M) administration  $\pm$  VP025 intervention on LTP in young rats in the first 2 min (0-2 min) and the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 4-6.

Units EPSP slope %	8 Days $A\beta_{1-40}/A\beta_{1-42}$ (63.8 $\mu$ M)	20 Days $A\beta_{1-40}/A\beta_{1-42}$ (63.8 $\mu$ M)	28 Days $A\beta_{1-40}/A\beta_{1-42}$ (63.8 $\mu$ M)
	40-45 min  89.20 $\pm$ 0.67	40-45 min  111.70 $\pm$ 1.03	40-45 min  85.36 $\pm$ 1.70

**Table 7** Maintenance of LTP is significantly impaired with increasing exposure time to  $A\beta_{1-40}/A\beta_{1-42}$  (63.8 $\mu$ M). Shown above are the figures for the last 5 min (40-45 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means  $\pm$  SEM; n = 4-6.

Arbitrary Units/ pg IL- 1 $\beta$ / mg	Acute $A\beta_{40-1}$  (200 $\mu$ M)	Acute $A\beta_{1-40}/A\beta_{1-42}$  (200 $\mu$ M)	8 Days $A\beta_{40-1}$ -VP025  (63.8 $\mu$ M)	8 Days $A\beta_{40-1}$ +VP025  (63.8 $\mu$ M)	8 days $A\beta_{1-40}/A\beta_{1-42}$ -VP025 (63.8 $\mu$ M)	8 days $A\beta_{1-40}/A\beta_{1-42}$ +VP025 (63.8 $\mu$ M)	20 Days $A\beta_{40-1}$ -VP025  (63.8 $\mu$ M)
CD86	0.68 $\pm$ 0.06	0.90 $\pm$ 0.02	0.46 $\pm$ 0.02	0.52 $\pm$ 0.04	0.66 $\pm$ 0.07	0.62 $\pm$ 0.07	0.39 $\pm$ 0.04
ICAM-1	0.35 $\pm$ 0.07	0.66 $\pm$ 0.10	0.33 $\pm$ 0.08	0.38 $\pm$ 0.13	0.76 $\pm$ 0.05	0.47 $\pm$ 0.10	0.15 $\pm$ 0.001
IL-1 $\beta$	171 $\pm$ 5.1	169 $\pm$ 3.1	383.3 $\pm$ 38.1	408.2 $\pm$ 35.22	406.5 $\pm$ 59.91	420.0 $\pm$ 50.90	688.4 $\pm$ 45.3
Arbitrary Units/ pg IL- 1 $\beta$ / mg	20 Days $A\beta_{40-1}$ +VP025  (63.8 $\mu$ M)	20 days $A\beta_{1-40}/A\beta_{1-42}$ -VP025 (63.8 $\mu$ M)	20 days $A\beta_{1-40}/A\beta_{1-42}$ +VP025 (63.8 $\mu$ M)	28 Days $A\beta_{40-1}$ -VP025  (63.8 $\mu$ M)	28 Days $A\beta_{40-1}$ +VP025  (63.8 $\mu$ M)	28 days $A\beta_{1-40}/A\beta_{1-42}$ -VP025 (63.8 $\mu$ M)	28 days $A\beta_{1-40}/A\beta_{1-42}$ +VP025 (63.8 $\mu$ M)
CD86	0.55 $\pm$ 0.10	0.76 $\pm$ 0.10	0.71 $\pm$ 0.12	0.73 $\pm$ 0.13	0.58 $\pm$ 0.13	0.70 $\pm$ 0.07	0.51 $\pm$ 0.04
ICAM-1	0.22 $\pm$ 0.05	0.65 $\pm$ 0.24	0.15 $\pm$ 0.024	0.51 $\pm$ 0.06	0.49 $\pm$ 0.06	0.59 $\pm$ 0.10	0.58 $\pm$ 0.10
IL-1 $\beta$	521.1 $\pm$ 37.07	641.8 $\pm$ 29.9	569.0 $\pm$ 43.05	138.4 $\pm$ 3.45		164.5 $\pm$ 12.5	

**Table 8** The effect of  $A\beta_{1-40}/A\beta_{1-42}$  administration  $\pm$  VP025 pre-treatment in hippocampus of rats. Values are expressed as means  $\pm$  SEM; n = 4-6.



	Acute A $\beta$ <sub>40-1</sub> (200 $\mu$ M)	Acute A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> (200 $\mu$ M)	8 Days A $\beta$ <sub>40-1</sub> -VP025 (63.8 $\mu$ M)	8 Days A $\beta$ <sub>40-1</sub> +VP025 (63.8 $\mu$ M)	8 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> -VP025 (63.8 $\mu$ M)	8 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> +VP025 (63.8 $\mu$ M)	20 Days A $\beta$ <sub>40-1</sub> -VP025 (63.8 $\mu$ M)
<b>Caspase-8</b>	-	-	845.5 $\pm$ 13.04	1021 $\pm$ 21.71	1360 $\pm$ 11.58	911.9 $\pm$ 16.28	912 $\pm$ 59.11
<b>Caspase-3</b>	771.0 $\pm$ 6.26	809.7 $\pm$ 5.10	604 $\pm$ 21.7	658.1 $\pm$ 25.35	799.5 $\pm$ 11.68	678 $\pm$ 24.99	1049 $\pm$ 127
<b>Sphingomyelinase</b>	-	-	481.7 $\pm$ 11.27	552.5 $\pm$ 15.96	558.8 $\pm$ 13.29	467.9 $\pm$ 7.56	828.2 $\pm$ 62.79
<b>sPLA<sub>2</sub></b>	-	-	42.55 $\pm$ 3.87	43.76 $\pm$ 3.81	55.68 $\pm$ 4.62	57.74 $\pm$ 3.60	15.20 $\pm$ 1.3
	20 Days A $\beta$ <sub>40-1</sub> +VP025 (63.8 $\mu$ M)	20 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> -VP025 (63.8 $\mu$ M)	20 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> +VP025 (63.8 $\mu$ M)	28 Days A $\beta$ <sub>40-1</sub> -VP025 (63.8 $\mu$ M)	28 Days A $\beta$ <sub>40-1</sub> +VP025 (63.8 $\mu$ M)	28 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> -VP025 (63.8 $\mu$ M)	28 days A $\beta$ <sub>1-40</sub> /A $\beta$ <sub>1-42</sub> +VP025 (63.8 $\mu$ M)
<b>Caspase-8</b>	986.4 $\pm$ 72.85	1348 $\pm$ 73.53	731.9 $\pm$ 53.58	2574 $\pm$ 29.23	2472 $\pm$ 31.30	3127 $\pm$ 56.47	2833 $\pm$ 52.30
<b>Caspase-3</b>	1684 $\pm$ 156.1	1936 $\pm$ 161.4	1836 $\pm$ 162.2	1370 $\pm$ 30.08	1224 $\pm$ 45.57	1863 $\pm$ 54	1548 $\pm$ 22.64
<b>Sphingomyelinase</b>	738.8 $\pm$ 158.1	1069.0 $\pm$ 132.2	836.9 $\pm$ 124.5	735.6 $\pm$ 43.56	722.9 $\pm$ 34.45	834.8 $\pm$ 34.2	788.1 $\pm$ 28.3
<b>sPLA<sub>2</sub></b>	13.74 $\pm$ 1.23	13.10 $\pm$ 1.4	16.48 $\pm$ 1.87	42.76 $\pm$ 4.27	41.31 $\pm$ 3.62	46.33 $\pm$ 4.78	43.15 $\pm$ 3.40

**Table 9** The effect of A $\beta$  administration  $\pm$  VP025 pre-treatment in rat cortex. Values are expressed as means  $\pm$  SEM; n = 4 - 6.

Absorbance 490 nm	DMSO - VP025	DMSO + VP025	A $\beta_{1-42}$ (10 $\mu$ M) - VP025	A $\beta_{1-42}$ (10 $\mu$ M) + VP025	Ceramide (100 $\mu$ M) - VP025	Ceramide (100 $\mu$ M) + VP025
		0.23 $\pm$ 0.012	0.19 $\pm$ 0.013	0.15 $\pm$ 0.004	0.13 $\pm$ 0.007	0.10 $\pm$ 0.005

**Table 10** The effect of A $\beta$  or ceramide  $\pm$  VP025 pre-treatment on cortical neuronal viability *in vitro*. Values are expressed as means  $\pm$  SEM; n = 7-12.

pg IL-1 $\beta$ / ml	DMSO - VP025	DMSO + VP025	A $\beta_{1-42}$ (10 $\mu$ M) - VP025	A $\beta_{1-42}$ (10 $\mu$ M) + VP025	Ceramide (100 $\mu$ M) - VP025	Ceramide (100 $\mu$ M) + VP025
		18.61 $\pm$ 7.9	24.99 $\pm$ 10.18	56.58 $\pm$ 11.08	26.86 $\pm$ 14.29	122.0 $\pm$ 46.49

**Table 11** The effect of VP025 pre-treatment on A $\beta$  or ceramide-induced IL-1 $\beta$  production by cortical neurons *in vitro*. Values are expressed as means  $\pm$  SEM; n = 3-5.

Absorbance 490 nm	DMSO	A $\beta_{1-42}$ (10 $\mu$ M)	GW4869 (20 $\mu$ M)	A $\beta_{1-42}$ (10 $\mu$ M) + GW4869
		0.23 $\pm$ 0.019	0.17 $\pm$ 0.007	0.23 $\pm$ 0.013

**Table 12** The effect of A $\beta_{1-42}$   $\pm$  GW4869 administration on cortical neuronal viability *in vitro*. Values are expressed as means  $\pm$  SEM; n = 6.

pg IL-1 $\beta$ / ml	DMSO	A $\beta$ <sub>1-42</sub> (10 $\mu$ M)	GW4869 (20 $\mu$ M)	A $\beta$ <sub>1-42</sub> (10 $\mu$ M) + GW4869
		24.57 $\pm$ 5.2	60.16 $\pm$ 21.15	19.93 $\pm$ 8.53

**Table 13** The effect of GW4869 administration on A $\beta$ -induced IL-1 $\beta$  production by cortical neurons *in vitro*. Values are expressed as means  $\pm$  SEM; n = 5-6.

pmol/min/mg pNA	DMSO	GW4869 (20 $\mu$ M)	A $\beta$ <sub>1-42</sub> (10 $\mu$ M)	A $\beta$ <sub>1-42</sub> (10 $\mu$ M) + GW4869	Ceramide (100 $\mu$ M)
		586.6 $\pm$ 7.88	372.9 $\pm$ 29.57	785.0 $\pm$ 30.86	628.4 $\pm$ 18.3

**Table 14** The effect of GW4869 administration on A $\beta$ - or ceramide- induced caspase-3 activation in cortical neurons *in vitro*. Values are expressed as means  $\pm$  SEM; n = 5-6.



## IX Appendix II. Addresses

Alzet	Alzet Osmotic Pumps Durect Corporation PO.Box 530 Cupertino CA 95015 – 0530 USA
Applied Biosystems	Applied Biosystems Dormstadt Germany
Biometra	Biometra biomedizinische Analytik GmbH Rudolf-Wissell-Str. 30 D-37079 Goettingen Germany
BD Labware	Becton Dickinson Labware The Danby Building Edmund Halley Road, Oxford Science Park Oxford, OX4 4DQ UK
Biomol	BIOMOL International, L.P. Palatine House Matford Court Exeter EX2 8NL UK
Biosource	Biosource International 542 Flynn Road Camarillo CA 93012 USA
Chance Propper	Chance Propper Ltd Uraniumweg 23 3812 RJ Amersfoort The Netherlands
GE Healthcare	GE Healthcare Life Sciences

	The Grove Centre White Lion Road Amersham Bucks HP7 9LL UK
Gibco	Gibco Ltd. 3 Fountain Drive Inchinnan Drive Paisley PA4 RF Scotlnd
GraphPad Software	GraphPad Software, Inc. 11452 El Camino Real, #215 San Diego CA92130 USA
Greiner Bio-One Ltd.	Greiner Bio-One Ltd. Brunel Way Stroudwater Business Park GL10 3SX Stonehouse UK
Invitrogen	Invitrogen Ltd 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF UK
Jencons	Jencons (Scientific) Ltd. Cherrycourt Way Stanbridge Road Leighton Buzzard Bedfordshire LU7 4UA UK
Labworks UVP	Ultra-Violet Products Ltd Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge CB4 1TG UK

MJ Research	MJ Research 590 Lincoln Street Waltham, MA 02451 USA
Molecular Probes	Molecular Probes Europe B.V. PoortGebouw Rijnsburgerweg 10 2333 AA Leiden The Netherlands
MTX Labsystems Inc	MTX Labsystems Inc. 8456 Tyco Road, Building D Vienna, Virginia 22182 U.S.A.
New England Biolabs	New England Biolabs (UK) Ltd. 75/77 Knowl Piece, Wilbury Way Hitchin, Herts. SG4 0TY UK
Pierce	Pierce Biotechnology Inc. 3747 N. Meridian Road P.O.Box 117 Rockford IL 61105 USA
Promega	Promega 2800 Woods Hollow Road Madison WI 53711 USA
R & D Systems	R&D Systems 614 McKinley Place NE Minneapolis MN 55413 USA
Santa Cruz	Santa Cruz Biotechnology Inc 2161 Delaware Ave Santa Cruz

CA 95060  
USA

Sarsdedt

Sarstedt Ltd  
Sinnottstown Lane  
Drinagh  
Wexford  
Ireland

Sigma

Sigma-Aldrich Company Ltd  
Fancy Road  
Poole  
Dorset BH12 4QH  
UK

Stoelting

Stoelting Co.  
620 Wheat Lane  
Wood Dale,  
Illinois 60191  
USA

Vasogen Inc

2505 Meadowvale Blvd.  
Mississauga,  
Ontario L5N 5S2  
Canada

Vector

Vector Laboratories  
30 Ingold Road  
Burlingame  
CA 94010  
USA



## X Appendix III. Solutions

The following solutions were used:

### **Krebs solution containing CaCl<sub>2</sub>**

NaCl, 136mM  
KCl, 2.54mM  
KH<sub>2</sub>PO<sub>4</sub>, 1.18mM  
MgSO<sub>4</sub>, 1.18mM  
NaHCO<sub>3</sub>, 16mM  
Glucose, 10mM  
Containing CaCl<sub>2</sub>, 2mM

### **Artificial cerebrospinal fluid**

Solution A:

Solution B:

NaCl, 149mM  
KCl, 3mM  
CaCl<sub>2</sub>.2H<sub>2</sub>O, 1.9mM  
MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.8mM

Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 0.8mM  
NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 0.2mM

### **Phosphate buffered saline, pH 7.3 for ELISA**

NaCl, 137mM  
KCl, 2.7mM  
Na<sub>2</sub>HPO<sub>4</sub>, 8.1mM  
KH<sub>2</sub>PO<sub>4</sub>, 1.4mM

### **Lysis buffer, pH 7.4**

Tris-HCL, 10mM  
NaCl, 50mM  
Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>.10H<sub>2</sub>O, 10mM  
NaF, 50mM  
IGEPAL, 1%  
Na<sub>3</sub>VO<sub>4</sub>, 1mM  
PMSF, 1mM  
Leupeptin, 2μg/ml  
Aprotinin, 2μg/ml  
Pepstatin, 2μg/ml



## XI Appendix IV. Publications

Miller A-M., Piazza A., Walsh M., Martin DSD., Mandel A., Bolton A., Lynch MA.(2005) A novel phospholipid-based drug formulation inhibits the acute and chronic effects of Beta-amyloid administration on long-term potentiation and microglia activation in rat hippocampus. *Society for Neuroscience Abstracts*. (910.3)

Miller A-M., Piazza A., Walsh M., Martin DSD., Mandel A., Bolton A., Lynch MA.(2006) A novel phospholipid-based drug formulation inhibits the acute and chronic effects of Beta-amyloid induced impairment in LTP and inflammation in urethane anaesthetised rat brain. *Neuroscience Ireland Abstracts*.

Miller A-M., Piazza A., Walsh M., Martin DSD., Mandel A., Bolton A., Lynch MA.(2007) A novel phospholipid-based drug formulation inhibits the chronic effects of amyloid- $\beta$  induced granzyme B, caspase-3 and sphingomyelinase activation in rat cortex. *British Neuroscience Association Abstracts*. (43.12)

Miller A-M., Piazza A., Walsh M., Martin DSD., Mandel A., Bolton A., Lynch MA.(2007) A novel phospholipid-based drug formulation inhibits the chronic effects of amyloid- $\beta$  induced granzyme B, caspases-3, - 8 and sphingomyelinase activation in rat cortex. *Annual Global Conference on Neuroprotection and Neuroregeneration (GCNN) Abstracts*.



## XII Appendix V. Amyloid- $\beta$ Preparation and Calculations

### Chronic Study

Peptide Species	Catalogue Number	Quantity	Molecular Weight
A $\beta$ <sub>1-40</sub>	03-136	1mg	4329.9
A $\beta$ <sub>1-40</sub> dissolved to 10mg/ml in 100 $\mu$ l molecular grade H <sub>2</sub> O then to 1mg/ml in 900 $\mu$ l PBS			
A $\beta$ <sub>1-40</sub> was incubated at 25°C for 30h			
A $\beta$ <sub>1-42</sub>	03-112	1mg	4515
A $\beta$ <sub>1-42</sub> dissolved to 6mg/ml in 160 $\mu$ l molecular grade H <sub>2</sub> O then to 1mg/ml in 840 $\mu$ l PBS			
A $\beta$ <sub>1-42</sub> was incubated at 37°C for 30h			
A $\beta$ <sub>40-1</sub>	03-245	1mg	4311
A $\beta$ <sub>40-1</sub> made up to 1mg/ml with 1ml molecular grade H <sub>2</sub> O			
A $\beta$ <sub>40-1</sub> was incubated at 25°C for 30h			

All A $\beta$  peptides were supplied by Biosource, USA

Alzet osmotic minipump; model 2004; total volume 200 $\mu$ l with a delivery rate of 0.25 $\mu$ l/hr = 6 $\mu$ l/day.

### A $\beta$ <sub>1-40</sub>

Pump Volume: 200 $\mu$ l

Rate: 6 $\mu$ l/day

A $\beta$  to be delivered/day: 0.7 $\mu$ g/day or 0.7 $\mu$ g in 6 $\mu$ l

Stock A $\beta$ <sub>1-40</sub> = 1mg/ml which is 1000 $\mu$ g/ $\mu$ l or 6 $\mu$ g/6 $\mu$ l

Stock = 6 $\mu$ g to a final of 0.7 $\mu$ g is a dilution factor of 1/8.57

For every 1 $\mu$ l of A $\beta$ <sub>1-40</sub> stock must add 7.57 $\mu$ l of aCSF

### Final Concentration

A $\beta$ <sub>1-40</sub> MW = 4329.9

1M = 4329.9g/L = 4329.9 $\mu$ g/ $\mu$ l

Have 0.7 $\mu$ g/6 $\mu$ l = 0.1167 $\mu$ g/ $\mu$ l

1M = 4329.9  $\rightarrow$  ?M = 0.1167 $\mu$ g

0.1167  $\div$  4329.9 = 2.69 $\times 10^{-5}$ M = 26.9 $\times 10^{-6}$ M = 26.9 $\mu$ M

### A $\beta$ <sub>1-42</sub>

Pump Volume: 200 $\mu$ l

Rate: 6 $\mu$ l/day

A $\beta$  to be delivered/day: 1 $\mu$ g/day or 1 $\mu$ g in 6 $\mu$ l

Stock  $A\beta_{1-42} = 1\text{mg/ml}$  which is  $1000\mu\text{g}/\mu\text{l}$  or  $6\mu\text{g}/6\mu\text{l}$   
 Stock =  $6\mu\text{g}$  to a final of  $1\mu\text{g}$  is a dilution factor of  $1/6$   
 For every  $1\mu\text{l}$  of  $A\beta_{1-40}$  stock must add  $5\mu\text{l}$  of aCSF

### Final Concentration

$A\beta_{1-40}$  MW = 4515  
 $1\text{M} = 4515\text{g/L} = 4329.9\mu\text{g}/\mu\text{l}$

Have  $1\mu\text{g}/6\mu\text{l} = 0.167\mu\text{g}/\mu\text{l}$   
 $1\text{M} = 4515 \rightarrow ?\text{M} = 0.167\mu\text{g}$   
 $0.167 \div 4515 = 3.69 \times 10^{-5}\text{M} = 36.9 \times 10^{-6}\text{M} = 36.9\mu\text{M}$

### $A\beta_{40-1}$

$A\beta_{40-1}$  delivered at the combined concentration of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  i.e.  $63.8\mu\text{M}$

### Final Concentration

$A\beta_{40-1}$  MW = 4331  
 $1\text{M} = 4331\text{g/L} = 4331\mu\text{g}/\mu\text{l}$   
 Want  $63.8\mu\text{M} = 4331 \div 1000 \times 63.8 \div 1000 = 0.276\mu\text{g}/\mu\text{l} = 1.657\mu\text{g}/6\mu\text{l}$

Stock =  $1\text{mg/ml} = 1000\mu\text{g}/\mu\text{l} = 6\mu\text{g}/\mu\text{l}$   
 Stock =  $6\mu\text{g}/\mu\text{l}$  want  $1.657\mu\text{g}/\mu\text{l} = 6/1.657 = 3.62$  dilution factor  
 For every  $1\mu\text{l}$  of  $A\beta_{40-1}$  stock must add  $2.62\mu\text{l}$  of aCSF

### Final Notes

$A\beta_{1-40}$  delivered at  $0.7\mu\text{g}/\text{day}$  and  $A\beta_{1-42}$  delivered at  $1\mu\text{g}/\text{day}$  is a 41% - 59% ratio of the pumps total  $200\mu\text{l}$  volume. To calculate how much actually goes into each pump see below:

$A\beta_{1-40}$  41% of  $200\mu\text{l}$  in  $1/8.57$  dilution factor =  $200 \div 8.57 = 23.33\mu\text{l}$

$A\beta_{1-42}$  59% of  $200\mu\text{l}$  in  $1/6$  dilution factor =  $200 \div 6 = 33.33\mu\text{l}$

$200\mu\text{l} - (23.33\mu\text{l} + 33.33\mu\text{l}) = 143.33\mu\text{l}$  which is the volume of aCSF to be added for a total of  $200\mu\text{l}/\text{pump}$ .

$A\beta_{40-1}$  use  $1/3.62$  dilution of  $200\mu\text{l}$  total capacity =  $200\mu\text{l} \div 3.62 = 55.25\mu\text{l}$

$200\mu\text{l} - 55.25\mu\text{l} = 144.75\mu\text{l}$  which is the volume of aCSF to be added to each pump in this instance.

## Acute Study

Total icv injection volume 5 $\mu$ l A $\beta$ 1-40/A $\beta$ 1-42 injected in a 41%-59% ratio.  
Want 200 $\mu$ M A $\beta$ 1-40 in a 2.1 $\mu$ l volume and 200 $\mu$ M A $\beta$ 1-42 in a 2.9 $\mu$ l volume.

### A $\beta$ 1-40

A $\beta$ 1-40 MW = 4329.9

1M = 4329.9g/L = 4329.9 $\mu$ g/ $\mu$ l

200 $\mu$ M = 4329.9  $\div$  1000  $\times$  200  $\div$  1000 = 0.866 $\mu$ g/ $\mu$ l = 1.82 $\mu$ g in 2.1 $\mu$ l

Stock 1mg/ml = 100 $\mu$ g/ $\mu$ l = 2.1 $\mu$ g in 2.1 $\mu$ l

Stock 2.1 $\mu$ g want final concentration of 1.82 $\mu$ g = Dilution factor of 2.1/1.82 = 1.15 of  
A $\beta$ 1-40

### A $\beta$ 1-42

A $\beta$ 1-42 MW = 4515

1M = 4515g/L = 4515 $\mu$ g/ $\mu$ l

200 $\mu$ M = 4515  $\div$  1000  $\times$  200  $\div$  1000 = 0.903 $\mu$ g/ $\mu$ l = 2.61 $\mu$ g in 2.9 $\mu$ l

Stock 1mg/ml = 100 $\mu$ g/ $\mu$ l = 2.9 $\mu$ g in 2.9 $\mu$ l

Stock 2.9 $\mu$ g want final concentration of 2.61 $\mu$ g = Dilution factor of 2.9/2.61 = 1.11 of  
A $\beta$ 1-42