Analysis of the effects of Beta-Amyloid and age on cell signalling in rat hippocampus: effect of treatment with polyunsaturated fatty acids

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II. Summary

Long-term potentiation (LTP) is considered to be a possible neurophysiological model for learning and memory and consistent with this, is the observation that LTP is sensitive to stress, injury and insult. This thesis investigates the effects of amyloid-β (Aβ) and subsequent cellular signalling pathways on expression of LTP in perforant-path granule cell synapses. Evidence suggests that an age-related impairment in LTP exists in hippocampus and that upregulation of pro-inflammatory signalling may underpin this effect. This observation indicates that hippocampus may be more susceptible to stress or insult with age. Consequently, the effects of Aβ administration on LTP in hippocampus of adult and aged rats was investigated.

The data presented here indicate that increased concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β) and interferon-γ (IFNγ), coupled with activation of the protein kinase c-Jun N-terminal kinase (JNK), in rat hippocampus, may underlie the impairment in LTP that occurs following Aβ administration and with age. Increased JNK activity in this study was also associated with apoptotic cell death and activation of the transcription factor c-Jun.

Significantly, supplementation with the omega-3 (n-3) polyunsaturated fatty acid, eicosapentaenoic acid (EPA), attenuated the Aβ-induced and age-related inhibition of LTP, along with the Aβ-stimulated and age-associated increase in IL-1β and IFNγ concentration. These data are consistent with the idea that Aβ has deleterious effects on neuronal function as evidenced by its inhibition of LTP and that hippocampus is more susceptible to insult such as Aβ administration since a subthreshold dose of Aβ that did not inhibit LTP in adult rats completely inhibited LTP in hippocampus of aged rats. Additionally, EPA exerted a neuroprotective role in rat hippocampus since it abrogated the Aβ and age-associated impairment of LTP and attenuated the Aβ-induced and age-related increases in the pro-inflammatory cytokines IL-1β and IFNγ and subsequent apoptotic changes.

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VI. List of Abbreviations

The following abbreviations are used:

AA	Arachidonic acid
Aβ	Amyloid-β
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of variance
APP	Amyloid-β precursor protein
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CNS	Central nervous system
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAA	Excitatory amino acid
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EGTA	Ethylene glycol bis (β-aminoethylether) N,N tetraacteic acid
ELISA	Enyzme linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
GLA	γ-Linolenic acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acid
HFS	High frequency stimulation
HPA	Hypothalamo-pituitary axis
HPETE	Hydroperoxyeicostetraenoic

HRP	Horseradish peroxidase
IκB	Inhibitor of κB
ICE	Interleukin-1β converting enzyme
ICV	Intracerebroventricular
IL-1β	Interleukin-1β
IL-1RI	Interleukin-1 type I receptor
IL-1RII	Interleukin-1 type II receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-10	Interleukin-10
IL-6	Interleukin-6
IL-4	Interleukin-4
IFNγ	Interferon-γ
IP	Intraperitoneal
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
LA	Linoleic acid
LPS	Lipopolysaccharide
LT	Leukotriene
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	metabotropic Glutamate receptor
mRNA	messenger Ribonucleic acid
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly- (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween

PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
PPARγ	Peroxisome proliferator activated receptor-γ
PG	Prostaglandins
PUFA	Polyunsaturated fatty acid
RAGE	Receptor for advanced glycation end-products
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TNF-α	Tumour necrosis factor-α
TUNEL	TdT-mediated dUTP nick-end-labelling
Tx	Thromboxanes
TZD	Thiazolidinediones

The great tragedy of Science – the slaying of a beautiful hypothesis by an ugly fact.

Thomas H. Huxley (1825 –1895)

Chapter 1

Introduction

1.1 The Hippocampus

1.1.1 Historical perspective

The term 'hippocampus' (Gr. *hippokampos*: *hippos*, horse; *kampos*, sea monster) was first applied to this region of the brain by the anatomist Arantius (1587) who considered the three-dimensional form of the grossly dissected human hippocampus to be reminiscent of a sea horse. Winslow (1732) likened the hippocampus to a ram's horn and de Garengeot (1742) named the hippocampus 'Ammon's horn' after the mythological Egyptian deity. The term 'cornu ammonis' (L., horn of Ammon) was appropriated by Lorente de Nó (1943) for his terminology of the hippocampus proper (CA1-CA4) and is used synonymously with the hippocampus today.

1.1.2 Anatomy of the hippocampal formation

The hippocampal formation, located on the medial aspect of each hemisphere beneath the cortical structures, comprises the dentate gyrus, the hippocampus proper and the subiculum (Figure 1.1), and is part of the limbic system. All 3 are composed of temporal lobe allocortex, tucked into an S-shaped scroll along the floor of the lateral ventricle.

The principal cell type in the dentate gyrus is the granule cell, found in the dense granule cell layer. Granule cells have unipolar dendrites that extend into the overlying molecular layer, which receives most of the afferent projections to the dentate gryrus, primarily from the entorhinal cortex. The polymorphic layer, or hilus of the dentate gyrus, contains cells that give rise primarily to ipsilateral association fibres within the dentate gyrus and do not extend into other hippocampal fields. The granule cell layer encloses a portion of the pyramidal cell layer of the cornu ammonis, which Lorente de Nó (1934) termed field CA4.

The human hippocampus proper (cornu ammonis) is trilaminar and has essentially a single cell layer, the pyramidal layer, with plexiform layers above and below it. It may best be divided into 3 distinct fields, following the nomenclature of Lorente de Nó (1934), namely CA1, CA2 and CA3. CA3 borders the hilus of the dentate gyrus at one end and CA2 at the other. In early accounts a field CA4 was also described, however, there appears to be no functional reason to distinguish field CA3

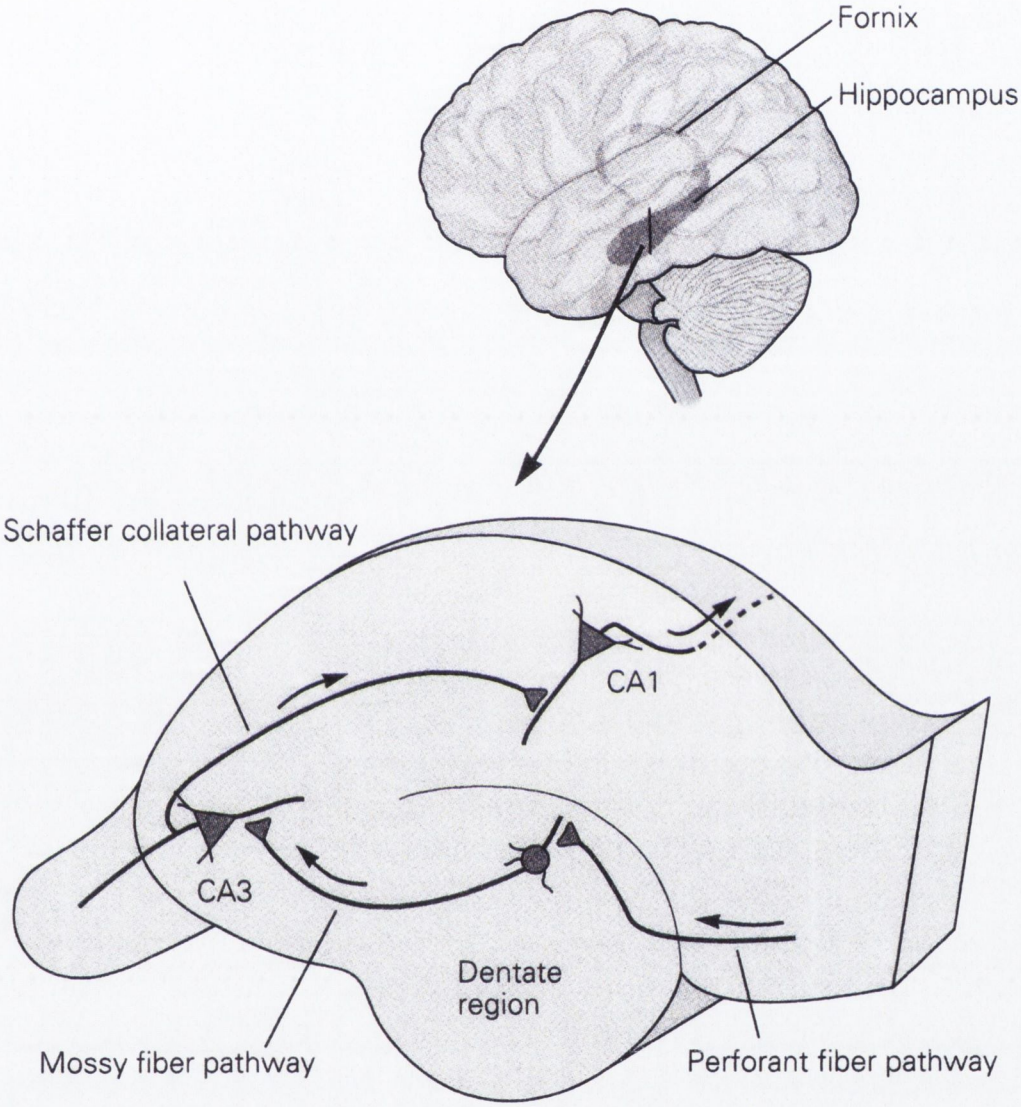


Figure 1.1 Hippocampal formation

The three major pathways in hippocampus (arrows denote the direction of impulse flow). The perforant fibre pathway from entorhinal cortex forms excitatory connections with the granule cells of dentate gyrus. The granule cells give rise to axons that form the mossy fibre 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.

(Adapted from Kandel, E.R. (2000) *Principles of Neural Science*).

from CA4 and so the term CA4 has been dropped from most contemporary descriptions of the hippocampal formation.

1.1.3 Afferent connections

The largest afferent connection of the hippocampal formation is the perforant path, which projects from layers II and III of the entorhinal cortex in the temporal lobe. The axons terminate in an orderly way in the outer two-thirds of the molecular layer of the dentate gyrus on the dendritic spines of granule cells. The dentate granule cells project heavily via their mossy fibres on to the proximal dendrites of CA3 pyramidal cells. These pyramidal cells of field CA3 give rise, via so-called Schaffer collaterals, to an equally impressive projection that terminates mainly in the stratum radiatum of the CA1 hippocampal field. In its turn, the CA1 field projects heavily to the subicular complex, which to complete the circuitry, projects to the entorhinal cortex.

1.1.4 Efferent connections

The largest efferent connection is a massive projection via the entorhinal cortex to the association areas of the neocortex. A second, forward projection is the fornix. The fornix is a direct continuation of the fimbria, which receives axons from the subiculum and hippocampus proper. The crus of the fornix arches up beneath the corpus callosum, where it joins its fellow to form the trunk and links with its opposite number through a small hippocampal commissure. Anteriorly, the trunk divides into two pillars. Each pillar splits around the anterior commissure, sending precommissural fibres to the septal area and postcommissural fibres to the anterior hypothalamus, mammillary body and medial forebrain bundle. The mammilary body projects into the anterior nucleus of thalamus, which projects in turn to the cingulate cortex, completing the circuit of Papez from cingulate cortex to hippocampus, which return to cingulate cortex via fornix, mammillary body and anterior thalamic nucleus.

1.1.5 Role of hippocampus in memory

It is now widely accepted that the hippocampus is central to the storage of memory (Kandel *et al.*, 2000). The importance of the temporal lobe in memory became apparent in the 1950's through the study of patients who had undergone bilateral removal of the hippocampus and neighbouring regions in the temporal lobe for treatment of epilepsy. H.M., a patient studied by Milner and Scoville, 1966 who suffered from untreatable bilateral temporal lobe seizures, had the hippocampal formation, amygdala and part of the temporal cortex removed bilaterally. Removal of these brain regions at surgery left H.M. with a significant memory deficit in the form of anterograde amnesia. This study identified the hippocampus and temporal lobe as having important roles in memory. Other studies involving lesion of the medial temporal lobe of monkeys demonstrated that these animals exhibited severe memory impairment (Mishkin, 1978) with the impairment being less severe when lesioning was confined to the hippocampus (Murray and Mishkin, 1998). More recently, evidence for a role for hippocampus in memory was demonstrated using magnetic resonance imaging and positron emission topography. These techniques assessed blood flow and oxygen consumption in the hippocampus and identified that these parameters fluctuated during learning tasks (Squire *et al.*, 1992).

1.2 Long-term Potentiation

1.2.1 First description of LTP

In 1973 Bliss and Lomo reported that trains of high frequency stimulation applied to any of the three major synaptic pathways in the anaesthetised rabbit increased the amplitude of the excitatory postsynaptic potentials (EPSPs) in the target hippocampal neurons. This sustained increase in synaptic efficacy was termed long-term potentiation (LTP; Figure 1.2). Bliss and Lomo's original report was followed by others, which indicated that the synaptic modifications that underlie certain forms of learning and memory resemble those that occur following LTP. Since this description it has become apparent that LTP is not limited to the hippocampus but may be a ubiquitous property of all excitatory synapses throughout the brain.

1.2.2 Properties of LTP

The basic properties of LTP were described in the 1970's and early 1980's and have been found to fit well with one of the most important hypotheses of neuroscience, Hebb's postulate. Hebb predicted that learning and memory would involve strengthening of synaptic efficacy through the coordinated firing of pre- and postsynaptic cells. There are three basic properties of LTP (Bliss and Collingridge, 1993). Firstly, when using high frequency stimulation to induce LTP, a critical number of fibres must be activated. Increasing the frequency of stimulation decreases the stimulation strength required to induce LTP – a property termed 'cooperativity' (Bliss and Collingridge, 1993). Secondly, when LTP is elicited at one set of synapses on a postsynaptic cell, adjacent synapses that were not activated do not undergo LTP – a property termed 'input specificity' (Andersen *et al.*, 1977; Lynch *et al.*, 1977). Thirdly, LTP can be induced in a set of synapses undergoing subthreshold low frequency stimulation if their activation is temporally concurrent with an LTP-inducing stimulus at another set of synapses on the same cell – a property termed 'associativity' (McNaughton *et al.*, 1978; Levy and Steward; 1979). This last property makes LTP an attractive mechanism for associating information carried by two different sets of afferents that synapse on the same postsynaptic cell. Each of the three key properties of LTP can be explained mechanistically by the biophysical properties of the *N*-methyl-D-aspartate (NMDA) receptor.

1.2.3 Induction of LTP

1.2.3.1 NMDA receptor

Glutamate receptors can be divided into two broad groups: ionotropic receptors that directly gate ion channels and metabotropic receptors, which indirectly gate ion channels through second messenger systems (Kandel *et al.*, 2000). There are three major subtypes of ionotropic receptors: AMPA, kainate and NMDA. Their nomenclature arises from the discovery of synthetic agonists, which selectively activate them (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, kainate and *N*-methyl-D-aspartate respectively).

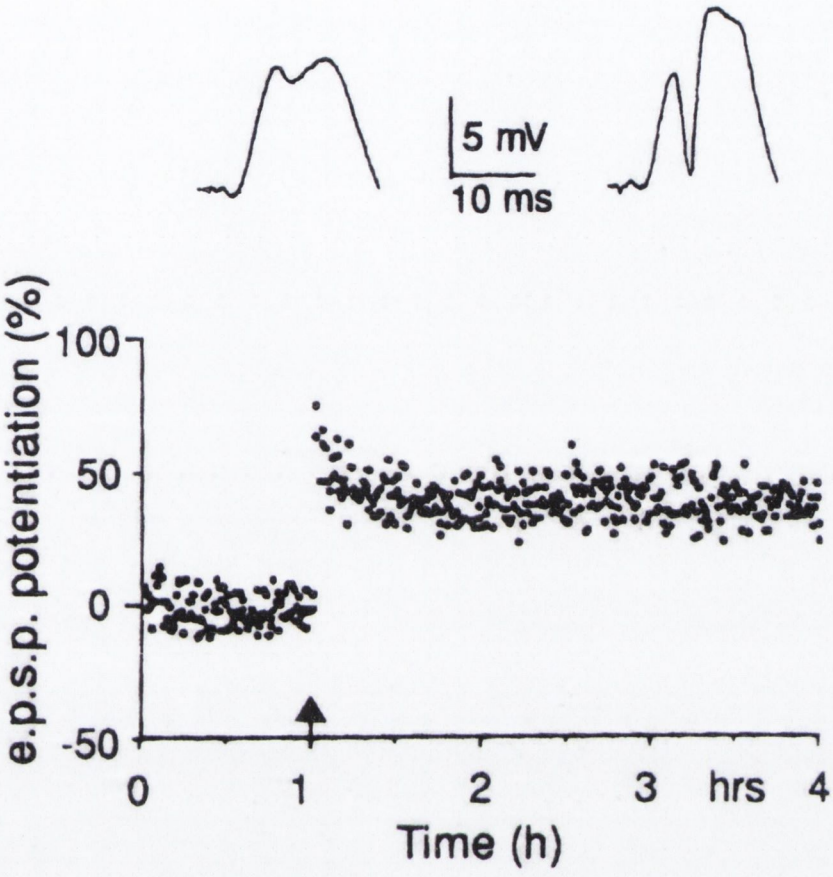


Figure 1.2 Long-term potentiation

An example for long-term potentiation (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 1 h before and 3 h following a tetanus (200Hz, 200msec) delivered at the time indicated by the arrow. (Adapted from Bliss, T.V.P. and Collingridge, G.L. (1993) *Nature*, **361**, 31-39)

Most hippocampal neurons have both non NMDA (AMPA and kainate) and NMDA receptors. The non NMDA receptors generate the early phase of the EPSP and gate ion channels with relatively low conductance that are permeable to both Na^+ and K^+ but usually not to Ca^{2+}. NMDA receptor-associated channels generate the late phase of the EPSP and have a higher conductance being permeable to both Na^+ and K^+ as well as to Ca^{2+}. NMDA receptor-channel functioning requires the presence of glycine in the extracellular fluid (MacDermott *et al.*, 1986). Glycine is normally present in sufficient quantities so as to allow efficient functioning of the channel.

The most interesting feature of the NMDA receptor-associated channel is its dual regulation being not only ligand-gated but also voltage-gated. The presence of extracellular Mg^{2+} in the pore of the channel explains the requirement for membrane depolarisation prior to influx of ions through the channel. Membrane depolarisation causes electrostatic repulsion of Mg^{2+} from its binding site in NMDA receptor-associated channel (Collingridge, 1983). The late phase of the EPSP is usually small after a single presynaptic action potential because of blockade of the channel by Mg^{2+}. If the presynaptic neuron fires repeatedly, the late phase of the EPSP is larger because of more extensive removal of Mg^{2+} due to the level of depolarisation of the membrane.

The characteristics of the NMDA receptor explain some of the observed properties of LTP: LTP requires activity of several afferent axons, a feature termed 'cooperativity' and a feature derived from the dual regulation of the NMDA receptor channel in that it requires both ligand binding and membrane depolarisation for its activation. Secondly, to elicit LTP, synaptic NMDA receptors must be activated, leading to a spatially restricted increase in intracellular Ca^{2+} in the relevant dendritic spine – this characteristic explains the 'input specificity' feature. Thirdly, 'associativity' refers to the ability of a weakly activated set of synapses to undergo LTP if a temporally concurrent set of synapses is undergoing an LTP-inducing stimulation. This property is explained by the fact that the LTP-inducing stimulation provides the requisite depolarisation, which is transmitted through the dendritic tree to those synapses in which the NMDA receptors are simultaneously activated by the subthreshold stimulus.

With the exception of the mossy fibre pathway, LTP in all subfields of the hippocampus is NMDA receptor dependent. Several elegant pharmacological experiments have led to the consolidation of this theory. The first such experiments employed the specific NMDA receptor antagonist AP5 (Collingridge, 1983) and the non-competitive NMDA associated channel blocker MK801 (Coan *et al.*, 1987) in CA1 *in vitro* and dentate gyrus *in vivo*. Use of both these pharmacological agents inhibited induction of LTP, demonstrating a pivotal role for NMDA receptor activation in LTP induction. However, LTP can be induced in CA1 without the participation of NMDA receptors requiring instead an increase in postsynaptic Ca^{2+} through activation of voltage-activated Ca^{2+} channels (Lynch and Baudry, 1984).

Furthermore, NMDA receptor activation alone does not induce LTP and use of thapsigargin, which depletes intracellular stores of calcium, inhibits LTP (Bortolotto and Collingridge, 1993). This finding suggests a requirement for augmentation of NMDA receptor mediated Ca^{2+} influx. NMDA receptor activation may be essential for many forms of LTP but not all, however a rise in intracellular Ca^{2+} concentration is a mechanism common to all forms of LTP described so far and it may be the critical element necessary for LTP induction.

1.2.3.2 Calcium

The evidence in support of a rise in intracellular Ca^{2+} as a trigger for LTP is compelling. Lynch and colleagues (1983) demonstrated that intracellular injection of the Ca^{2+} chelator, EGTA, prevented the induction of LTP. Additionally, increasing concentrations of postsynaptic Ca^{2+} by photolysis of caged Ca^{2+} can mimic LTP – these data indicate a postsynaptic site for expression of LTP that is Ca^{2+} dependent. Direct evidence for the role of postsynaptic Ca^{2+} in LTP induction came from imaging studies that demonstrated an increase in Ca^{2+} in dendritic spines resulting from NMDA receptor activation (Regehr and Tank, 1990). Currently it is believed that a short-lasting (1-3sec) threshold level of Ca^{2+} must be reached in order to trigger LTP (Bliss and Collingridge, 1993). Whether influx of extracellular Ca^{2+} alone is adequate or amplification due to Ca^{2+} release from intracellular stores is required to trigger LTP remains unclear. However some experimental evidence does exist which

indicates a requirement for release of Ca^{2+} from intracellular stores to induce LTP. Alford and colleagues (1993) found that intracellular injection of thapsigargin, a drug that depletes intracellular stores of Ca^{2+}, decreased the NMDA receptor-associated transient increase in Ca^{2+}. Furthermore, thapsigargin has also been shown to inhibit induction of LTP (Bortoletto and Collingridge, 1993).

Another important issue surrounding the involvement of Ca^{2+} in LTP is whether Ca^{2+} alone is sufficient to trigger LTP or whether additional factors, provided by synaptic activity, are required (Malenka and Nicoll, 1999). A family of G protein-coupled receptors known as metabotropic glutamate receptors (mGluR) have been proposed as a candidate for modulating the induction for LTP.

1.2.3.3 Metabotropic glutamate receptors

mGluRs are a group of amino acid receptors that have been implicated in the modulation of the induction of LTP. mGluRs mediate their effect through a G-protein associated with the receptor. Eight mGluR subtypes have been identified to date (Anwyl, 1999).

Experiments using the nonselective mGluR agonist 1-amino1,3 cyclopentanedicarboxylic acid (ACPD) revealed an enhancement of LTP with application of ACPD suggesting a role for mGluRs in LTP (McGuinness *et al.*, 1991). Subsequently, ACPD has been shown to enhance the synaptic response in CA1 (Bortolotto *et al.*, 1994) and dentate gyrus (O'Connor *et al.*, 1995). This enhancement occurred as a result of Ca^{2+} dependent changes and activation of protein kinase C (PKC) since both thapsigargin and staurosporine prevented the effect (Bortolotto *et al.*, 1998). mGluRs may have a differential role in NMDA-dependent and independent pathways as they have had differential effects in CA1, dentate gyrus and mossy-fibre CA3 synapses where LTP was unaffected. The mGluR5 subunit has been proposed to have a modulatory role in NMDA-dependent LTP since potentiation of the NMDA response was absent in mGluR5 mutant mice (Lu *et al.*, 1997). The role of mGluR1 has not been clearly defined, as conflicting data has been reported with respect to LTP in mossy-fibre CA3 LTP with Conquet and coworkers (1994) reporting its absence in mGluR1 deficient mice and no effect reported by Hsia

and coworkers (1995). The modulatory role of mGluRs on LTP may result from their ability, via the G-protein to activate phospholipase C-γ (PLC-γ) and phospholipase A_2 (PLA_2) and adenylate cyclase – all of which are important second messenger systems implicated in LTP.

1.2.4 Maintenance of LTP

1.2.4.1 Protein kinases

Upon Ca^{2+} influx into the postsynaptic cell a number of events must occur to translate the Ca^{2+} signal into a long-lasting increase in synaptic efficacy. The literature on this topic indicates a very long list of signalling molecules that are involved (Lynch, 2004). However, compelling evidence exists to implicate α-calcium-calmodulin-dependent protein kinase II (CaMKII) as a key component of the signal transduction pathway. CaMKII is found in very high concentrations in the postsynaptic density where it is ideally located to respond to fluctuations in postsynaptic Ca^{2+} concentrations. In 1989 two independent groups reported that inhibitors of CaMKII had the capacity to block LTP in CA1 (Malenka *et al.*, 1989; Malinow *et al.*, 1989). Since then this observation has been confirmed many times using a number of different approaches – in 1992, Silva and co-workers reported that genetic depletion of CaMKII in mice blocked LTP. Furthermore it has been shown that by increasing concentrations of constitutively active CaMKII in CA1 cells, synaptic transmission was enhanced and sensitivity to tetanic stimulation occluded (Pettit *et al.*, 1994).

Upon autophosphorylation at threonine-286, CaMKII becomes persistently activated with the autophosphorylation occurring after LTP induction (Fukunaga *et al.*, 1995). Mutations at this residue have lead to impairment of LTP and spatial memory (Giese *et al.*, 1998). Since the proposal of the "silent synapse" theory of LTP, additional evidence has supported the importance of CaMKII as a key component of the molecular machinery underlying the expression of LTP. Increased responsiveness to applied glutamate following LTP, due largely to increased AMPA conductance occurs, as a result of CaMKII-induced phosphorylation of the GluRI subunit of the AMPA receptor on serine-381 (Derkach *et al.*, 1999). Antagonists of CaMKII prevent

phosphorylation of this subunit (Cai *et al.*, 2002). Furthermore, delivery of AMPA receptors to the dendritic spine following LTP, a process required for their transition from functionally silent to non-silent synapses, may depend on CaMKII activation (Shi *et al.*, 1999; Liao *et al.*, 2001; Shi *et al.*, 2001).

Several other protein kinases including PKC, cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (PKA), the tyrosine kinase, Src, and mitogen-activated protein kinases (MAPK) have been proposed to initiate signalling pathways that contribute to LTP. However, the evidence in support of these kinases is considerably weaker than that of CaMKII. Inhibitors of PKC have been shown to block LTP (Klann *et al.*, 1991) and increasing PKC activity can enhance postsynaptic transmission (Hu *et al.*, 1987) giving rise to the suggestion that PKC plays a similar role to CaMKII. PKA has been proposed to "boost" the activity of CaMKII because of its ability to suppress the activity of the competing protein phosphatase (Blitzer *et al.*, 1998). Src activation may exert some effect on LTP through modulation of NMDA receptor function (Lu *et al.*, 1998). The group of proteins known as mitogen-activated protein kinases have also been shown to perform some function in LTP (English and Sweatt, 1996).

1.2.4.2 MAP kinases and LTP

Mitogen-activated protein kinases (MAPK) are a family of serine-threonine protein kinases that act within a multi-tiered protein kinase cascade to coordinate cellular responses to external stimuli (Mielke and Herdegen, 2000).

One of the downstream consequences of an increase in cAMP concentration is activation of the MAPK, extracellular-signal regulated kinase (ERK). A role for ERK in expression of LTP was first delineated by the finding that its inhibition resulted in suppression of LTP in CA1 (English and Sweatt, 1997; Impey *et al.*, 1998) and dentate gyrus (McGahon *et al.*, 1999a). Indeed induction of LTP in dentate gyrus leads to phosphorylation and consequently activation of ERK (Davis *et al.*, 2000). ERK has a number of different substrates including the potassium channel Kv4.2 (Sweatt, 2001) and synapsin I (Jovanovic *et al.*, 1996) – either of which may help to explain its role in LTP. ERK regulation of Kv4.2 decreases its voltage-dependent activation and

consequently enhances LTP while phosphorylation of synapsin I induces vesicle movement to the active zone thereby enhancing transmitter release (Greengard *et al.*, 1993). Inhibition of ERK by PD98059 reduces KCl-stimulated release of glutamate in dentate gyrus (Gooney and Lynch, 2001; Gooney *et al.*, 2002) and results in inhibition of LTP in perforant path granule cell synapses.

Other substrates of ERK include cytoskeletal proteins MAP-2 and Tau, nuclear proteins c-Myc, c-fos and c-Jun, CREB and ATF-2 and the signalling proteins PLA_2 and ribosomal S6 kinase (RSK). The long-term effects of ERK activation involve translation and transcription. The LTP-induced increases in ERK, CREB and Elk-1 have been shown to be accompanied by activation of immediate early gene Zif268 – an effect that can be blocked by the MEK inhibitor SL327 indicating a role for ERK in activation of Zif268 (Davis *et al.*, 2000). Consistently Zif268 mRNA is rapidly induced following LTP in dentate gyrus (Cole *et al.*, 1989; Wisden *et al.*, 1990) and is attenuated by pre-treatment with PD98059 (Davis *et al.*, 2000; Rosenblum *et al.*, 2002). Activation of ERK and CREB have been implicated in the protein synthesis associated with LTP in dentate gyrus (Gooney and Lynch, 2001).

1.2.4.3 Retrograde messengers

Members of another class of signal transduction molecules related to LTP are the postulated retrograde messengers. If the locus for expression of LTP exists both pre- and postsynaptically then a retrograde messenger must be released from the postsynaptic cell to modify presynaptic function since the initial triggering of LTP clearly resides in the postsynaptic cell (Bliss and Collingridge, 1993). To be considered as a retrograde messenger, a molecule must satisfy certain criteria – (a) it must be released from the postsynaptic cell in response to tetanic stimulation or following the application of the appropriate neurotransmitter; (b) it must appear in the perfusate following induction of LTP and (c) it must act on the presynaptic terminal to evoke an increase in neurotransmitter release. Several molecules have been proposed as possible candidates including nitric oxide, carbon monoxide, arachidonic acid, NGF and platelet-activating factor (Bliss and Collingridge, 1993). NO has been the most prominent candidate being generated in a Ca^{2+}-dependent

manner in response to NMDA receptor activation. However, studies from the early 1990's have revealed that NO is not absolutely required for LTP expression (Haley *et al.*, 1993; Williams *et al.*, 1993; Cummings *et al.*, 1994). Another particularly strong candidate is arachidonic acid (AA). Williams and colleagues (1989) demonstrated that weak activation of the perforant path leads to a persistent increase in synaptic efficacy when administered in the presence of AA and this potentiation is accompanied by an increase in glutamate release. Additionally, inhibitors of phospholipase A_2 (PLA_2) which cleaves AA from phospholipids have been shown to block LTP (Williams and Bliss, 1989). Although there is a great deal of evidence indicating that retrograde signalling occurs during LTP, it is currently difficult to conclude whether any of these molecules are absolutely required for the generation of LTP.

1.2.4.4 AMPA receptors and silent synapses

One of the most important debates surrounding LTP is whether the observed increase in synaptic efficacy is a pre- or postsynaptic modification. The simplest postsynaptic change that might explain LTP would be a modification in AMPA receptor function or number, whereas the simplest presynaptic change would be an increase in the probability of transmitter release (Malenka and Nicoll, 1999). Several groups have reported that the AMPA receptor-mediated excitatory postsynaptic current (EPSC) is increased to a greater extent following LTP than is the NMDA receptor-mediated EPSC (Kauer *et al.*, 1988; Liao *et al.*, 1995). Evidence indicates that this increase in AMPA receptor responsiveness results from enhanced phosphorylation of the GluR1 subunit of the receptor by CaMKII at serine-831, which increases the single-channel conductance of homomeric GluR1 AMPA receptors (Roche *et al.*, 1996). In support of this proposal is the finding that CaMKII inhibitors block induction of LTP (Barria *et al.*, 1997).

An extensively reported finding is that following induction of LTP the proportion of synaptic failures decreases, however much evidence has indicated that the probability of transmitter release is unaffected by LTP. These results posed a difficult question – how could results that argued against an increase in transmitter

release and for a postsynaptic change explain the decrease in synaptic failures? The answer appears to lie in the existence of synapses that contain only NMDA receptors and so are functionally silent at hyperpolarised membrane potentials (Isaac *et al.*, 1995; Liao *et al.*, 1995). The change in failure rate could be explained by the conversion of these silent synapses to synapses that also express AMPA receptors without invoking any change in neurotransmitter release.

There is now reasonably strong evidence supporting the silent synapse theory. Several groups have reported recordings of EPSCs mediated only by NMDA receptors but following LTP induction AMPA receptor-mediated EPSCs can also be recorded (Isaac *et al.*, 1995; Liao *et al.*, 1995). Additionally all hippocampal synapses appear to express NMDA receptors as evidenced by immunocytochemical analysis in native hippocampal tissue and in cell cultures while only a certain proportion express AMPA receptors - NMDA receptor-dependent increases in dendritic spines of cells containing green fluorescent protein-GluR1 fusion protein have been reported (Shi *et al.*, 1999). Conversely, NMDA receptor-dependent long-term depression (LTD), a depression of synaptic function, in hippocampal cultures has been associated with a loss in synaptic AMPA receptors (Lee *et al*, 1998; Carroll *et al.*, 1999). Proteins involved in membrane fusion can interact with AMPA receptors and thus provide a mechanism for increasing the number of AMPA receptors at the synapse (Nishimune *et al.*, 1998). Proteins that interfere with membrane fusion in the postsynaptic cell impair LTP (Lu *et al.*, 1995).

The expression of LTP results from delivery of AMPA receptors to the membrane or clustering of AMPA receptors within the synaptic membrane (Shi *et al.*, 1999). These modifications may occur at synapses that already contain AMPA receptors as well as synapses that contain NMDA receptors alone.

1.2.4.5 Protein Synthesis

LTP can be divided into two phases (1) *early* LTP, which lasts between 1 and 3 hours and does not require protein synthesis and (2) *late* LTP, which is a more persistent phase that lasts for at least 24 hours and requires new protein and RNA synthesis. Recent studies have suggested that the late phase of LTP involves the

activation of additional presynaptic machinery for transmitter release and the insertion of new clusters of postsynaptic receptors as well as morphological changes that occur in the pre- and post-synaptic cell. Findings from several studies have indicated that tetanus-induced potentiation of the synaptic response in CA1 and dentate gyrus was relatively short-lived in animals injected with protein synthesis inhibitors (Krug *et al.*, 1984; Mullany and Lynch, 1997). However, actinomycin D (Otani *et al.*, 1989; Frey *et al.*, 1996), an mRNA synthesis inhibitor, exerted no effect during the first 3 hours of LTP and exerted an inhibitory effect after 5 hours suggesting a dependence on both RNA and protein synthesis at this later time point.

It is probable that the increase in protein synthesis associated with LTP contributes to the establishment of morphological changes that have been reported. These morphorlogical changes include increases in postsynaptic surface area (Desmond and Levy, 1990), spine number (Chang and Greenough, 1984) and spine area (Fifkova and Vanharreveld, 1977). LTP has been shown to be accompanied by protein synthesis in both granule cells (M.A. Casey and M.A. Lynch, unpublished data) and entorhinal cortex (Mullany and Lynch, 1998; Kelly *et al.*, 2000) and the presynaptic proteins, synapsin, synaptophysin and synaptotagmin (Lynch *et al.*, 1994), all of which are involved in vesicular trafficking and fusion. Protein synthesis in presynaptic cells of cultured CA1 and CA3 has also been demonstrated by an increase in the number of active presynaptic terminals with this effect being blocked by anisomycin – an inhibitor of protein synthesis (Krug *et al.*, 1984). These data are consistent with LTP-associated recruitment of previously 'silent synapses'.

1.2.5 LTP and stress

Stress is best described as a disturbance of physiological and psychological homeostasis ultimately controlled by activity of the hypothalamo-pituitary-adrenal (HPA) axis and resulting in secretion of corticosteroids from the adrenal cortex (Lynch, 2004). The hippocampus has the highest concentration of glucocorticoid receptors and, as such, is particularly vulnerable to stress. Interestingly, stress levels of glucocorticoids have been shown to induce a profound inhibitory effect on hippocampal cell activity (Talmi *et al.*, 1993) while low levels enhance activity (Joels

et al., 1995) – a similar effect is observed with regard to LTP (Diamond *et al.*, 1994, 1996; Pavlides *et al.*, 1996). The effects of stress are not confined to increased plasma levels of glucocorticoids but are also mediated by neurohormones and neurotransmitters. LTP is impaired by many stress paradigms including irradiation (Tolliver and Pellmar, 1987; Lonergan *et al.*, 2002) lipopolysaccharide (LPS; Vereker *et al.*, 2000a; Lonergan *et al.*, 2004), and oxidative stress (Kelly *et al.*, 2001). Cognitive deficits have been reported in aged rats particularly in spatial memory processing which is a hippocampal process (Barnes, 1988).

1.3 Ageing

1.3.1 Anatomy/neurophysiology of ageing

The brain is one of the organs most profoundly affected by ageing, with the hippocampal formation being one of the most susceptible brain regions (Hasan and Glees, 1973). Ageing is associated with impairments in learning and memory (Barnes *et al.*, 1980), effects that are comparable to impairments induced by hippocampal damage. Anatomical and electrophysiological studies indicate that hippocampal function is altered by the ageing process which particularly leads to decreases in synapse number and function (Geinisman *et al.*, 1992), NMDA-receptor mediated responses (Rao *et al.*, 1994) and an alteration in Ca^{2+} regulation (Landfield and Pitler, 1984; Campbell *et al.*, 1996). These changes may underlie the observed age-related impairments of synaptic plasticity and subsequently cognition (Jarrard, 1993). Deficits in LTP in aged rats have been reported several times (de Toledo-Morrell and Morrell, 1985; Lynch and Voss, 1994; O'Donnell *et al.*, 2000) and have been correlated with deficits in spatial information processing (Barnes *et al.*, 1980). Coupled with these changes is the finding that synaptophysin, a synaptic vesicle protein involved in transmitter release, is decreased with age (Mullany and Lynch, 1998) and this may contribute to the observed age-related decline in transmitter release. The underlying mechanism leading to age-related impairment of synaptic plasticity is unclear however much evidence has led to the development of two theories of ageing – the "free radical theory of ageing" and the "membrane hypothesis".

1.3.2 The free radical theory of ageing

In 1956, Harman proposed that reactive oxygen species (ROS) generated by metabolism may cause cumulative damage over a lifetime (Harman, 1981). The discovery of the enzyme superoxide dismutase (SOD; McCord *et al.*, 1969) and an understanding of antioxidant defences (Yu, 1994) provided considerable support for this theory. Oxidants are generated at numerous sites in the body (Beckman *et al.*, 1998). Since oxidative damage can cause considerable harm at the cellular level there are a number of antioxidant defences employed involving antioxidant enzymes and scavengers. SOD, catalase and glutathione peroxidase constitute the antioxidant enzymes that are involved in detoxification of ROS. There are three main antioxidant scavengers that contend with free radicals – glutathione, vitamin E and vitamin C. Indeed, age-related increases in accumulation of ROS and age-related changes in anti-oxidant defence systems have been reported by several groups (Yu, 1994; Gabbita *et al.*, 1997; de la Asuncion *et al.*, 1996).

ROS can cause cellular damage through the indiscriminate oxidation of proteins – a process that induces denaturation and leaves proteins susceptible to digestion by endogenous proteases (Stadtman and Berlett, 1997). Age-related increases in peroxidation of proteins in cerebral cortex and in cerebellum have been shown to be associated with a decrease in cognitive function and motor skills in mice (Forster *et al.*, 1996). Additionally, the reaction of free radicals with lipids in the membrane leads to damage of the membrane and subsequent disruption of the membrane lipid bilayer (Grinna, 1977).

Mitochondria have long been considered a primary site of oxidative damage due to its high ROS concentration (Yu, 1994), and oxidative damage has been shown to contribute to the mitochondrial dysfunction associated with age (de la Asuncion *et al.*, 1996). The age-related increase in ROS accumulation has been shown to result, at least in part, to the upregulation in activity of SOD with no concomitant changes in catalase or glutathione peroxidase (O'Donnell and Lynch, 1998) – this effect leads to the generation of O_2^- and H_2O_2 without subsequent conversion to O_2 and H_2O. Studies involving the use of transgenic mice overexpressing SOD reveal impairments in LTP with an accompanying impairment in spatial learning associated with

upregulation of this enzyme. The consequences of a chronic increase in ROS are profound and lead to lipid peroxidation (Murray and Lynch, 1998), which has a deleterious effect on the fluidity of the membrane.

1.3.3 The membrane hypothesis of ageing

An age-related decrease in membrane fluidity occurs as a result of increases in membrane concentrations of cholesterol (Zs-Nagy, 1994), sphingomyelin (Giusto *et al.*, 1992) and dicholol (Pullarkat *et al.*, 1984) coupled with decreases in membrane polyunsaturated fatty acids (Suzuki *et al.*, 1989; Lynch and Voss, 1994). Cholesterol regulates mobility of phospholipid fatty acyl chains in the membrane determining membrane fluidity/rigidity ratio (Zs-Nagy, 1994). Since cholesterol concentration and the cholesterol/phospholipid ratio are highest in hippocampus (Zhang *et al.*, 1996) the hippocampus may be particularly susceptible to age-related changes in cholesterol concentration.

In general, the 1-acyl position in phospholipids is saturated while the 2-acyl position contains unsaturated fatty acids. In brain tissue the degree of unsaturation at the 2-acyl position is extremely high and particularly rich in arachidonic acid concentration especially in hippocampus (Gatti *et al.*, 1986). This observation suggests that any alteration in phospholipid metabolism may have considerable impact in hippocampus. Phospholipid metabolism is decreased and alterations in the degree of unsaturation of the phospholipid acyl chains occur with age (Gatti *et al.*, 1986; Bazan, 1989; Fonlupt *et al.*, 1994). Incorporation of arachidonic acid into neuronal membranes is markedly impaired in aged brains (Terracina *et al.*, 1992) consistent with the observed decrease in membrane fluidity in membrane prepared from brains of aged rats. The deleterious effects of the alteration in degree of unsaturation on membrane fluidity is further compounded by the age-related accumulation of ROS which leads to lipid peroxidation of fatty acids in the membrane and consequently increased membrane rigidity (Mullany and Lynch, 1998). Consistent with the idea that accumulation of ROS impacts on membrane composition by decreasing arachidonic acid concentration is the finding that the age-related decline in spatial learning and similarly the impairment of LTP is coupled

with decreased arachidonic acid concentration in hippocampus (Lynch and Voss, 1994).

The appropriate lipid environment of the plasma membrane is critical for the optimum functioning of membrane proteins however this lipid environment is negatively affected with age and so it is not surprising that impairments in signal transduction and transmitter function have been associated with age (Mullany *et al.*, 1996; Murray *et al.*, 1997).

1.3.4 Cellular changes associated with ageing

Physiological stress induces increases in secretion of hormones such as corticosteroids from the adrenal cortex (Yau *et al.*, 2002). Stress levels of glucocorticoids have a profound inhibitory effect on learning, memory and LTP - effects that have largely been attributed to the high concentration of glucocorticoid receptors present in the hippocampus (McEwen, 1994). It is not surprising that the age-related decline in cognition is accompanied by a concomitant increase in plasma levels of corticosterone and glucocorticoids (Talmi *et al.*, 1993). However, the effects of stress and age are not confined to an increase in glucocorticoid production but include a myriad of effects on several neurohormones and neurotransmitters such as opioids, noradrenaline, adrenaline, and vasopressin – all of which modulate hippocampal function. Recent evidence has highlighted pro-inflammatory cytokines as key mediators of many forms of stress. Indeed, expression of certain cytokines such as the pro-inflammatory cytokine interleukin-1β (IL-1β) is altered by stress (Vereker *et al.*, 2001) and also with age (Murray *et al.*, 1997; Murray and Lynch, 1998). These data indicate that increased IL-1β may contribute to the stress-associated increase in corticosteroids since intrahippocmapal administration of IL-1β has been shown to result in activation of the hypothalamo-pituitary axis (HPA; Melik *et al.*, 1999).

IL-1β attenuates LTP in all major pathways of the hippocampal formation (Katsuki *et al.*, 1990; Bellinger *et al.*, 1993; Murray and Lynch, 1998) and impairs spatial learning in rats (Gibertini *et al.*, 1995). Many stress paradigms induce increases in central IL-1β concentration including behavioural stress (Vereker *et al.*,

2001), LPS administration (Vereker *et al.*, 2000a; Lonergan *et al.*, 2004) and γ-irradiation (Lonergan *et al.*, 2002). Consistent with the similarity between the effects of age and hippocampal damage on learning and memory is the finding that an increase in IL-1β also occurs with age (Murray and Lynch, 1998; Martin *et al.*, 2002). Interestingly, evidence indicates that the effects of isolation stress on SOD activity occur as a result of increased IL-1β concentration (Vereker *et al.*, 2001) suggesting that IL-1β may be a key mediator in the age-related accumulation of ROS that has been reported. Chronic treatment of aged rats with aspirin, which ameliorates age-related evidence of inflammation, improves performance in spatial learning tasks (Smith *et al.*, 2002). These data point to a central role for pro-inflammatory cytokines in the deleterious effects of age on synaptic plasticity.

Changes in membrane composition and fluidity have been described with age and occur, at least in part, as a result of the age-related changes in inflammatory processes. These changes are likely to impact on cellular function. Interestingly, NMDA receptor binding and signalling are impaired with age (Bonhaus *et al.*, 1990; Tamaru *et al.*, 1991; Ingram *et al.*, 1992) indicating that a dysregulation of calcium homeostasis may exist in the aged brain. Indeed the activities of certain calcium sensitive enzymes such as PKC and CaMKII are decreased with age (Mullany *et al.*, 1996) – enzymes which are important in the expression phase of LTP. However, pro-inflammatory cytokines induce a myriad of effects independent of their effects on the membrane and an investigation of the cellular cascades induced or modulated by cytokines may help to further elucidate the underlying mechanisms of the age-related impairments in learning, memory and LTP.

1.4 Cytokines

1.4.1 Cytokine classfication and function

Cytokines encompass three broad groups of proteins – growth factors such as epidermal and hepatocyte growth factor, immunomodulatory cytokines such as the interleukins and interferons, and chemokines such as macrophage inflammatory proteins. Cytokines are signalling proteins, produced transiently after cellular activation, that act as humoral regulators which modulate the functions of individual

cells under normal, developmental and pathological conditions (Dinarello *et al.*, 1990; Meager, 1998). These proteins are produced and act locally via specific receptors located primarily on the surface of their target cells (Miyajima *et al.*, 1992). Cells producing cytokines are not organised into specific glands as in the case of hormones but act systemically to affect inflammation, wound healing, organogenesis and oncogenesis (Foster, 2001). While hormones ensure the efficient function of normal tissues and the whole organism, cytokines with a physiological role in the circulation are concerned with restoring normal function to the tissue in which they were produced. When tissues are severely challenged, and larger concentrations of cytokines enter the systemic circulation, they may be responsible for altering systemic homeostasis through induction of fever, sickness behaviour, cachexia and a variety of endocrine hormone imbalances (Waage *et al.*, 1989; Slifka and Whitton, 2000). Cytokines have important roles in chemically-induced tissue damage repair, cancer development; regression, control of cell replication and apoptosis and in the modulation of immune reactions.

The term cytokine was initially derived from the Greek *kytos* (meaning 'hollow') and *kinein* (meaning 'to move') to separate the classical immunomodulatory cytokines from growth factors, however, evidence over the last few decades has revealed a considerable overlap in function between growth factors and cytokines (Foster, 2001). Chemokines are the third group associated with this family of proteins and act as soluble chemo-attractant cytokines concerned with the recruitment and activation of immune and inflammatory cells to sites of cell damage (Meager, 1998). Different cytokines can exert similar effects on a single cell type - a phenomenon that allows one factor to compensate for another (Foster, 2001). Additionally most cytokines induce multiple effects in multiple cell types – this ubiquitous activity makes it difficult to delineate the role of specific cytokines in the regulation of normal physiology (Foster, 2001). Similarly, a single cytokine may induce opposing effects under different circumstances (Sun *et al.*, 1999) depending on the growth state of the recipient cells, the extracellular environment determined by neighbouring cells, cytokine concentration and combinations of other cytokines present (Hasbold *et al.*, 1999).

Interaction of cytokines with specific cell surface membrane receptors is required for modulation of biological activity. The precise mechanisms invoked by cytokine binding at receptor sites is not clearly understood, however most cytokine binding induces a conformational change or oligomerisation of receptors which allows the intracellular domain of the receptor to interact with accessory molecules. In all cases, receptor binding results in the activation of downstream effectors and activation of protein kinases which phosphorylate protein substrates (Foster, 2001). There are two major pathways of signal transduction involved in cytokine activation – the first pathway, used by mitogenic cytokines utilises tyrosine kinases as the signal transducers, either directly or indirectly linked to the intracellular domain of the receptor. The second pathway involves activation of phospholipases that produce small mediators that activate serine-threonine kinases. Different cytokines and their receptors can share common signal transduction pathways – a finding that explains the functional redundancy between family members and the pleiotropic response that some cytokines elicit (Foster, 2001). Regulation of cytokine function is not clearly understood, however there exists a hierarchical order of cytokine actions such that some cytokines preactivate cells so as to respond to later acting cytokines (McKay *et al.*, 2000).

1.4.2 Pro-inflammatory cytokine signalling

Immunomodulatory cytokines such as the interleukins and interferons can be subdivided into two classes – the class I cytokines which are considered to be pro-inflammatory cytokines and the class II cytokines which have anti-inflammatory properties. As indicated by their name pro-inflammatory cytokines promote inflammation and anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines; they suppress genes for pro-inflammatory cytokines such as IL-1 and interferon-γ (IFNγ). While IFNγ possesses anti-viral activity its ability to augment pro-inflammatory cytokine activity and induce NO have lead to its classification as a pro-inflammatory cytokine (Dinarello, 2000).

1.4.2.1 IL-1β - superfamily and receptors

One of the most investigated pro-inflammatory cytokines in terms of its effects in the CNS is IL-1β. Since cytokines exert profound effects on cellular (and in some cases endocrine) homeostasis it is not surprising that communication between the CNS and the endocrine system exists. One proposed mediator of this type of communication is IL-1β, a cytokine capable of modulating hypothalamo-pituitary-adrenal (HPA) activity (Sapolsky *et al.*, 1987). IL-1β is produced by most immune cells but also by glia and neurons in the CNS (Breder *et al.*, 1988; Sheng *et al*, 1998; Pearson *et al.*, 1999). IL-1 exerts its effects by binding to high affinity receptors expressed on many cell types including neurons, astrocytes, microglia and tissue macrophages in addition to immune cells (Takao *et al.*, 1990; Ban *et al.*, 1991; Dower *et al.*, 1992). Indeed, the IL-1 type I receptor is expressed in highest density in hypothalamus (Hammond *et al.*, 1999) highlighting a role for IL-1β in neuroendocrine modulation. Classically, the IL-1 superfamily was considered to consist of three members – IL-1α, IL-1β and the endogenous receptor antagonist IL-1ra (Dinarello, 1996). IL-1α and IL-1β exist as precursors of molecular weight 31kDa and are cleaved to an active 17kDa form by IL-1β-converting enzyme (ICE; caspase-1). IL-18 has since been added to this family due to the discovery of its existence as a large precursor molecule that must be cleaved by ICE to yield an active form. Recently four novel genes (FIL-1δ, FIL-1ε, FIL-1ξ and FIL-1η; FIL for 'family of IL-1') have been cloned which have been added to the IL-1 family as a result of the sequence homology that exists between them (Smith *et al.*, 2000).

Two primary receptors for IL-1β have been identified – IL-1 receptor type I and type II (IL-1RI and IL-1RII; Sims *et al.*, 1988; McMahan *et al.*, 1991). IL-1β must interact with IL-1RI to exert its biological effects. IL-1RI is an 80kDa receptor that binds IL-1α and IL-1β with high affinity; it also binds IL-1ra however unlike IL-1α and IL-1β, IL-1ra binding fails to recruit IL-1 receptor accessory protein (IL-1RAcP) and therefore cannot activate signalling cascades induced by the other two IL-1 isoforms (Dinarello, 1997a, 1997b). In this manner, IL-1ra serves as an endogenous antagonist to IL-1RI by preventing interaction of IL-1α and IL-1β with IL-1RI (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). Recruitment of IL-1RAcP to the IL-1RI

forms the first step in a cascade that results in formation of a complex involving IL-1RI, IL-1RAcP, the adaptor proteins MyD88, MyD88-adaptor-like (MAL) and Tollip and IL-1 receptor-associated serine/threonine kinase (IRAK; Wesche *et al.*, 1997a, b). The IRAKS are a family of 4 kinases – two active (IRAK-1 and IRAK-4) and two inactive (IRAK-2 and IRAK-M) kinases that are involved in IL-1 and tumour necrosis factor (TNF) signalling. On IL-1 binding to IL-1R1, IRAK-1 is phosphorylated possibly by IRAK-4 and dissociates from the receptor complex, associating with the signal transducer TNF receptor-associated factor 6 (TRAF6; Cao et al., 1996) to mediate activation of nuclear factor (NF)κB and mitogen-activated protein kinases (MAPK).

The second receptor for IL-1 is IL-1RII, which exists in two forms – as a soluble or membrane-bound protein. The soluble form, IL-1RII, appears to act as a decoy receptor by reducing free IL-1 concentration and limiting its interaction with IL-1RI (Colotta *et al.*, 1994, 1995). Recent evidence has indicated that upon IL-1 binding IL-1RII recruits IL-1RAcP and in this way may modulate IL-1RI function (Malinowsky *et al.*, 1998). Other receptors for IL-1 have been identified including IL-1Rrp1, IL-1Rrp2, T1/ST2, IL-1RAcP, IL-18RAcP, SIGIRR and IL-1RAPL (O'Neill and Dinarello, 2000). In the context of this study it is important to note that IL-1RmRNA has been identified in mouse hippocampus by autoradiography (Parnet *et al.*, 1994) and IL-1RI protein has been detected in rat hippocampus (Lynch *et al.*, 2003).

1.4.2.2 IL-1β-induced signalling cascades

Expression of IL-1β has been shown to be high in hippocampus and IL-1β-induced impairment in LTP has been consistently reported (see Section 1.2.3 LTP and stress). Precisely how IL-1β exerts its deleterious effect on plasticity is unclear, however, a huge number of molecules become activated in response to IL-1β. Among the downstream effects of IL-1β stimulation is activation of the mitogen-activated protein kinases (MAP kinases), c-Jun N-terminal kinase (JNK) and p38. IL-1β increases activity of p38 in Chinese hamster CC139 (Guay *et al.*, 1997) and HeLa (Raingeaud *et al.*, 1995) cells and IL-1β-induced activation of JNK has been reported in human glomerular mesangial (Uciechowski *et al.*, 1996) and HeLa (Raingeaud *et*

al., 1995) cells. The complex formed upon binding of IL-1β to IL-1RI requires activation of TNF receptor-associated factor-6 (TRAF-6) to exert its downstream effects (Figure 1.3) – data from studies of mice deficient in MyD88, IRAK or TRAF-6 have identified these signalling proteins in IL-1β-induced signalling cascades (Kawai *et al.*, 1999; Naito *et al.*, 1999; Thomas *et al.*, 1999). The functions of JNK and p38 remain largely unclear however they have both been implicated in the neuronal stress response (Mielke and Herdegen, 2000). Together with ERK, the JNK and p38 form the MAP kinases – a family of proteins involved in modulation of neuronal function.

1.4.2.3 MAP Kinases

While the physiological functions of MAP kinases are relatively unknown, there is compelling evidence that JNK and p38 are potent effectors of apoptosis and degeneration of neurons *in vitro* and *in vivo* (Mielke and Herdegen, 2000). They are activated in response to a number of cell stresses, including deprivation of trophic factors, ionising radiation, free radicals, hypoxia ischaemia, heat shock, production of lipid messengers such as ceramide or activation of death domain receptors (Mielke and Herdegen, 2000). These potentially deleterious stimuli can provoke intracellular signalling that results either in apoptosis or protective adaptations (Herdegen *et al.*, 1997).

1.4.2.4 JNK isoforms

The JNK kinases are highly conserved during evolution with an amino acid homology of more than 90% in mammals. JNK isoforms are encoded by three separate genes *jnk1*, *jnk2* and *jnk3*. The *jnk1* and *jnk2* genes are ubiquitously expressed while expression of the *jnk3* gene is confined to the brain, testis and heart (Mielke and Herdegen, 2000). The transcripts of each of these genes can give rise to a variety of splicing products that differ subtly in their interactions and in the catalytic alteration of their substrates *in vitro* (Gupta *et al.*, 1996).

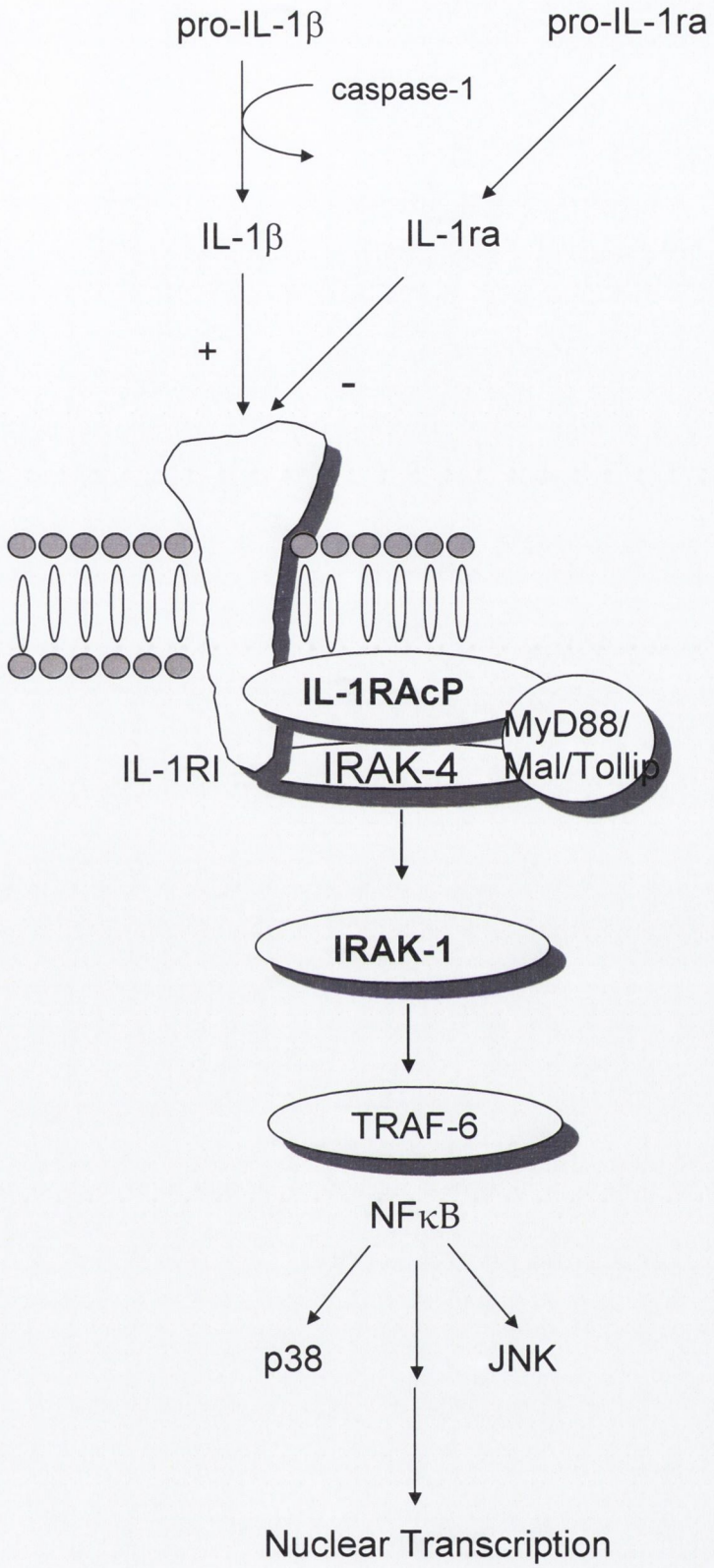


Figure 1.3 Events in interleukin-1β signalling

Schematic representation of the current view of signalling events, which occur following

1.4.2.5 Substrates of JNK

JNK phosphorylates a variety of cytoplasmic substrates including cytoskeletal proteins, p53 (Fuchs *et al.*, 1998) and Bcl-2 (Maundrell *et al.*, 1997). Many of these substrates are essential to the physiological function and apoptotic action of JNK in the nervous system. JNK antagonises the anti-apoptotic function of Bcl-2 compromising the integrity of the mitochondrial membrane (Park *et al.*, 1997). The JNK signalling pathway also results in stabilisation and modulation of tumour suppressor p53 (Fuchs *et al.*, 1998), a pro-apoptotic transcription factor that suppresses Bcl-2 and enhances Bax induction (Miyashita *et al.*, 1994). These last two findings implicate JNK as initiator of a pro-apoptotic cascade mediated by mitochondrial dysfunction.

Additionally JNK has a number of nuclear substrates. JNK is the only known kinase that can phosphorylate the inducible transcription factor c-Jun at serine 63 and 73 *in vivo* (Gupta *et al.*, 1996). JNK also catalyses the phosphorylation of other nuclear substrates such as transcription factors ATF-2 (Gupta *et al.*, 1995) and Elk-1 (Cavigelli *et al.*, 1995). Following dissociation in the cytoplasm and nuclear translocation, JNK associates with its substrates. Subsequent to substrate activation, JNK can dissociate from its substrates. However it is unclear whether it returns to the cytoplasm or is degraded in the nucleus (Kallunki *et al.*, 1996).

JNK functions as a mediator of neuronal cell death and degeneration in response to a number of stressful stimuli including excitotoxicity, ischaemia, withdrawal of trophic factors, oxidative stress, cytokines e.g. IL-1, Fas ligand, UV irradiation and many others (Mielke and Herdegen, 2000). Several of these stressful stimuli are potent inducers of cell death with concomitant activation of JNK and c-Jun phosphorylation. However, the role of c-Jun as a downstream target of JNK and other stress-activated signal pathways in brain remains to be precisely elucidated. c-Jun can be activated independently of JNK possibly through a calcium-triggered mechanism independent of c-Jun phosphorylation (Cruzalegui *et al.*, 1999). This finding provides evidence that c-Jun is a nuclear effector of other signalling cascades e.g. Ca^{2+}-regulated signal cascades. It has been demonstrated that degeneration and

apoptosis induced by JNK are mediated by c-Jun e.g. in degenerating hippocampal neurons following application of kainic acid (Yang *et al.*, 1997; Behrens *et al.*, 1999).

1.4.3 Interferon-γ

Interferon-γ (IFNγ) was first described in 1965 by Wheelock and is one of the most intensively studied cytokines to date. IFNγ is a 40kDa homodimeric cytokine expressed primarily by T helper lymphocytes type 1 (Th1) but also by cytotoxic T cells and natural killer cells (Boehm *et al.*, 1997) and has an immunoregulatory role. Following IFNγ binding to its receptor, the α and β-chains dimerise leading to phosphorylation of the associated JAK1 and JAK2 kinases resulting in receptor phosphorylation. Phosphorylation of the receptor triggers the recruitment and phosphorylation of 'signal transducer and activator of transcription' 1 (STAT1) proteins that subsequently dissociate from the receptor and undergo nuclear translocation. Activated STAT1 homodimers bind to DNA sequences in promoters of IFNγ inducible genes containing the gamma-activating site (GAS) motif and thereby activate transcription (Decker *et al.*, 1991; Lew *et al.*, 1991). One of the primary genes activated by STAT1 in response to IFNγ is interferon regulatory factor 1 (IRF-1) which is itself a transcription factor that can bind to promoter regions in many IFNγ-inducible secondary genes (Pine *et al.*, 1994; Figure 1.4). Some of the genes upregulated by stimulation with IFNγ include inducible nitric oxide synthase (iNOS; Harris *et al.*, 1995), caspase-1 (Kim *et al.*, 2002), major histocompatibility complex I (MHC-I; Molina and Huber, 1991) and ICAM-1 (Renkonen *et al.*, 1992).

IFNγ has many functions but some of the most important include macrophage activation, Th-cell differentiation and stabilisation, MHC expression and anti-viral effect (Belardelli, 1995; Boehm *et al.*, 1997). Consequently, IFNγ has a considerable effect on the production of other pro-inflammatory cytokines through its stabilisation of Th1 cells and its activation of macrophages – cells which are both capable of synthesising and releasing pro-inflammatory cytokines in response to stressful stimuli. Additionally, IFNγ antagonises the functions of the anti-inflammatory cytokine IL-4 (Nakamura *et al.*, 1997).

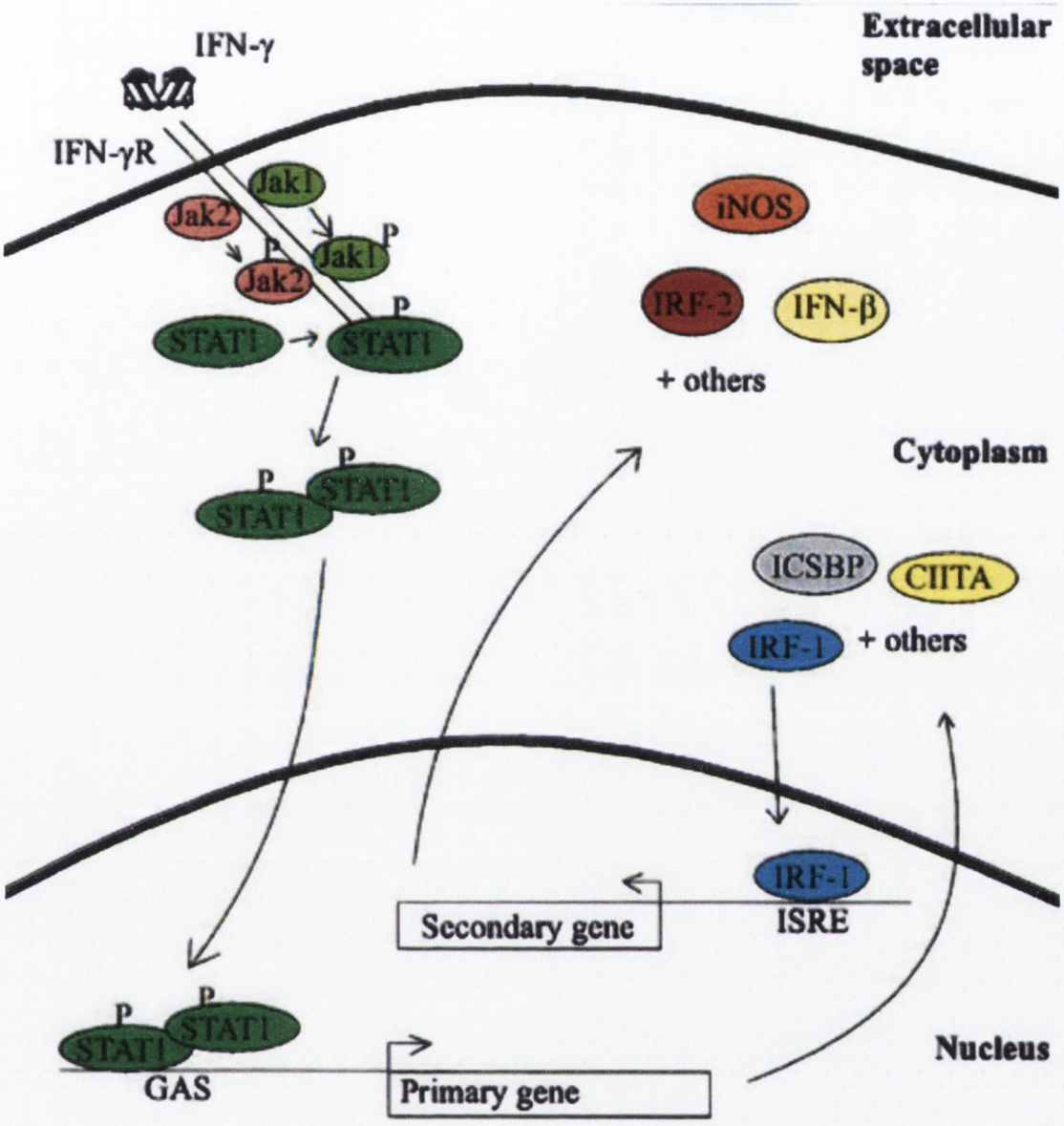


Figure 1.4 Events in interferon-γ signalling

Schematic representation of the current view of signalling events, which occur following interferon-γ (IFNγ) interaction with IFNγ receptor.

(Adapted from Paludan, S.R., (1998) *Scandinavian Journal of Immunology*, **48**, 459-468).

1.4.4 Anti-inflammatory cytokines

The regulation of immune responses is accomplished by a network of soluble molecules, including cytokines (Oppenheim and Neta, 1994; Kishimoto *et al.*, 1994) that can function in a synergistic or antagonistic manner. Th cells secrete distinct subsets of cytokines (Mosmann *et al.*, 1986) with the Th1 cells having a bias towards secretion of pro-inflammatory cytokines and Th2 cells secreting anti-inflammatory cytokines such as IL-10, IL-5 and IL-4. Th2 derived cytokines promote humoral immunity and oppose Th1-dependent activities. Anti-inflammatory cytokines activate a number of signalling pathways that influence gene expression and can result in the suppression of pro-inflammatory cytokines, chemokines and certain cell surface molecules. Anti-inflammatory cytokines interact with their respective receptors in a similar manner to pro-inflammatory cytokines and the JAK-STAT signalling pathways are also employed to induce downstream effects (Keegan *et al.*, 1994).

IL-4 was originally described as a B-cell stimulating factor (Howard *et al.*, 1982) inducing B-cell activation and proliferation. Since then the number of functions attributed to IL-4 has increased and includes modulation of macrophage functions and T-cell differentiation. IL-4 activates at least four distinct signalling pathways to influence gene expression – phosphatidylinositol-3 kinase (Hirasawa *et al.*, 2000), phosphorylation of insulin receptor substrates by IL-4Rα chain (Zamorano *et al.*, 1996), activation of Ras/MAP kinases including ERK (David et al., 2001), and activation of the JAK/STAT pathway (Keegan *et al.*, 1994).

Binding of IL-4 to its receptor results in the translocation of STAT-6 to the nucleus (Nelms *et al.*, 1999) through a similar mechanism to that described for IFNγ. Once in the nucleus, STAT-6 binds to STAT-binding elements to activate gene transcription (Decker *et al.*, 1997) Genes induced by IL-4 include IL-4Rα, IL-1ra, IL-4 and MHC II (Paludan, 1998). Additionally IL-4 inhibits activation of genes associated with inflammation including IL-1β, IL-12, tumour necrosis factor-α (TNFα) and iNOS which generates NO (Paludan, 1998). While the mode of action of IL-4 is largely unknown many of these activities involve STAT-6 activation and translocation. Ultimately, IL-4 binding results in the suppression of macrophage activity and the

differentiation of Th cells towards Th2 cell type further promoting the bias towards anti-inflammatory cytokines (Paludan, 1998).

1.4.5 Cytokines and neurodegenerative events

Cytokines are involved in the communication of systemic injury, infection, inflammation, modulation of responses to peripheral nerve injury, control of behaviour, mediation of physiological sleep and synaptic plasticity and in the progression or inhibition of neurodegeneration to the CNS (Merrill and Benveniste, 1996). Cytokines affecting the CNS have two possible origins: (1) they originate from peripheral immune organs and cross the blood-brain barrier or (2) they are produced by glial cells and certain neurons. These inflammatory responses, generally characterised by a pronounced activation of glia aim to remove pathogenic triggers and inhibit the neurodegenerative process. However, instigation of an uncontrolled inflammatory response may promote the neurodegenerative process (Viviani *et al.*, 2004). In general it is accepted that pro-inflammatory cytokines exacerbate and sustain neurodegenerative processes while anti-inflammatory cytokines promote regeneration, protection and cell survival. Within the CNS, pro-inflammatory cytokine expression increases after damage resulting from exposure to neurotoxic agents (Viviani *et al.*, 1998, 2001; Sriram *et al.*, 2002) or from the onset of diseases such as Alzheimer's disease (Griffin *et al.*, 1989) and brain injury.

1.5 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the elderly population. AD is a chronic neurodegenerative disease that causes progressive deterioration in a broad range of cognitive functions including language, visual-spatial abilities and characteristically memory (McKhann *et al.*, 1984). One of the hallmarks of AD is the accumulation of amyloid-β (Aβ) peptide in the brain and its deposition as extracellular plaques, AD is also characterised by the presence of intraneuronal neurofibrillary tangles – observations first described buy Alois Alzheimer in 1907. Loss of neurons and synapses in the neocortex, hippocampus and other subcortical regions of the brain are also common features of AD. The

mechanisms underlying progression of the disease are largely unknown however a dysregulation in the control of a number of different signalling pathways has been reported.

1.5.1 Neuropathology

AD is histopathologically characterised by the presence of numerous senile plaques, neurofibrillary tangles and marked atrophy of the brain (Katzman and Saitoh, 1991). The mature senile plaque consists of a central core of extracellular Aβ (Glenner and Wong, 1984; Masters *et al.*, 1985; Glenner, 1988) surrounded by degenerating nerve endings. Additonally, neurons exhibit fibrillary accumulation in the cytoplasm, including neurofibrillary tangles (NFT; neurofibrillary pathology in cell bodies and proximal dendrites), neutrophil threads (filamentous accumulation in dendrites) and dystrophic neuritis (filament-containing neuronal processes). NFTs consist of paired helical filaments composed of hyperphosphorylated micro-tubule-associated protein tau (Grundke-Iqbal *et al.*, 1986, Kosik *et al.*, 1986; Nukina and Ihara, 1986; Wood *et al.*, 1986). Although classically defined inflammation, which includes oedema and neutrophil invasion, is not a characteristic feature of the AD brain, numerous acute-phase reactants and immune-related markers are present with Aβ deposition. Microglial activation is central to the inflammatory response in AD (McGeer and McGeer, 1995). Loss of synapses and neurons has been reported in AD and synaptophysin density is markedly reduced (Terry *et al.*, 1991). Intracellular neurofibrillary lesions correlate well with dementia (Arriagada *et al.*, 1992; Neve and Robakis, 1998) while dysfunction of cholinergic neurons occurs early in the disease progression and appears to contribute to cognitive deficits (Coyle *et al.*, 1983). Mechanisms leading to the deposition of Aβ and the generation of NFTs are not known, however, some risk factors for the disease have been identified including mutations of Aβ-precursor protein (APP), apolipoprotein E (ApoE; Strittmatter *et al.*, 1993) and presenilins 1 and 2 (Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995). These genes exert their effects on the processing of APP to Aβ leading to enhanced Aβ production.

1.5.2 Physiological functions of Amyloid-β precursor protein

Aβ is processed from the amyloid-β precursor protein (APP)- a transmembrane glycoprotein with a large extracellular domain, a membrane-anchoring domain and a short intracellular C-terminal widely distributed in the CNS and peripheral tissues. In brain, APP is expressed primarily by neurons. Three different isoforms exist encoding proteins of three different amino acid lengths – 695, 751 and 770. APP695 is the most abundant form in the brain. Whilst a link between abnormal APP metabolism and AD pathology has been established, the question of the normal biological function of APP remains largely unanswered – a number of theories have been postulated. Firstly, it has been identified as having a regulatory role in cell growth and adhesion (Schubert *et al.*, 1989). APP has been found colocalised with other cell adhesion molecules such as β-1-integrin. Additionally, APP has a number of heparin, laminin and collagen binding domains that may mediate its role in cell adhesion (Turner *et al.*, 2003). APP is produced and axonally transported to both peripheral and central synapses. Studies in neonatal rat brain have shown APP to be localised to growing tips of nerve fibers (Masliah *et al.*, 1992) – suggesting involvement of APP in presynaptic structure and function. Studies from APP transgenic mice have highlighted the importance of APP in synaptic transmission and plasticity – APP null mice show gliosis (Zheng *et al.*, 1995), decreased expression of synaptophysin in neocortex and hippocampus, decreased dendritic lengths, decreased survival of cultured neurons and impaired LTP (Seabrook *et al.*, 1998). APP null mice develop age-dependent deficits in cognitive function and impairments in LTP (Seabrook *et al.*, 1999) suggesting that normal APP may serve an essential role in the maintenance of synaptic function during ageing. Compromise of this important function of APP may exacerbate the Aβ-induced progression of memory decline and neurodegenerative changes observed in AD. In this way, enhanced cleavage of APP, as occurs in AD, may have a detrimental effect on cognition in two ways – through the increased production of neurotoxic Aβ peptides and the concomitant decreased expression of neuroprotective full-length APP.

APP is one of a multigene family that contains at least two other homologues known as amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2; Wasco *et al.*, 1992; Sprecher *et al.*, 1993; Slunt *et al.*, 1994). The APLPs contain most of the domains and motifs of APP, including a hydrophobic membrane spanning region, N-glycosylation sites, copper and zinc binding domains and a Kunitz-type protease inhibitor domain (only APLP2). Considerable functional redundancy exists between these family members probably as a result of the considerable sequence homology. 80% of APP/APLP2 knockout mice die within the first week after birth suggesting the requirement of these proteins for early postnatal development (von Koch et al., 1997). The APLPS undergo the same secretory/cleavage pathway as APP (Slunt *et al.*, 1994).

APP may also function as a G-protein coupled receptor as it is coupled to a G_o subunit and consequently may affect signalling cascades which require activation of adenylyl cyclase (Carter and Medzihradsky, 1993) and PLC (Moriarty *et al.*, 1990) while modulating activation of voltage-dependent Ca^{2+} channels (Hescheler *et al.*, 1987) and apoptotic pathways (Giambarella *et al.*, 1997). No endogenous ligand has yet been identified which can bind APP.

1.5.3 Processing of APP

APP is processed by two distinct proteolytic pathways – the non-amyloidogenic pathway generating soluble APPα (sAPPα; Figure 1.5) and the amyloidogenic pathway yielding Aβ peptides (Figure 1.5, Selkoe, 2001). APP is cleaved by a number of different proteases termed secretases. α-Secretase cleaves APP in its extracellular domain between Lysine-613 and Leucine-614 of the peptide sequence – a region which lies within the Aβ sequence of APP and thus cleavage by α-secretase precludes the liberation of Aβ peptides. Processing of APP in this manner generates sAPPα which has a number of neuroprotective properties (Turner *et al.*, 2003). The remaining carboxy-terminal fragment can be cleaved by γ-secretase yielding a highly hydrophobic fragment (p3) and a carboxy-terminal fragment – the roles of both of these remain largely unknown. A number of different proteins have been proposed as candidates for α-secretase including TNFα-converting enzyme

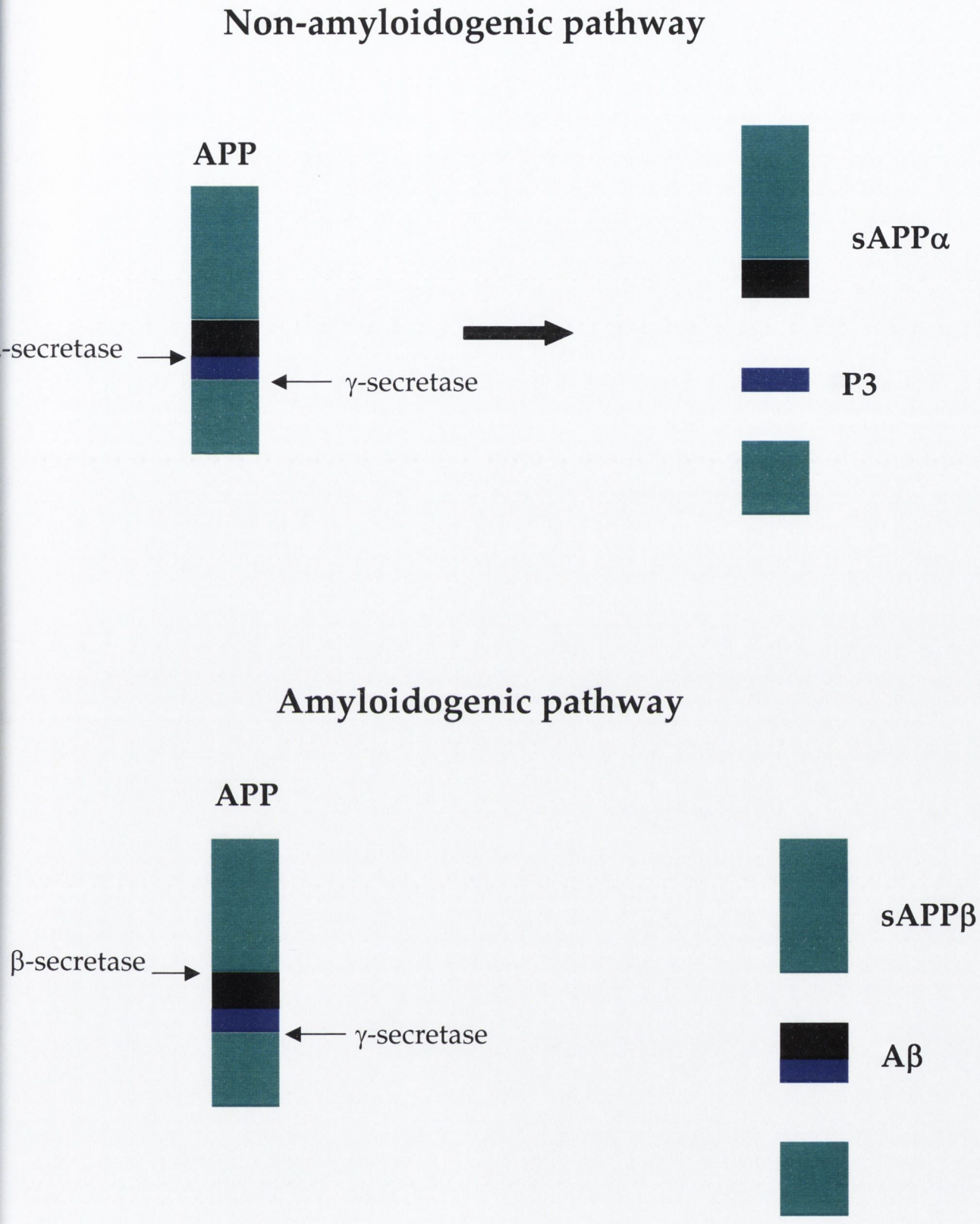


Figure 1.5 Processing of amyloid-β precursor protein

Schematic representation of non-amyloidogenic and amyloidogenic processing of amyloid-β precursor protein (APP).

(TACE; Black *et al.*, 1997), which releases TNFα from its receptor form. The α-secretase pathway is sensitive to PKC activity (Nitsch *et al.*, 1997) and cAMP-mediated signals (Kirasov *et al.*, 1997).

The amyloidogenic proteolytic pathway requires the concerted actions of two enzymes to generate Aβ peptides. The first of these is β-secretase which cleaves at position 596 and 597 of APP695. Cleavage at this site liberates sAPPβ whose proliferative effects are 100-fold less potent than those of sAPPα. γ-Secretase is then able to cleave APP within the cell membrane to liberate Aβ peptides. A number of different candidates have been proposed as β-secretases including the aspartyl proteases β-APP cleaving enzymes 1 (Yan *et al.*, 1999) and 2 (Farzan *et al.*, 2000) and carboxypeptidase B (Matsumoto *et al.*, 2000). Candidates proposed as γ-secretases include presenilins (Wolfe *et al.*, 1999) and nicastrin (Esler *et al.*, 2002) – evidence indicates that activity of γ-secretases depends upon the formation of a complex (Leem *et al.*, 2002). APP molecules can be cleaved within cellular compartments such as the Golgi apparatus, and to a lesser extent the endoplasmic reticulum - Aβ peptides generated in these intracellular spaces can secreted into the extracellular space (Greenfield *et al.*, 2000). The majority of secreted peptides are 40 amino acids in length (Aβ1-40) although a smaller fraction have received greater attention due to the propensity of these peptides to nucleate and drive production of amyloid fibrils (Jarrett *et al.*, 1993).

1.5.4 Presenilins

Mutations in two novel genes, presenilin 1 and 2, have been causally associated with the pathology of AD. These transmembrane proteins appear to be essential for normal somite and skeletal formation (Yankner, 1996). The absence of PS1 (in knockouts) may be the direct cause of loss of neural progenitor cells and neurons during development (Handler *et al.*, 2000). These presenilins are expressed at high levels in regions that are affected by AD such as hippocampus, cerebral cortex and amygdala. PS1 can alter the processing of APP toward Aβ42 formation. There have been a number of suggestions made to explain this fact. Presenilins may be involved in the regulation of APP intracellular trafficking thereby guiding APP to

compartments of γ-secretase activity (Naruse *et al.*, 1998). Alternatively an interaction between the two proteins in the endoplasmic reticulum offers the simplest mechanistic explanation for the modification of APP processing (Cook *et al.*, 1997). Presenilins may modify the structure of the APP/presenilin complex thereby changing either directly APP cleavage or interfering with APP trafficking. Some groups have suggested that PS1 may actually be γ-secretase (Wolfe *et al.*, 1999). Besides modification of APP processing presenilins may have additional mechanisms that contribute to neuronal degeneration.

1.5.5 Aβ and neurotoxicity

Recent lines of experimental evidence have indicated that excessive amounts of Aβ are deleterious to neuronal function, aside from its proposed neurotoxic effects. Addition of Aβ, in various aggregation states, to neuronal preparations has been shown to impact on electrophysiological activity (Cullen *et al.*, 1997; Hartley *et al.*, 1999; Freir *et al.*, 2001). However, the findings of these studies must be examined within the context of the difficulties in working with peptides whose biological properties depend on aggregation states and peptide size and composition (Walsh *et al.*, 1999; Fezoui *et al.*, 2000). Additionally the relevant subcellular sites and pathophysiological concentrations of Aβ that exist are unknown and therefore difficult to mimic with application of exogenous Aβ. Studies involving the use of animals overexpressing APP have limited capacity to yield information on the effects of Aβ alone since a number of domains within APP have been reported to have important physiological functions (Cao and Sudhof, 2001; Kamal *et al.*, 2001).

Natural oligomers of human Aβ are formed soon after the generation of the peptide and microinjection of cell medium containing these oligomers markedly inhibits hippocampal LTP in rats *in vivo* (Walsh *et al.*, 2002). The underlying mechanism through which Aβ impairs neuronal function is not well understood however recent findings indicate that Aβ impacts upon the fluidity of cell membranes and subcellular fractions through perturbation of the acyl-chain layer (Muller *et al.*, 2001). Other data indicates that Aβ has an amplifying effect on cellular Ca^{2+} signalling and also induces oxidative stress (Mattson *et al.*, 1993; Small and

McLean, 1999; Kawahara and Kuroda, 2000). Elucidation of the signalling pathways activated by Aβ depends on investigation of the receptors and binding proteins with which Aβ interacts.

1.5.6 Aβ receptors/binding proteins

Numerous receptors and proteins have been proposed to bind to Aβ but the subsequent signalling events have not been fully delineated. Given that Aβ impacts upon numerous cellular cascades the cellular response is extremely complex and consequently the impact of any one pathway is difficult to isolate. Much evidence indicates a strong relationship between the deterioration of brain lipid homeostasis, vascular changes and the pathogenesis of AD. Both the generation and clearance of Aβ are regulated by cholesterol – elevated cholesterol levels increase Aβ in cellular and animal models of AD and drugs that inhibit cholesterol synthesis lower Aβ in these animals (Puglielli *et al.*, 2003). Disribution of cholesterol within neurons impacts on Aβ biogenesis. ApoE is one of the major apolipoproteins in the plasma and the principal cholesterol carrier in the brain. Three alleles of this carrier exist in the brain e2, e3 and e4 – the latter being consistently confirmed as a prevalent risk factor for AD and deposition of Aβ being most severe with this allele. It has been suggested that the e4 allele of ApoE binds Aβ with highest affinity and may increase the rate of Aβ aggregation extracellularly. Additionally it may enhance the internalisaton of Aβ and thus promote its intracellular aggregation, however the mechanims underlying the link between ApoE and AD remain controversial. The class A scavenger receptors (El Khoury et al., 1996) and the serpin-enzyme complex receptor (Boland et al., 1996) also bind Aβ however in these cases Aβ is believed to be internalised and degraded.

AD patients show marked decreases in cortical nicotinic cholinergic receptor (nAchR) binding and a selective loss of certain subtypes of nicotinic receptors. Aβ can bind several subtypes of nAchR (Dineley et al., 2001) and induce several effects – persistent activation of ERK followed by downregulation of this MAP kinase may explain the ability of Aβ to impair memory, internalisation and cellular accumulation of Aβ and inhibiton of glutamate uptake by astrocytes which may induce

excitotoxicity and oxdative stress through persistent activation of the NMDA receptor.

Finally the receptor for advanced glycation end-products (RAGE) present on both microglial and neurons has been purported to mediate cellular effects of Aβ - activation of RAGE can induce oxidative stress and production of free radicals and pro-inflammatory cytokines mediated by MAP kinases (Schmidt *et al.*, 2001).

1.6 Apoptosis

Several lines of evidence point towards a role of apoptosis or general cellular stress in the massive neuronal loss observed in AD pathology. In AD brains there is abundant staining for fragmented DNA in both neuronal and glial cells of the hippocampus (Smale *et al.*, 1995). The demonstration that Aβ peptides are neurotoxic in cell culture gave rise to the hypothesis that Aβ may be a primary cause of neuronal degeneration in AD (Yankner *et al.*, 1989). Elucidation of the molecular mechanism of Aβ neurotoxicity has been confounded by the fact that Aβ has many effects on cells in culture. Aβ can potentiate excitatory amino acid (EAA)-induced toxicity (Kaneko *et al.*, 2000), glucose deprivation (Copani *et al.*, 1991) and oxidative stress (Lockhart *et al.*, 1994). However the direct toxicity of Aβ is not mediated by EAAs. Aβ has the ability to impair mitochondrial redox activity and increase the generation of free radicals (Behl *et al.*, 1994). Neuronal apoptosis may be a consequence of persistent binding and activation of cell surface receptors by amyloid fibrils (Kang *et al.*, 1987). Alternatively, Aβ may initiate an inflammatory response through microglial activation and production of pro-inflammatory cytokines. Activated microglia that express complement receptors and major histocompatibility antigens are closely associated with amyloid plaques (McGeer *et al.*, 1994). AD has been associated with increased expression of IL-1 – both elevated tissue levels of IL-1 and increased numbers of glia immunoreactive for IL-1 have been reported (Griffin *et al.*, 1989). Aβ may also act by forming a membrane pore, leading to increased ion influx in artificial lipid membranes (Arispe *et al.*, 1993). However, it remains to be determined whether Aβ neurotoxicity is mediated by a single central mechanism or by several mechanistically distinct pathways.

1.6.1 Mitochondrial induction of apoptosis

There is accumulating evidence that mitochondria play an essential role in many forms of apoptosis (Green and Reed, 1998) by releasing apoptogenetic factors such as cytochrome *c* (Yang *et al.*, 1997) from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis. The Bcl-2 family of proteins is thought to regulate mitochondrial changes (Fu *et al.*, 2002).

Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphoma. At least 19 Bcl-2 family members have been identified in mammalian cells (Zimmermann *et al.*, 2001). Each of these members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1-4). The members can be subdivided into three catagories depending on their structure and function. (1) anti-apoptotic members such as Bcl-2, Bcl-X_L and Bcl-w each of which possess anti-apoptotic activity and contain at least one BH1 and one BH2 domain. (2) Pro-apoptotic members such as Bax, Bak and Bok that share sequence homology in BH1-3 domains. (3) 'BH3 only' pro-apoptotic members such as Bid, Bad, Bim and Bik (Zimmermann *et al.*, 2001).

Pro-apoptotic members can reside in the cytosol or on the mitochondrial membrane whereas anti-apoptotic members exist on the mitochondrial membrane alone. During apoptosis, the pro-apoptotic Bcl-2 members are activated, presumably undergo a conformational change (Desagher *et al.*, 1999) leading to exposure of the pro-apoptotic BH3 domains and translocate to the mitochondria. The translocation of Bax, Bad or Bid to the mitochondria can then induce the organelle to release proteins contained within the intermembrane space, including one key protein, cytochrome *c* (Gross *et al.*, 1998; Desagher *et al.*, 1999).

Each of the pro-apoptotic members may be selectively activated by a specific stress. However, cytochrome *c* release appears to be a universal pathway utilised by most stresses to induce cell death. Following cytochrome *c* release caspases are activated and the cell undergoes apoptosis (Green and Reed, 1998). This occurs through the formation of an 'apoptosome' that consists of cytochrome *c*, apoptotic protease activating factor-1 (APAF-1) and procaspase-9 (Rodriguez *et al.*, 1999). The apoptosome can then recruit and activate procaspase-3 and release it to mediate

apoptosis – mice deficient in caspase-9 display reduced apoptosis and cytochrome *c* mediated caspase activation (Kuida *et al.*, 1998).

1.6.2 The Fas receptor signaling pathway

Fas and Fas ligand (FasL) belong to the tumour necrosis factor (TNF) superfamily. The Fas receptor is a glycosylated cell surface molecule of 45kDa (Oehm *et al.*, 1992). It is a transmembrane protein that is ubiquitously expressed and its expression may be augmented by cytokines and lymphocyte activation (Zimmermann *et al.*, 2001). In contrast FasL is more tightly regulated and its expression is restricted to immune cells (Zimmermann *et al.*, 2001). Both Fas and FasL are expressed on immune-privileged sites with their primary function being the attenuation of immune responsiveness through the deletion of activated peripheral lymphocytes (Green & Scott, 1994).

Binding of ligands to death receptors causes the rapid formation of a death inducing signaling complex (DISC). Formation of this DISC is enabled by the presence of a death domain on the Fas receptor and on the Fas-associated death domain (FADD) adaptor molecule (Chinnaiyan *et al.*, 1996). FADD's unique structure enables it to proximally bind Fas at one end and to couple to downstream effectors via a death effector domain (DED) at the other (Sharma *et al.*, 2000). The DED recruits pro-caspase-8. Activation of caspase-8 occurs via aggregation of a number of pro-caspase-8 molecules (Muzio et al., . Ordinarily pro-caspase-8 has a low level of activity but when in close proximity to each other, two pro-caspase-8 molecules can process each other to the mature, active form (Zimmermann *et al.*, 2001). Caspase-8 can then trigger the apoptotic caspase cascade. Caspase-8 serves as a link between the classic non-mitochondrial death pathway and the mitochondrial pathway as it has the ability to cleave Bid and thus induce its translocation to mitochondria where it causes cytochrome *c* to be released (Li *et al.*, 1998).

Caspase-2 is similarly activated through death-receptor ligation. Upon trimerisation of TNFR1 receptors, receptor-interacting protein (RIP) can associate with TNFR1 and proximally bind procaspase-2 at the other end via the adaptor molecule RAIDD (Duan and Dixit, 1997). Active caspase-2 is generated in a similar

fashion to caspase-8. This represents another death-inducing mechanism whereby active caspase-2 acts as initiator and effector, leading to cellular destruction (Sharma *et al.*, 2000).

In addition to binding procaspase-8, FADD can associate with Fas death domain associated protein (DAXX). Recruitment of DAXX to the cytosol leads to activation of MAP kinases (Yang *et al.*, 1997). JNK can upregulate FasL expression thus, completing a positive feedback loop (Wajant, 2002).

It is clear that Fas and FasL are critical in controlling cellular homeostasis of the immune system and disease processes (Sharma *et al.*, 2000). Underexpression of Fas or FasL may result in lymphoproliferation and susceptibility to lymphoid carcinomas. In contrast, overexpression of either Fas or FasL results in uncontrolled apoptosis, which may contribute to neurodegenerative disorders, failure to clear infectious pathogens and cardioretinopathologies.

1.7 Therapies targeted at AD

Since AD is characterised by cholinergic hypofunction, one of the primary therapeutic targets is inhibition of acetylcholinesterase to prevent degradation of acetylcholine upon its release. Additionally nAchR agonists, in a seeming contradiction to the actions of Aβ, induce neuroprotective mechanisms and have some effect on cholinergic dysfunction and memory impairment – cholinergic activation promotes release of neuroprotective sAPPα (Nitsch *et al.*, 1992).

While NMDA receptor binding is downregulated in AD brains, the use of the NMDA receptor antagonist memantine is currently under investigation for treatment of AD (Danysz *et al.*, 2003). Memantine is a channel blocking agent which at high concentrations inhibits synaptic plasticity and LTP. However, at lower concentrations, memantine can enhance memory in animal models of AD (Miguel-Hidalgo *et al.*, 2002). Additionally it may prevent the destruction of cholinergic neurons by inhibiting 'weak' NMDA receptor-dependent excitotoxicity and Aβ production .

The neurotrophin nerve growth factor (NGF) is decreased in hippocampus and cortex of AD patients. NGF enhances cholinergic neuron survival and promotes

release of Ach – however its central infusion has considerable side-effects and causes stimulation of other adjacent sensitive neuronal populations. Drugs that induce NGF synthesis within specific brain regions may prove more beneficial (Salehi *et al.*, 2004). Currently, clinical trials with AIT-082 (Grundman *et al.*, 2003) and NGF-mimetic drugs are underway.

Finally some studies show that long-term use of anti-inflammatory drugs (non-steroidal ant-inflammatory drugs; NSAIDs) reduces the risk of AD (McGeer and Rogers, 1998). NSAIDs can block Aβ-induced activation of microglia and subsequent inflammation (Netland *et al.*, 1998) – a phenomenon known to be important in the cellular deterioration associated with Aβ treatment and in the pathogenesis of AD. Other anti-inflammatory agents would therefore be of considerable interest for prevention or treatment of the cognitive decline associated with AD.

1.8 Polyunsaturated Fatty Acids

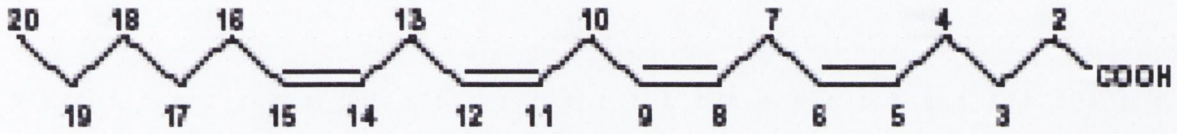
In 1929, Burr and Burr reported the essentiality in mammals of the fatty acids linoleic (LA; 18:2n-6) and α-linolenic acid (LNA; 18:3n-3). Fatty acids usually contain an even number of carbon atoms and the nomenclature assigned to each fatty acid indicates the number of carbon atoms and also the number of carbon-carbon double bonds present in the fatty acid (Youdim *et al.*, 2000). The abbreviations n-6 and n-3 for LA and LNA refer to the position of the first double bond when counting from the methyl carbon atom at the distal end of the fatty acid (FA) chain (Youdim *et al.*, 2000). Both of these FAs are required for the synthesis of longer chain polyunsaturated fatty acids, and since they cannot be synthesised in the body *de novo* they are referred to as essential fatty acids. The essentiality of FAs raised many questions as to their precise biological function and numerous theories have been postulated – structural integrity and fluidity of membranes (Stillwell *et al.*, 1993; Ehringer *et al.*, 1990), enzyme activities (Martin, 1998), lipid-protein interactions (Salem and Niebylski, 1995) and their role as precursors for eicosanoids such as prostaglandins, leukotrienes and thromboxanes (Schror, 1993).

Reports of the important roles of FAs has prompted investigators to determine the requirements of FAs for optimum health (Allison *et al.*, 1999). Classically, n-3 FAs were only deemed essential because of their limited ability (in comparison to n-6 FAs) to ameliorate some classic symptoms of EFA deficiency such as dermatitis (Andreassi *et al.*, 1997), growth retardation (Zhang, 1997) and reproductive failure (Cerolini *et al.*, 1997). However the prominence of FAs in neural and retinal tissues has prompted investigation of their other roles. Profound effects of FAs on CNS have been reported (Okuyama, 1992; Kaplan and Greenwood, 1998). Additionally, n-6 and n-3 fatty acids such as arachidonic acid (AA; 20:4n-6; Figure 1.6) and eicosapentaenoic acid (EPA; 20:5n-3; Figure 1.6) are precursors for eicosanoid synthesis, molecules that have considerable impact on inflammation and immunity. PUFA composition within the membrane has also been reported to modulate the efficiency of numerous membrane transporters and enzymes (Okuyama, 1992; Goldberg and Zidovetski, 1997; Pearce *et al.*, 1997).

1.8.1 Dietary sources and synthesis of essential fatty acids

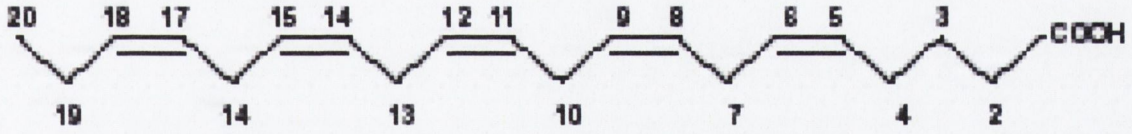
Since the optimal requirements for EFAs are not known, recommendations for dietary intake are somewhat limited (Youdim *et al.*, 2000), however, intake of 3-6% of total fat is generally recommended (Bjerve, 1991). Within the n-6 family, LA is the major EFA and based on animal studies about 1-2% of total caloric intake is required to relieve symptoms induced by its deficiency (Bjerve *et al.*, 1991). LNA is required at 0.5-1% of total fat and is also required as a source for EPA and docosahexaenoic acid (DHA) synthesis for neuronal and retinal tissues. Thus the requirements of EPA and DHA would be about 0.4% and the optimum dietary intake would be around 900mg/day for EPA and 400mg/day for DHA (Bjerve *et al.*, 1991). Plants, particularly seeds provide rich sources of triglycerides containing PUFAs. However, fresh and processed fish have been shown to be the major dietary sources of 20 and 22-carbon PUFAs in the western diet (Salem, 1989). EFAs must be consumed in sufficient quantities to cross intestinal epithelium before they can reach different tissues and digestion and absorption constitute further problems to their availability (Carlier *et al.*, 1991). Carlier and colleagues showed relatively high

Arachidonic Acid
(20:4n-6)



Arachidonic Acid ($C_{20}H_{32}O_2$, 20:4ω-6, MW: 304.467)

Eicosapentaenoic acid
(20:5n-3)



Eicosapentaenoic Acid ($C_{20}H_{30}O_2$, 20:5ω-3, MW: 302.451)

Figure 1.6 Polyunsaturated fatty acids

Structures of long-chain polyunsaturated fatty acids Arachidonic acid (AA) and eicosapentaenoic acid (EPA).

(Adapted from SanGiovanni, J.P. and Chew, E.Y. (2005) *Prog Retin Eye Res*, **24**, 87-138)

proportions of EFAs consumed appear as components of phospholipids. The majority of PUFAs are synthesised from LA and LNA via a series of desaturation and elongation reactions (Sprecher, 1989; Voss *et al.*, 1991). Brenner clearly demonstrated that Δ6 and Δ5 desaturases are present in rat liver microsomes (Brenner, 1969) and Sinclair demonstrated that desaturation of LA and LNA to their 20- and 22-carbon PUFA forms occur in rat liver and brain (Sinclair, 1975). Other studies have verified both Δ6 and Δ5 desaturation of n-6 fatty acids in rat liver and shown that LNA may be a competitive inhibitor of these processes (Blond and Lemarchal, 1984).

1.8.2 PUFAs and brain fatty acid composition

General composition in the phospholipid fractions of the brain contains very little LA (Tinoco, 1982). AA was found to be an important component but DHA was the major component present (Youdim and Deans, 1999). High levels of DHA have also been reported in subcellular fractions such as synaptosomes (Viani *et al.*, 1991; McGee *et al.*, 1994), synaptic vesicles, mitochondria (Willumsen *et al.*, 1996), microsomes (Srinivasarao *et al.*, 1997) and nerve growth cones (Martin and Bazan, 1992). Modifications of brain membrane fatty acid composition have been reported with dietary oils (Bourre *et al.*, 1990; Fernstrom, 1999).

Fish oils, which contain high levels of 20- and 22-carbon PUFAs exert the most profound in influence on brain PUFA concentrations. Data from several studies indicates that a high degree of unsaturation is required in brain and that a reduction in n-3 FAs is reversible within a few weeks (Galli *et al.*, 1971; Connor *et al.*, 1990; Anderson and Connor, 1994). One of the functional roles of PUFAs is in maintaining an appropriate state of membrane fluidity so as to preserve lipid-protein interactions, and certain enzyme activities.

1.8.3 PUFAs and neuronal function

The term membrane fluidity refers to the physical state of the fatty acyl chains comprising the membrane bilayer structure, as well as a measure of the different rates of motion of molecule elements within the membrane (Youdim *et al.*, 2000). DHA is one of the fatty acyl chains that exerts most influence. The replacement of a

single double bond is sufficient to exert a profound effect on the physical properties of the membrane (Cohen and Zubenko, 1985; Mason *et al.*, 1997). Indeed, age-related declines in PUFAs result in a decrease of Na^+-K^+ ATPase activity (Viani *et al.*, 1991).

Elucidation of a role for PUFAs in neuronal function has followed two separate lines of investigation. The first line has investigated the effects of dietary supplementation with PUFAs or dietary restriction of PUFAs. Deprivation of PUFAs in the diet has resulted in deleterious effects on cAMP-dependent protein kinase A (PKA) and PKC activities (Speizer *et al.*, 1991), brain membrane lipid composition (Delion *et al.*, 1997), LTP and neurotransmission (McGahon *et al.*, 1999b) and learning ability in rats (Gamoh *et al.*, 1999). Additionally, increasing dietary intake of n-3 FAs can improve learning and memory tasks in young rats and overcome age-related deficits in LTP (McGahon *et al.*, 1999b, 1999c). It has been suggested that these beneficial effects of n-3 FAs occur as a result or physical alterations in characteristics of the membrane and by affecting neurotransmitter release as well as receptor and channel function (Nishikawa *et al.*, 1994).

An alternative investigative approach has focused on the effect of direct application of n-3 fatty acids on cellular biochemistry and synaptic transmission. PUFAs have also been shown to regulate neuronal excitability - PUFAs modulate Na^+ and Ca^{2+} currents in CA1 neurons (Vreugdenhil *et al.*, 1996). Vreugddenhil and colleagues (1996) demonstrated that extracellular application of EPA to freshly isolated hippocampal CA1 neurons produced a concentration-dependent shift of the voltage-dependence of inactivation of Na^+ and Ca^{2+} currents to hyperpolarising potentials. This accelerated the inactivation and retarded the recovery from inactivation, which may have potential anticonvulsive effects *in vivo*.

Mirnikjoo and colleagues (2001) investigated the effects of DHA and EPA on the activities of several protein kinases in CNS. The authors report a PUFA-induced reduction in activity of PKC, PKA, ERK and CaMKII. Impaired induction of LTP was also reported following perfusion of DHA and EPA onto hippocampal slices *in vitro* presumably as a result of the reported decrease in activity of the investigated protein kinases (Mirnikjoo *et al.*, 2001). DHA-induced inhibition of LTD in hippocampal CA1 slices has been reported (Young *et al.*, 1998) and intracerebroventricular injection of

DHA in anaesthetised rat inhibited LTP induction in area CA1 while having no effect on LTP in dentate gyrus (Itokazu *et al.*, 2000). These data suggest a region-specific effect on hippocampal transmission for PUFAs. Taken together, these data indicate a certain confusion surrounding the precise role of PUFAs in neuronal function.

1.8.4 PUFAs and eicosanoid production

In addition to functioning as physical and chemical barriers separating aqueous compartments, membrane lipids are involved in many aspects of cell function and response. Inflammation is classically associated with *rubor, tumor, calor and dollar* (L. redness, swelling, heat and pain). This clinical pathology results from the release of inflammatory mediators, predominantly from activated leukocytes that migrate to the area of inflammation.

Eicosanoids, like cytokines, are a group of chemical messengers that act within the immune system (Youdim *et al.*, 2000). These compounds link PUFAs, inflammation and immune function. Upon stimulation, membrane lipids are remodelled to generate biologically active lipid mediators that can serve as intracellular or extracellular signals. Some FAs are precursors for eicosanoids, which include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), intermediate hydroperoxyeicostetraenoic (HPETE) and hydroxyeicosatetraenoic (HETE) acids. The synthesis of eicosanoids begins with hydrolysis of FAs from the 2-acyl position of phospholipids, usually by the action of phospholipase (PL)-A_2 (Smith, 1992).

PGs and LTs act in paracrine and autocrine manner to affect cell function through a variety of transmembrane receptors (Smith, 1992; Brooks and Summers, 1996) modulating numerous aspects of cell function. Indeed, epidemiogical evidence suggests that diets rich in PUFAs may protect against cardiovascular disease by lowering plasma lipid levels and affecting eicosanoid biosynthesis (Nieuwenhuys and Hornstra, 1998; Adan *et al.*, 1999). Supplementation with EPA is associated with increased activity of 5-lipoxygenase enzyme since EPA is the preferred substrate for the enzyme as compared with AA. The relevance of this finding becomes clear when the products of enzymatic activity are compared. Metabolism of EPA yields leukotriene B5 (LTB5) while metabolism of AA yields LTB4, which has considerably

greater potentiating effect on inflammation than LTB5. Additionally, DHA and EPA are precursors for the 3-series of prostanoids and the 5-series of leukotrienes – the corresponding AA metabolites have considerably greater aggregatory and inflammatory properties. Diets providing high concentrations of PUFAs not only have a beneficial effect on the membrane but reduce synthesis of harmful eicosanoids derived from AA (Youdim *et al.*, 2000).

1.8.5 Ageing, inflammation and PUFAs

Tissue concentrations of LA, LNA and n-3 PUFAs decrease with age (Youdim and Deans, 1999). Studies have shown that this may occur as a consequence of age-associated decreased Δ6 and Δ5 desaturase activities (Maniongui *et al.*, 1993). Consequently supplementation with 20- and 22-carbon PUFAs may bypass this age-related change and restore PUFA concentration. While changes in PUFA synthesis occur with age, the concomitant increase in lipid peroxidation and oxidative stress have considerable impact on membrane PUFA concentration (Youdim and Deans, 1999). Increased lipid peroxidation is a feature associated with many neurodegenerative diseases (Pearce *et al.*, 1997; Prasad *et al.*, 1998). Supplementation with low doses of EPA and DHA, rather than providing fuel for lipid peroxidation, modifies the membrane composition of FAs without increasing susceptibility to oxidative stress (Calviello *et al.*, 1997).

Increased production of pro-inflammatory cytokines in the absence of inflammatory stimulus is a frequent phenomenon that occurs in the elderly (McGeer and McGeer, 1995). Similarly TNFα is produced in higher amounts in cytotoxic reactions in the elderly (Chang *et al.*, 1996). The deleterious effects of pro-inflammatory cytokines have been discussed (section 1.4.2) and interestingly LA and other FAs have been found to influence TNFα induced apoptosis (Toberek *et al.*, 1997). This study was carried out in endothelial cells, however similar effects may occur in brain cells.

Supplementation with EPA and DHA results in decreased AA in membrane phospholipids of cells involved in inflammation and immunity (Endres *et al.*, 1989). Additionally, n-3 PUFAs appear to inhibit the release of AA from membrane

phospholipids possibly by inhibition of phospholipases (Calder, 2001). EPA also competes with AA for the active sites of cyclooxygenase and 5-lipooxygenase (5-LOX; Calder, 2001). Furthermore EPA acts as a substrate for these enzymes generating different eicosanoid derivatives which are considered less biologically potent than the analogues synthesised from AA.

Data from several studies have indicated that EPA and DHA exhibit potent anti-inflammatory and immunosuppressive effects. Both EPA and DHA inhibit production of IL-1β and TNFα by human monocytes *in vitro* (Baldie *et al.*, 1993) and IL-6 production by rat peritoneal macrophages (Tappia *et al.*, 1995). Dietary supplementation with fish oil decreased *ex vivo* production of TNFα, IL-1β and IL-6 by rodent macrophages (Yaqoob and Calder, 1995; Wallace *et al.*, 2000). Decreased peak plasma TNFα, IL-1β and IL-6 in response to LPS administration have been reported following dietary manipulation with fish oil in mice (Sadeghi *et al.*, 1999). Evidence from human studies also indicates decreased production of IL-1 and TNFα following dietary supplementation with EPA and DHA (Endres *et al.*, 1989; Meydani *et al.*, 1991; Caughey *et al.*, 1996). Taken together these studies indicate that addition of high levels of PUFAs *in vitro*, in animal and human studies exerts potent anti-inflammatory effects, through inhibition of pro-inflammatory cytokines, that may prove beneficial in protecting against stress-induced cellular deterioration.

1.9 Objectives

Central administration of Aβ exerts a myriad of effects in the brain and age-associated inflammatory changes may leave the hippocampus more susceptible to stress or insult. The objectives of this study therefore were to assess the functional and biochemical changes that occur in hippocampus of adult and aged rats following intracerebroventricular injection of Aβ and to determine whether hippocampus of aged rats was more susceptible to insult from Aβ due to underlying inflammation. Since considerable evidence exists indicating that PUFAs may have putative anti-inflammatory effects, the ability of PUFAs to protect against the deleterious effects of Aβ and to reverse the age-associated changes was investigated.

Chapter 2

Materials and Methods

2.1 Materials

Acrylamide	Sigma
Actin antibody	Santa Cruz
Amonium persulfate	Sigma
β-Amyloid	BioSource
Anti-active caspase-3 antibody	Promega
Anti-goat IgG HRP	Vector
Anti-mouse FITC conjugated antibody	Vector
Anti-mouse IgG HRP	Sigma
Anti-rabbit IgG HRP	Amersham
Anti-rabbit rhodamine conjugated antibody	Vector
Aprotinin	Sigma
Bio-Rad dye reagent concentrate	Bio-Rad
Bis-acrylamide	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
B-27 supplement	Gibco
Calcium chloride	Lennox
Caspase-1 inhibitor (Ac-YVAD-CMK)	Calbiochem
CD40 antibody	Santa Cruz
CD11b antibody	Serotec
Cytochrome *c* antibody	Santa Cruz
Cytosine arabinofuranoside	Sigma
Dimethyl sulphoxide	Sigma
Dithiothreitol	Sigma
DJNKI1	Alexis
DNase	Sigma
Dulbecco's modified Eagle media	Gibco
EDTA	Sigma
EGTA	Sigma
Ethanol	Lennox

Ethyl-eicosapentaenoic acid	Laxdale
Fluorescent mounting medium	Vector
Foetal bovine serum	Gibco
Glucose	Lennox
Glutamax	Gibco
Glycerol	Sigma
Glycine	Sigma
Horse serum (heat-inactivated)	Gibco
HEPES	Sigma
Hydrochloric acid	Lennox
Hyperfilm	Amersham
IFNγ DuoSet	R&D Systems
Recombinant rat IL-1β	R&D Systems
IL-1β ELISA DuoSet	R&D Systems
IL-4 ELISA DuoSet	R&D Systems
JNK1 antibody	Santa Cruz
Leupeptin	Sigma
Magnesium sulphate	Sigma
Magnesium chloride	Sigma
β-Mercaptoethanol	Sigma
Methanol	Lennox
Minocycline hydrochloride	Sigma
Neurobasal media	Gibco
Nitrocellulose membrane	Sigma
Normal goat serum	Vector
PARP antibody	Santa Cruz
phospho-c-Jun antibody	Santa Cruz
Penicillin/Streptomycin	Gibco
Pepstatin A	Sigma
Phenylmethylsulphonyl fluoride	Sigma
Sterile phosphate-buffered saline (10X)	Sigma

Phospho-JNK antibody	Santa Cruz
Poly-L-lysine	Sigma
Potassium chloride	Sigma
Potassium hydroxide	Sigma
Potassium phosphate	Sigma
PPARγ antibody	Cell Signalling
Prestained molecular weight standard	Santa Cruz
Prestained molecular weight standard (broad range)	Sigma
RAGE antibody	Chemicon
ReBlot Plus strong antibody stripping solution	Chemicon
Sodium azide	Sigma
Sodium carbonate	Sigma
Sodium chloride	Sigma
Sodium dodecylsulphate	Sigma
Sodium hydrogen carbonate	Lennox
Sodium hydroxide	Lennox
Sodium phosphate (monobasic)	Sigma
Sodium phosphate (dibasic)	Sigma
Standard grade No. 3 filter paper	Whatman
Sucrose	Lennox
Sulphuric acid	Lennox
SuperSignal West Dura extended duration substrate	Pierce
Tris-base	Sigma
Tris-HCl	Sigma
Troglitazone	Calbiochem
Trypsin	Sigma
Trypsin Inhibitor	Sigma
Tween-20	Lennox
Urethane	Sigma
Vectastain ABC kit	Vector

2.2 Animals

2.2.1 Housing of animals

All adult animals used were an inbred strain of male Wistar rat aged between 2 and 4 months supplied by the Bio Resources Unit, Trinity College, Dublin, weighing between 200 and 300g. Middle-aged and aged animals were aged between 12 and 14 months and 22 and 24 months respectively supplied by Bantham & Kingham (UK). Young animals were housed in groups of either 4 or 6 per cage and middle-aged/aged animals were housed in groups of 2. All rats were maintained under a 12 hr light-dark cycle in the Bio Resources Unit, Trinity College, Dublin and food (normal laboratory chow, unless otherwise stated) and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C. All animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC.

2.2.2 Preparation of Amyloid-β peptide

Amyloid-β ($A\beta_{(1-40)}$; BioSource, USA) was aggregated according to manufacturer's instructions. The lyophilised peptide was dissolved in sterile dH_2O at 6mg/ml and then diluted to a 1mg/ml stock solution with calcium-free sterile PBS (Sigma, UK). The peptide was allowed to aggregate for 48 hr at 37°C. Stock solution was aliquoted and stored at –20°C until required. For intracerebroventricular injection, $A\beta_{(1-40)}$ was diluted to a final concentration of 20, 60 or 200μM and 5μl were injected. $A\beta_{(1-40)}$ was used in these experiments since it is the most predominant Aβ species in the brain and has been shown to have inhibitory effects on LTP. Hereafter all $A\beta_{(1-40)}$ treatments will be referred to as Aβ treatment.

2.2.3 Aβ administration

Rats were anaesthetised by intraperitoneal injection of urethane (1.5g/kg; 33% w/v). The absence of a pedal reflex was used to confirm deep anaesthesia and if needed a further top-up dose was administered (to a maximum of 2.5g/kg). Anaesthetised rats were injected intracerebroventricularly, 3mm posterior to bregma

with sterile dH_2O or Aβ (5μl; 20, 60 or 200μM; BioSource, USA). In the initial study, rats were killed by cervical dislocation six hours after vehicle/Aβ treatment and in subsequent studies, rats were assessed for their ability to sustain long-term potentiation three hours following vehicle/Aβ treatment (LTP; see Section 2.3).

2.2.4 Polyunsaturated fatty acid treatment

In one series of experiments rats were treated for 4 weeks with eicosapentaenoic acid (EPA). The composition of the EPA preparation was 20:5n-3 ethyl-eicosapentaenoic acid (greater than 95% pure) containing 0.2% DL-tocopherol as an antioxidant (Laxdale, UK). Food intake was measured for 2 weeks prior to the period of experimental treatment to establish daily food intake. At the end of this period, rats were randomly assigned to a group that received normal laboratory chow (Red Mills, Ireland) supplemented with either 125mg/rat/day EPA (hereafter referred to as 125mg EPA treatment) or control treatment of normal laboratory chow enriched in monounsaturated fatty acids (hereafter referred to as control treatment). All treatments had an isocaloric value. Treatments were freshly prepared and rats were offered their full daily requirement each day.

2.3 Induction of LTP *in vivo*

2.3.1 Preparation of rats

Rats were anaesthetised as described above (see Section 2.2.3). Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame (ASI Instruments, UK). A midline incision was made with a scalpel to reveal the skull. The periosteum was scraped clear and a dental drill was used to remove a window of skull to allow correct placement of the electrodes. The dura mater was peeled away so as to expose the brain. The recording chamber consisted of a stereotaxic unit attached to the laboratory bench and surrounded by a Faraday cage to isolate it from interference from the external environment. All instruments in the cage were grounded to eliminate 50Hz cycle noise.

2.3.2 Electrode implantation

Bipolar stimulating electrodes and unipolar recording electrodes (Clark Electromedical, UK) were used. The stimulating electrode was placed on the surface of the brain, 4.4mm lateral to lambda. The recording electrode was placed on the surface of the brain 2.5mm lateral and 3.9mm posterior to bregma. The positions of the stimulating and recording electrodes were carefully monitored as they were lowered in increments through the cortical and hippocampal layers into the perforant path and granule cell layer of the dentate gyrus respectively, until the characteristic perforant path granule cell synapse response was observed. The depth of the electrodes was finely adjusted so as to maximise the response. This was carried out by generating 0.1 msec duration, 2 msec delay, 4V pulse through the stimulating electrode at a frequency of 0.1Hz. Evoked responses were picked up by the recording electrode and displayed on an Apple Macintosh computer (Performa 5200). The final depth of the recording electrode was between 2.5 and 3.5mm and for the stimulating electrode was between 2.5 and 3mm. Stimuli were delivered at 30 sec intervals.

2.3.3 EPSP recordings

The population field excitatory post-synaptic potential (field EPSP) was used as a measure of excitatory synaptic transmission in the dentate gyrus. EPSPs were recorded by passing a single square wave of current at low frequency (0.033Hz, 0.1 sec, 2 msec delay) generated by a constant isolation unit (IsoFlex, UK), to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (DAM 50; differential amplifier; gain 75, World Precision Instruments, USA) to an analogue-to-digital converter (Maclab/2e, Analog Digital Instruments). This was a digitised system linked to an Apple Macintosh computer (Performa 5200), which interfaced with the converter via a specifically written software package (Scope, Version 3.36). This was customised to control both the generation of the square wave pulses and recording of the evoked potentials. The field EPSPs were displayed on-line and could be analysed at the time of recording or, in the case of these studies, at a later date. The slope of the EPSP was taken as the indicator of excitatory synaptic transmission. After a period of stabilisation, test shocks at 1/30 sec were recorded for

a 10 min control period to establish stable baseline recordings. This was followed by delivery of 3 trains of stimuli (250Hz for 200 msec) at 30 sec intervals. Recording at test shock frequency then resumed for 40 min. Recordings in the 40 min following tetanus were expressed as a percentage of control baseline recordings prior to tetanus.

2.4 Preparation of tissue

2.4.1 Dissection

Rats were killed by cervical dislocation and decapitation and the brains were rapidly removed and dissected on ice. The hippocampus was dissected free. This procedure took approximately 2 min.

2.4.2 Preparation of slices for freezing

Freshly dissected tissue was sliced bidirectionally to a thickness of 350μm using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., UK) and placed in microfuge tubes containing 1ml Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10) with added $CaCl_2$ (2mM final concentration). Slices were allowed to settle and were rinsed once more with Krebs. The slices were rinsed a further two times with Krebs solution containing 10% dimethyl sulphoxide (Haan and Bowen, 1981) and stored at –80°C until required for later analyses.

2.5 Protein quantification

Protein quantification was assessed according to Bradford (1976). Standards were prepared from a stock solution of 1000μg/ml bovine serum albumin (BSA; Sigma, Dorset, UK). This was diluted in dH_2O to prepare a range of standards from 500μg/ml to 0μg/ml. Duplicate aliquots (10μl) of standard and samples were added to the 96-well plate (Sarstedt, Ireland) and Bio-Rad dye reagent concentrate (200μl; 1:5 dilution in dH_2O; Bio-Rad, UK) was added to both samples and standards. Absorbance was assessed at 600nm using a 96-well plate reader (Labsystems

Multiskan RC). A regression line was plotted (GraphPad Prism, USA) and the concentration of protein was calculated.

2.6 Analysis of cytokines *ex vivo*

2.6.1 Preparation of samples

Hippocampal slices were thawed rapidly, washed 3 times in Krebs solution and homogenised (x30 strokes) in Krebs solution (500µl) 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. Samples were stored at –80°C until required.

2.6.2 Analysis of interleukin-1β concentration

An Enzyme Linked ImmunoSorbent Assay (ELISA) method was used to determine the concentration of interleukin-1β (IL-1β). 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100µl; 1 µg/ml; goat anti-rat IL-1β in phophate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, and 1.5mM KH_2PO_4 pH 7.3; R&D Systems, USA) and incubated overnight at room temperature (RT). Plates were washed in PBS containing 0.05% Tween-20, pH 7.4 (PBS-T) 3 times, a blocking buffer was added to the wells (300µl; PBS containing 1% bovine serum albumin (BSA), 5% sucrose and 0.05% NaN_3) and the plates were incubated for 2 hr at RT. Standards (0-1000pg/ml) were made from recombinant rat IL-1β (R&D Systems, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in PBS-T and 100µl standard and samples (see section 2.6.1) were incubated for 2 hr at RT. After this incubation period the plates were washed 3 times in PBS-T and the detection antibody (100µl; 350ng/ml; biotinylated goat anti-rat IL-1β in PBS containing 1% BSA and 2% normal goat serum; R&D Systems, USA) was added and incubation continued for 2 hr at RT. Plates were washed 3 times in PBS-T and streptavidin-horseradish peroxidase conjugate (streptavidin-HRP; 100µl; 1:200 dilution in PBS containing 1% BSA; R&D Systems, USA) was added and incubation continued for 20min at RT. Plates were washed 3 times in PBS-T, and substrate solution (100µl; 1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D

Systems, USA) was added to the wells and incubated in the dark for 1 hr creating a colour change to blue. Stop solution (50µl; 1M H_2SO_4) was added and the plates were read at 450nm within 30 min (Labsystems Multiskan RC). A standard curve was constructed and results were expressed as pg IL-1β/mg tissue corrected for protein (see section 2.5 for protein analysis).

2.6.3 Analysis of interferon-γ concentration

Interferon-γ (IFN-γ) concentration was determined by ELISA. 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100µl; 2µg/ml; monoclonal mouse anti-rat IFN-γ in PBS; R&D Systems, USA) and incubated overnight at RT. Plates were washed 3 times in PBS-T, blocking buffer (300µl; PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3) was added to the wells and incubated for 1.5 hr at RT. Standards (0-2500pg/ml) were prepared from rat recombinant IFN-γ (R&D Systems, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in PBS-T and 100µl standard and samples were incubated for 2 hr at RT. Following this incubation period plates were washed 3 times in PBS-T and detection antibody (100µl; 150ng/ml; biotinylated goat anti-rat IFN-γ in PBS containing 1% BSA; R&D Systems, USA) was added and incubation continued for 2 hr at RT. Plates were washed 3 times in PBS-T and streptavidin-HRP (100µl; 1:200 dilution in PBS containing 1% BSA; R&D Systems, USA) was added and incubation continued for 1 hr at RT. Plates were washed 3 times in PBS-T, substrate solution (100µl; 1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems, USA) was added to the wells and incubated in the dark for 30 min creating a colour change to blue. Stop solution (50µl; 1M H_2SO_4) was added and the plates were read at 450nm within 30 min (Labsystems Multiskan RC). A standard curve was constructed and results were expressed as pg IFN-γ/mg tissue corrected for protein (see section 2.5 for protein analysis).

2.6.4 Analysis of interleukin-4 concentration

The ELISA method was used to determine the concentration of interleukin-4 (IL-4). 96-well plates (Nunc-Immuno plate with MaxiSorp surface, UK) were coated

with capture antibody (100µl; 2µg/ml; monoclonal mouse anti-rat IL-4 in PBS; R&D Systems, USA) and incubated overnight at RT. Plates were washed in PBS-T 3 times, a blocking buffer was added to the wells (300µl; PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3) and plates were incubated for 1 hr at RT. Standards (0-1000pg/ml) were made from rat recombinant IL-4 (R&D Systems, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in PBS-T and 100µl standard and samples (see section 2.6.1) were incubated for 2 hr at RT. After this incubation period the plates were washed 3 times in PBS-T and the detection antibody (100µl; 50ng/ml; biotinylated goat anti-rat IL-4 in PBS containing 1% BSA; R&D Systems, USA) was added and incubation continued for 2 hr at RT. Plates were washed 3 times in PBS-T and streptavidin-HRP (100µl; 1:200 dilution in PBS containing 1% BSA; R&D Systems, Minneapolis, USA) was added and incubation continued for 1 hr at RT. Plates were washed 3 times in PBS-T and substrate solution (100µl; 1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems, USA) was added to the wells and incubated in the dark for 1 hr creating a colour change to blue. Stop solution (50 µl; 1M H_2SO_4) was added and the plates were read at 450nm within 30 min (Labsystems Multiskan RC). A standard curve was constructed and results were expressed as pg IL-4/mg tissue corrected for protein (see section 2.5 for protein analysis).

2.7 SDS-polyacrylamide gel electrophoresis

2.7.1 Preparation of samples

2.7.1.1 Preparation of whole cell lysate for assessment of JNK and c-Jun phosphorylation, JNK1, PARP, FasL, RAGE and CD40 expression

Hippocampal slices were thawed rapidly, washed 3 times in Krebs solution containing 2 mM $CaCl_2$ and homogenised (x30 strokes) in lysis buffer (400µl; 20mM HEPES; 10mM KCl; 1.5mM $MgCl_2$; 1mM EGTA; 1mM EDTA; 1mM DTT; 0.1mM PMSF; 2µg/ml leupeptin; 2µg/ml aprotinin; 200mM sucrose; pH 7.4) using a 1ml glass homogeniser (Jencons, UK). Protein concentrations were assessed (see section 2.5) and equalised with lysis buffer. Sample buffer (0.5M Tris-HCl pH 6.8; 20% glycerol (v/v); 2% SDS (w/v); 5% β-mercaptoethanol (v/v); 0.05% bromophenol blue (w/v)) was added to a final concentration of 1mg/ml and samples were boiled for 5 min.

2.7.1.2 Preparation of nuclear fraction for assessment of PPARγ expression

Nuclear fractions were prepared by homogenising hippocampal slices in permeabilisation buffer (composition in mM: sucrose 250, KCl 70, NaCl 137, Na_2HPO_4 4.5, KH_2PO_4 1.4, PMSF 0.1, with leupeptin 10 μg/ml, aprotinin 2μg/ml, digitonin 200μg/ml), incubating for 20 min on ice and centrifugation (750*g* for 10 min at 4°C). The supernatant was removed and the pellet (nuclear fraction) resuspended in permeabilisation buffer. Samples were equalised, sample buffer was added to a final concentration of 1mg/ml and samples were boiled for 5 min.

2.7.1.3 Preparation of mitochondrial fraction for assessment of Bax expression and cytosolic fraction for assessment of JNK phosphorylation and cytochrome *c* expression

A mitochondrial fraction was prepared by centrifuging (10,000*g* for 10 min at 4°C) the supernatant yielded from extraction of the nuclear fraction. The supernatant (cytosolic fraction) was removed and the pellet (mitochondrial fraction) resuspended in mitochondrial buffer (composition in mM Tris-Base 50, NaCl 150, EGTA 2, EDTA 2, PMSF 0.1, with Triton 0.2%, Igepal P-40 0.3%, leupeptin 10μg/ml, aprotinin 2μg/ml). All fractions were equalised, sample buffer was added to a final concentration of 500μg/ml and samples were boiled for 5 min.

2.7.2 Preparation of polyacrylamide gels

Polyacrylamide separation gels (1mm thick) with a monomer concentration of either 10% or 12% overlaid with 4% stacking gel (see Appendix III) were cast between 10cm wide glass plates and mounted on an electrophoresis unit (Sigma Techware, UK; Laemmli, 1970). The upper and lower reservoirs of the unit were filled with electrode running buffer (25mM Tris-base; 200mM glycine; 17mM SDS). Samples (10μl) were loaded into the wells using a Hamilton MicroLiter syringe. Pre-stained molecular weight standard (5μl; Sigma, UK or Santa Cruz, California, USA) was also loaded. Proteins were separated by application of 32mA current and

migration of the bromophenol blue was monitored. The current was switched off when the blue dye band reached the bottom of the gel (approximately 30 min).

2.7.3 Semi-dry electrophoretic blotting of proteins

The gel was removed from the gel apparatus, placed on top of a sheet of nitrocellulose membrane (0.45μm pore size; Sigma, UK), moistened in transfer buffer (25mM Tris-base; 192mM glycine; 20% methanol (v/v); 0.05% SDS (w/v)) and cut to the size of the gel. Filter paper (Standard Grade No. 3, Whatman, UK) was placed on top and beneath the nitrocellulose/gel forming a sandwich. The sandwich was soaked in transfer buffer and placed on the platinum-coated titanium electrode (anode) of a semi-dry blotter (Sigma, UK), moistened with transfer buffer. Air bubbles were removed from the sandwich by gently rolling a Pasteur pipette over it. The lid of the blotter (stainless steel cathode) was placed down firmly on top of the sandwich. The uncovered portion of the cathode was shielded with a mylar cutout (Sigma, UK), ensuring all applied current passed directly through the sandwich. A constant current of 225mA was applied for 90 min.

2.8 Western immunoblotting

The nitrocellulose membrane was blocked for non-specific binding as indicated in text and probed with an antibody raised against the appropriate protein. The membrane was washed and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody. A chemiluminescent detection agent was added and the membrane was exposed to 5 x 7 inch photographic film (Hyperfilm ECL, Amersham, UK) and developed using a Fuji FPM 800A X-ray processor.

2.8.1 JNK phosphorylation

JNK phosphorylation was assessed in whole-cell lysate and the cytosolic fraction. Non-specific binding was blocked by incubating nitrocellulose membranes overnight at 4°C in tris-buffered saline (TBS; 20mM Tris-HCl; 150mM NaCl; pH 7.6) containing 5% BSA. Membranes were washed for 15 min 4 times in TBS containing 0.05% Tween-20 (10ml; TBS-T). The primary antibody used was a mouse monoclonal

IgG_1 antibody raised against a peptide corresponding to a short amino acid sequence phosphorylated on Threonine-183 and Tyrosine-185 of JNK1 of human origin (10ml; 1:300 dilution in TBS containing 1% BSA; Santa Cruz, USA). Membranes were incubated for 2 hr at RT in the presence of the antibody and washed for 15 min 4 times in TBS-T. The secondary antibody (10ml; 1:400 dilution; goat anti-mouse IgG HRP in TBS containing 1% BSA; Sigma, UK) was added and incubation proceeded for 2 hr at RT. Membranes were washed for 15 min 4 times in TBS-T, incubated with SuperSignal (Pierce, USA) for 5 min and membranes were exposed to photographic film for 1 sec after which time the film was developed.

2.8.2 Total JNK1 expression

Following Western immunoblotting for JNK phosphorylation, blots were stripped with an antibody stripping solution (10ml; 1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon, USA) and reprobed for total JNK1 to ensure equal protein loading. The primary antibody used was a mouse monoclonal IgG_1 antibody raised against a recombinant protein corresponding to amino acids 1-384 representing full length JNK1 of human origin (10ml; 1:300 dilution in TBS containing 1% BSA; Santa Cruz, USA). Blotting proceeded as described above (section 2.8.1).

2.8.3 c-Jun phosphorylation

c-Jun phosphorylation was assessed in whole-cell lysate using a mouse monoclonal IgG_1 antibody raised against a peptide corresponding to amino acids 56-59 of c-Jun of human origin (10 ml; 1:200 dilution in TBS-T containing 1% BSA; Santa Cruz, USA). Blotting proceeded as described above (see section 2.8.1).

2.8.4 Fas Ligand expression

Fas Ligand (FasL) expression was assessed in whole-cell lysate. Membranes were blocked for non-specific binding as described previously and probed for FasL using a mouse monoclonal primary antibody raised against a peptide mapping at the amino terminus of FasL of rat origin (10ml; 1:500 dilution in TBS-T containing 1%

BSA; Santa Cruz, USA) for 2 hr at RT. Membranes were washed in TBS-T for 15 min 4 times and incubated in the presence of secondary antibody (10ml; 1:2000 dilution; sheep anti-rabbit IgG HRP in TBS containing 0.1% BSA; Sigma, UK) for 1 hr at RT. Membranes were washed for 15 min 4 times in TBS-T. SuperSignal (Pierce, USA) was added for 5 min and membranes were exposed to photographic film for 5 sec in the dark after which time the film was developed.

2.8.5 Bax expression

Bax expression was assessed in a mitochondrial fraction. Non-specific binding was blocked as described above (see section 2.8.1) and membranes were washed in TBS-T for 15 min 4 times in TBS-T (10ml). Bax expression was assessed using a mouse monoclonal antibody mapping at amino acids 1-171 of Baxα of human origin (10ml; 1:200 dilution in TBS-T containing 1% BSA; Santa Cruz, USA) for 2 hr at RT. Blotting proceeded as described in section 2.8.1.

2.8.6 Cytosolic cytochrome *c* expression

Cytochrome *c* expression was assessed in a cytosolic fraction. Non-specific binding was blocked by incubating membranes overnight at 4°C in PBS-T containing 6% non-fat dried milk (10ml). Membranes were washed for 15 min 3 times in PBS-T (10ml). The primary antibody used was a rabbit polyclonal antibody raised against recombinant protein corresponding to amino acids 1-104 representing full length cytochrome *c* of horse origin (10ml; 1:250 dilution in PBS-T containing 2% non-fat dried milk; Santa Cruz, USA). Membranes were incubated overnight at 4°C in the presence of the antibody and washed for 15 min 4 times in PBS-T. The secondary antibody (10ml; 1:600 dilution; sheep anti-rabbit IgG HRP in PBS-T containing 2% non-fat dried milk; Amersham, UK) was added and membranes were incubated for 1 hr at RT. Membranes were washed for 15min 8 times in PBS-T. SuperSignal (Pierce, USA) was added for 5 min and membranes were exposed to photographic film for 30 sec in the dark after which time the film was developed.

2.8.7 PARP cleavage

Expression of the intact form of poly(ADP-ribose)-polymerase (PARP) (116kDa) was assessed in whole-cell lysate. Non-specific binding was blocked as described in section 2.8.6. The primary antibody used was a rabbit polyclonal antibody corresponding to amino acids 764–1014 mapping at the carboxy terminus of PARP of human origin (10ml; 1:500 dilution in PBS-T containing 2% non-fat dried milk; Santa Cruz, USA). Blotting proceeded as described in section 2.8.6.

2.8.8 CD40 expression

CD40 expression was assessed in whole-cell lysate, blotting proceeded as described in section 2.8.1. CD40 expression was assessed using a mouse monoclonal IgG_{2a} antibody raised against the extracellular domain of CD40 of human origin (10ml; 1:250 dilution in TBS-T containing 0.1% BSA; Santa Cruz, USA).

2.8.9 RAGE expression

Receptor for advanced glycation end-products (RAGE) expression was assessed in whole-cell lysate. RAGE expression was assessed using a goat polyclonal antibody raised against a peptide corresponding to amino acids 42-59 of RAGE of human origin (10ml; 1:1000 dilution in PBS-T containing 2% non-fat dried milk; Chemicon, USA). Blotting proceeded as described in section 2.8.6.

2.8.10 PPARγ expression

Peroxisome proliferator-activated receptor-γ (PPARγ) expression was assessed in a nuclear fraction using a rabbit polyclonal antibody raised against a peptide corresponding to amino acids surrounding Serine-84 of PPARγ of human origin (10ml; 1:1000 dilution in PBS-T containing 2% non-fat dried milk; Cell Signalling, USA). Blotting proceeded as described in section 2.8.6.

2.8.11 Actin expression

Following Western immunoblotting for Bax, CD40, FasL, c-Jun, CD40, cytochrome *c*, RAGE, PARP and PPARγ, blots were stripped with an antibody

stripping solution as described before (see Section 2.8.2) and reprobed for analysis of actin expression to confirm equal loading of protein. The primary antibody was a mouse monoclonal IgG_1 antibody corresponding to amino acid sequence mapping at the carboxy terminus of actin of human origin (10ml; 1:200 dilution in TBS containing 1% BSA; Santa Cruz, USA). Blotting proceeded as described in section 2.8.1.

2.8.12 Densitometry

In all cases quantification of protein bands was achieved by densitometric analysis using the ZERO-Dscan Image Analysis System (Scanalytics, USA). Values are expressed as arbitrary units.

2.9 Preparation of cultured cells

2.9.1 Preparation of sterile coverslips

Glass coverslips (13mm diameter; Chance Propper, UK) were soaked in 70% ethanol for 1 hr followed by overnight exposure to ultraviolet light. Coverslips were coated with poly-L-lysine (40μg/ml in sterile dH_2O; Sigma, UK) for 1 hr at 37°C so as to provide a suitable surface for cells to adhere. Coated coverslips were air-dried, placed in 24-well plates (Greiner, Austria) and stored at 4°C until required.

2.9.2 Preparation of primary cultures of cortical neurons

Primary cortical neurons were isolated and prepared from wistar rats 1 day postpartum (BioResources Unit, Trinity College, Dublin). Rats were killed by decapitation in a laminar flow hood and cerebral cortices were dissected free and placed in sterile PBS (Sigma, UK). Meninges were removed using a fine forceps and the tissue was chopped into 3-4mm pieces using a sterile disposable scalpel (Schwann-Mann, UK). Tissue was incubated in 3ml of PBS containing 0.3% trypsin (Sigma, UK) for 20 min at 37°C. Tissue was triturated 5-7 times in PBS containing 0.1% soyabean trypsin inhibitor (Sigma, UK), DNase (0.2mg/ml; Sigma, UK) and $MgSO_4$ (0.1M). Cell suspensions were passed through a sterile nylon mesh filter (40μm; Becton Dickinson Labware, France) and centrifuged (2500*g* for 3 min at 20°C). The 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). Resuspended neurons were placed on the centre of each coverslip at a density of 1 x 10^5 cells and allowed to adhere to the glass coverslip for 2 hr in a humidified incubator containing 5% CO_2: 95% air at 37°C (Jencons, UK) before 400µl of pre-warmed NBM was added to each well. Cells were grown for 3 days. Media were replaced with NBM containing cytosine arabino-furanoside (5µg/ml; Sigma, UK) to prevent proliferation of non-neuronal cells. Media supplemented with cytosine arabino-furanoside was removed after 24 hr and replaced with NBM.

2.9.3 Primary culture of cortical glia

Glia were isolated from cerebral cortices of wistar rats 1 day postpartum (BioResources Unit, Trinity College, Dublin). Rats were decapitated and cortices dissected as described above (see section 2.9.1). Cortices were placed in 3ml Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100U/ml; Gibco, UK). Tissue was triturated (x7), passed through a sterile nylon mesh filter and centrifuged (2500*g* for 3 min at 20°C). The pellet was resuspended in DMEM. Resuspended glia were placed on the centre of each coverslip and allowed to adhere to the glass coverslip for 2 hr in a humidified incubator containing 5% CO_2: 95% air at 37°C before 400µl of pre-warmed DMEM was added to each well. Cells were grown for 14 days prior to treatment and media replaced every 3 days.

2.10 Cell Treatments

2.10.1 $A\beta_{(1-40)}$

β-Amyloid ($A\beta_{(1-40)}$; BioSource, USA) was aggregated according to manufacturer's instructions (see section 2.2.2). For cell treatment, $A\beta_{(1-40)}$ was diluted to a final concentration of 2µM (10ng/ml in media). Cells were treated with $A\beta_{(1-40)}$ for 24 hr.

2.10.2 IL-1β

Recombinant rat IL-1β (R&D Systems, USA) was prepared as a stock solution of 1μg/ml in sterile PBS and used at a final concentration of 5ng/ml in NBM. Cells were treated with IL-1β for 21 hr.

2.10.3 DJNKI1

The cell permeable JNK1 inhibitor DJNKI1 (Alexis, UK) was diluted to a 1mM stock solution in sterile dH_2O and stored at –20°C until required. DJNKI1 was diluted to a final concentration of 1μM in NBM for cell treatment. Cells were treated with DJNKI1 for 20 min prior to IL-1β/Aβ treatment.

2.10.4 Caspase-1 Inhibitor

Caspase-1 inhibitor (Ac-YVAD-CMK; Calbiochem, UK) was dissolved in dimethyl sulfoxide (DMSO; 100mM stock solution) and diluted to a final concentration of 100nM for cell treatment. Cells were treated with caspase-1 inhibitor for 20 min prior to IL-1β/Aβ treatment.

2.10.5 Minocycline hydrochloride

Minocycline hydrochloride (Sigma, UK) was dissolved in DMSO (5mg/ml stock solution) and stored at -20°C until required. Cells were treated with minocycline (10μg/ml in DMEM) 20 min prior to Aβ treatment and co-incubated in the presence of Aβ for 21 hr.

2.10.6 Troglitazone

Troglitazone was dissolved in DMSO (5mM stock solution) and stored at -20°C until required. Cells were treated with troglitazone (50μM in DMEM) 20 min prior to Aβ treatment and co-incubated in the presence of Aβ for 21 hr.

2.11 Immunocytochemistry

2.11.1 TdT-mediated-UTP-end nick labelling (TUNEL)

Following treatment of cultured neurons, coverslips were washed in TBS, fixed in 4% (w/v) paraformaldehyde for 30 min at RT and stored at 4°C in TBS until required for analyses. Apoptotic cell death was assessed by monitoring DNA fragmentation, using the DeadEnd colorimetric apoptosis detection system according to the manufacturer's instructions (Promega, USA). Cells were permeabilised with Triton X-100 (0.1%, v/v), proteinase-K (1μg /ml) in TBS and refixed in 4% paraformaldehyde for 10 min. Cells were incubated in equilibration buffer (100μl/coverslip; 200mM potassium cacodylate (pH 6.6 at 25°C), 25mM Tris-HCl (pH 6.6 at 25°C), 0.2mM DTT, 0.25mg/ml BSA, 2.5mM cobalt chloride) for 10 min. A reaction buffer (100μl/coverslip; 1μl biotinylated nucleotide mix (25μM biotinylated nucleotide mix, 10mM Tris-HCl, pH7.6, 1mM EDTA), 1μl Terminal deoxynucleotidyl Transferase (TdT) enzyme and 98μl equilibration buffer, Promega, USA) was applied for 1 hr at 37°C in order to incorporate the biotinylated nucleotide to the 3' end of fragmented DNA strands. Strep-HRP was bound to the biotinylated nucleotide (100μl; 1:100 dilution in PBS for 1 hr at RT) and detected using a diaminobenzidine solution. Incubation proceeded for approximately 10 min. Coverslips were washed in dH_2O, dehydrated through graded alcohols and mounted on slides with DPX mounting medium. Cells were viewed under light microscopy (Nikon Labophot) at x100 magnification, where the nuclei of TUNEL positive cells stained dark purple.

2.12. Fluorescent Immunocytochemistry

2.12.1 Glial fibrillary acidic protein

Cells were permeabilised with Proteinase K (20μg/ml) in 0.1% TritonX100 in TBS (w/v) for 8 min, washed, refixed in 4% paraformaldehyde for 10 min, washed, blocked with blocking buffer (10% NGS in TBS; v/v) for 2 hr and incubated with a glial fibrillary acidic protein antibody (GFAP; 1:800 in 2.5% NGS in TBS, Chemicon, UK) overnight at 4ºC. Cells were stained by incubating with fluorescently tagged secondary antibody for 1 hr at 37ºC; goat anti rabbit IgG L-Rhodamine linked antibody (1:100 in 2.5% NGS in TBS, BioSource, UK). Coverslips were sealed using

fluorescent mounting medium (Vectastain, Vector, UK) and stored in the dark at 4ºC until viewed under a Zeiss fluorescence microscope with the appropriate filter (L-Rhodamine – excitation 574nm and emission 602nm).

2.12.2 JNK phosphorylation

Cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and non-reactive sites were blocked with goat serum (blocking buffer; 5% in PBS). To determine the intracellular distribution of phosphorylated JNK cells were incubated overnight with an anti-active JNK antibody (100µl; 1:400 dilution in 10% blocking buffer; Santa Cruz, USA) purified from mouse serum. This antibody was raised against a peptide corresponding to a short amino acid sequence of JNK1 and JNK2 of human origin containing phosphorylated Threonine-183 and Tyrosine-185. Coverslips were washed 3 times in PBS and secondary antibody was added (100µl; 1:100 dilution; anti-mouse IgG conjugated to FITC; Sigma, UK) for 1 hr at RT. Coverslips were washed several times in dH_20, before being mounted onto microscope slides using a mounting medium for fluorescence (Vector, USA) and the perimeter of each coverslip was sealed using nail varnish. Mounted coverslips were viewed under x40 magnification by fluorescence microscopy (Leitz Orthoplan Microscope) using Improvision software (Improvision, UK). Cells were observed under excitation, 490nm; emission, 520nm for FITC labelled antibodies.

2.12.3 Caspase-3 activity

Cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and non-reactive sites were blocked with goat serum (blocking buffer; 5% in PBS). Cells were incubated overnight with a polyclonal anti-active caspase-3 antibody (100µl; 1:250 in 10% blocking buffer; Promega, USA) at 4°C. This antibody was purified from rabbit serum and recognizes the 17kDa cleaved and active fragment of caspase-3. Cells were washed 3 times in PBS and incubated with secondary antibody added (100µl; 1:50 in 10% blocking buffer; goat anti-rabbit rhodamine conjugate, Vector, USA) for 2 hr at RT. Coverslips were washed several times in dH_20 before being mounted onto microscope slides using a mounting

medium for fluorescence (Vector, USA) and the perimeter of each coverslip was sealed using nail varnish. Mounted coverslips were viewed under x40 magnification by fluorescence microscopy (Leitz Orthoplan Microscope) using Improvision software (Improvision, UK). Cells were observed under excitation, 490nm; emission, 600nm for rhodamine-labelled antibodies.

2.13 Statistical Analysis

All mean data is included in table format as an appendix (pages xvi-xxiii). Data are expressed as means ± standard error of the means. A Student's t-test for Independent means or a one-way analysis of variance (ANOVA) was performed where appropriate to determine whether significant differences existed between conditions. When this analysis indicated significance (at the 0.05 level), post hoc student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other (GraphPad Prism).

Chapter 3

Results

3.1 Aβ administration is associated with an increase in the concentration of interleukin-1β

The concentration of the proinflammatory cytokine interleukin-1β (IL-1β) was assessed in hippocampal homogenate prepared from vehicle-treated and Aβ-treated rats. Figure 3.1 shows that IL-1β concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated rats (215.7pg/mg ± 17.65 SEM) compared with tissue prepared from vehicle-treated rats (132.8pg/mg ± 11.28 SEM; *$p<0.01$; Student's *t* test for independent means).

3.2 Aβ administration is associated with an increase in JNK phosphorylation

Figure 3.2A shows an immunoblot indicating increased JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.2B shows mean data obtained from densitometric analysis; this reveals a significant increase in JNK phosphorylation in hippocampal tissue prepared from Aβ-treated rats (11.84 arbitrary units ± 1.77) compared with vehicle-treated rats (8.32 arbitrary units ± 1.13; *$p<0.001$; Student's *t* test for independent means). In contrast to this Aβ-induced effect Figure 3.2C shows a sample immunoblot indicating that there was no change in the expression of total JNK (46kDa) in whole-cell lysate of hippocampal tissue prepared from vehicle-treated rats (lane 1) compared with tissue prepared from Aβ-treated rats (lane 2). Analysis of the mean data (Figure 3.2D) confirmed that mean JNK1 expression was similar in hippocampal tissue prepared from both Aβ-treated (2.73 arbitrary units ± 0.34) and vehicle-treated rats (2.51 arbitrary units ± 0.46). In parallel with the Aβ-induced increase in JNK phosphorylation, Figure 3.3A shows one sample immunoblot indicating increased c-Jun phosphorylation in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.3B shows mean data obtained from densitometric analysis; this reveals a significant increase in c-Jun phosphorylation in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (5.22 arbitrary units ± 0.79) compared with vehicle-treated rats (2.98.00 arbitrary units ± 0.11; *$p<0.05$; Student's *t* test for independent means).

3.3 Aβ administration is associated with disruption of mitochondrial function

Evidence suggests that JNK activation affects the integrity of the mitochondrial membrane, therefore Bax expression and cytosolic cytochrome *c* expression were assessed. Figure 3.4A shows one sample immunoblot indicating increased Bax expression in a mitochondrial fraction of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.4C shows mean data obtained from densitometric analysis; this reveals a significant increase in Bax expression in a mitochondrial fraction of hippocampal tissue prepared from Aβ-treated rats (9.43 arbitrary units ± 1.14) compared with vehicle-treated rats (5.51 arbitrary units ± 0.12; *$p<0.05$; Student's *t* test for independent means). Figure 3.5A shows one sample immunoblot indicating increased cytochrome *c* expression in cytosol of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.5C shows mean data obtained from densitometric analysis; this reveals a significant increase in cytochrome *c* in cytosol of hippocampal tissue prepared from Aβ-treated rats (5.08 arbitrary units ± 0.53) compared with vehicle-treated rats (3.62 arbitrary units ± 0.16; *$p<0.05$; Student's *t* test for independent means).

3.4 Aβ administration is associated with a decrease in intact PARP (116kDa)

Data from several studies indicates that translocation of cytochrome c from mitochondria to cytosol results in activation of the caspase cascade and accumulation of DNA fragmentation, through cleavage of DNA repair enzymes such as PARP. Figure 3.6A shows one sample immunoblot indicating decreased expression of intact poly(ADP-ribose)-polymerase (PARP; 116kDa) in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.6C shows mean data obtained from densitometric analysis; this reveals a significant decrease in intact PARP expression in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (3.95 arbitrary units ± 0.56) compared with vehicle-treated rats (5.37 arbitrary units ± 0.29; *$p<0.05$; Student's *t* test for independent means) suggesting that PARP cleavage occurs.

3.5 Aβ administration is associated with an increase in FasL expression

In an effort to consolidate the findings that indicated that cell viability was compromised, Fas ligand (FasL), which is an indicator of cell deterioration, was assessed. Figure 3.7A shows one sample immunoblot indicating increased FasL expression in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with vehicle-treated rats (lane 1). Figure 3.7C shows mean data obtained from densitometric analysis; this reveals a significant increase in FasL expression in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (5.35 arbitrary units ± 0.43) compared with vehicle-treated rats (3.32 arbitrary units ± 0.38; *$p<0.01$; Student's *t* test for independent means).

3.6 Aβ treatment is associated with an increase in release of IL-1β *in vitro*

The data reported above indicate that Aβ administration is associated with cell deterioration mediated by IL-1β and JNK. Consequently, primary neuronal cultures were used to assess the effect of Aβ on cell signalling and to determine whether IL-1β or JNK were intimately involved in this process. The concentration of interleukin-1β (IL-1β) was assessed in supernatant from control- and Aβ-treated cultured cortical neurons. Figure 3.8 shows that IL-1β release was significantly increased from cortical neurons treated with Aβ (7.13pg/mg ± 0.54) compared with IL-1β release from control-treated cortical neurons (3.99pg/mg ± 0.72; *$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in IL-1β release between control-treated cortical neurons pre-treated with vehicle (3.99pg/mg ± 0.72) and control- (2.74pg/mg ± 0.83) and Aβ-treated cortical neurons (4.5pg/mg ± 01.4) pre-treated with caspase-1 inhibitor.

In an effort to address whether the Aβ-induced increase in JNK phosphorylation occurred as a result of increases in IL-1β concentration, primary cortical neurons were incubated with Aβ in the presence of a caspase-1 inhibitor. Figure 3.9 shows that JNK phosphorylation was increased in cultured cortical neurons treated with Aβ compared with control-treated cortical neurons. There was no difference in JNK phosphorylation between control-treated and Aβ-treated

cortical neurons pre-treated with the caspase-1 inhibitor indicating that Aβ-induced activation of JNK was mediated by IL-1β.

3.7 IL-1β treatment is associated with increases in c-Jun phosphorylation and FasL expression *in vitro*

Primary cortical neurons were incubated with IL-1β in the presence of a JNK1 inhibitor to determine whether the effects of Aβ could be mimicked by IL-1β and if inhibition of JNK would prevent these effects. Figure 3.10A shows one sample immunoblot indicating increased c-Jun phosphorylation in whole-cell lysate prepared from cultured cortical neurons treated with IL-1β (lane 2) compared with control-treated neurons (lane 1), Aβ-treated neurons pre-treated with DJNKI1 (lane 4) and control-treated cortical neurons pre-treated with DJNK1I (lane 3). Figure 3.10C shows mean data obtained from densitometric analysis; this reveals a significant increase in c-Jun phosphorylation in whole-cell lysate prepared from cortical neurons treated with IL-1β (488.10 arbitrary units ± 46.21) compared with control-treated cortical neurons (319.8 arbitrary units ± 22.2; *$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in c-Jun phosphorylation in whole-cell lysate prepared from control-treated cortical neurons (319.8 arbitrary units ± 22.2), Aβ-treated cortical neurons pre-treated with DJNKI1 (371.5 arbitrary units ± 0.3) or control-treated cortical neurons pre-treated with DJNK1I (331.9 arbitrary units ± 2.3).

Figure 3.11A shows one sample immunoblot indicating increased FasL expression in whole-cell lysate prepared from cortical neurons treated with IL-1β (lane 2) compared with control-treated cortical neurons (lane 1), Aβ-treated cortical neurons pre-treated with DJNKI1 (lane 4) and control-treated cortical neurons pre-treated with DJNK1I (lane 3). Figure 3.11C shows mean data obtained from densitometric analysis; this reveals a significant increase in FasL expression in whole-cell lysate prepared from cortical neurons treated with IL-1β (511.90 arbitrary units ± 25.81) compared with control-treated cortical neurons (342.90 arbitrary units ± 21.49; *$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in FasL expression in whole-cell lysate prepared from control-treated cortical neurons (342.90 arbitrary units ± 21.49), Aβ-treated cortical neurons pre-treated with DJNKI1 (387.10

arbitrary units ± 51.19) or control-treated cortical neurons pre-treated with DJNK1I (362.60 arbitrary units ± 39.51). These data indicate that IL-1β mimics the effects of Aβ and that the observed effects of both Aβ and IL-1β on c-Jun and FasL are mediated by JNK.

3.8 Aβ treatment is associated with apoptotic changes *in vitro*

Caspase-3 activity and DNA fragmentation were assessed in cultured cortical neurons to investigate whether the Aβ-induced increases in IL-1β and JNK phosphorylation resulted in cell deterioration. Caspase-3 activity was increased in cultured cortical neurons treated with Aβ (Figure 3.12B) compared with control-treated cortical neurons (Figure 3.12A). There was no difference in caspase-3 activity between control-treated neurons (Figure 3.12A) and Aβ-treated neurons (Figure 3.12C) pre-treated with the caspase-1 inhibitor or DJNK1I (Figure 3.12D).

DNA fragmentation was increased in cultured cortical neurons treated with Aβ (Figure 3.13B) compared with control-treated cortical neurons (Figure 3.13A). There was no difference in DNA fragmentation between control-treated neurons (Figure 3.13A) and Aβ-treated (Figure 3.13C) neurons pre-treated with the caspase-1 inhibitor or DJNK1I (Figure 3.13D). These findings indicate that Aβ induces apoptosis in cultured cortical neurons – an effect that is mediated by IL-1β and JNK since inhibition of both of these proteins occluded the Aβ-stimulated increases in caspase-3 activity and DNA fragmentation.

3.9 Aβ administration and age do not affect stimulus strength or baseline synaptic transmission

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups of adult (3 months) rats (1.45g/kg ± 0.02 for vehicle-treated rats and 1.4g/kg ± 0.06; 1.58g/kg ± 0.12 and 1.47g/kg ± 0.02 for rats treated with 20, 60 and 200μM Aβ respectively; data not shown).

The mean stimulus strength needed to induce a spike was similar in all groups of adult rats (5.96V ± 1.23 for vehicle-treated rats and 5.60V ± 1.18; 6.0V ± 0.7

and 6.18V ± 1.3 for_rats treated with 0.1nmol, 0.3nmol and 1nmol Aβ respectively; data not shown).

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups for middle-aged (14 months) rats (2.68g/kg ± 0.13) for vehicle-treated rats and (2.53g/kg ± 0.06) rats treated with 20μM Aβ (data not shown). The mean stimulus strength needed to induce a spike was similar in both groups of middle-aged rats - 6.48V ± 1.54 for vehicle-treated rats and 6.03V ± 1.56 for rats treated with 20μM Aβ (data not shown).

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups for aged (24 months) rats (2.52g/kg ± 0.16 for vehicle-treated rats and 2.50 ± 0.11 for rats treated with 20μM Aβ; data not shown). The mean stimulus strength needed to induce a spike was similar in all groups of rats (5.40V ± 0.98 for vehicle-treated rats and 6.33V ± 1.65 for rats treated with 20μM Aβ; data not shown).

3.10 Aβ administration and age are associated with impaired LTP

Delivery of a high frequency train of stimuli to the perforant path (at time point 0) resulted in an immediate increase in the mean population excitatory post-synaptic potential (EPSP) slope in all groups of adult (Figure 3.14), middle-aged (Figure 3.16) and aged rats (Figure 3.17). Changes in EPSP slope in the first 2 min immediately following tetanic stimulation which are indicative of the induction phase of LTP (compared with the mean value in the 5 min prior to tetanus) were decreased in adult rats treated with 60μM (117.89 ± 3.15) and 200μM (118.91 ± 4.70) Aβ compared with vehicle-treated adult rats (140.00 ± 5.62; *$p<0.001$; ANOVA; n=5 in all cases; Figure 3.15A). There was no difference in EPSP slope between vehicle-treated adult rats (140.00 ± 5.62) and adult rats treated with 20μM Aβ (143.90 ± 10.57). Changes in EPSP slope in the last 5 min of recording following tetanic stimulation which are indicative of the maintenance phase of LTP (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in adult rats treated with 60μM (95.18 ± 3.70) and 200μM (95.19 ± 3.81) Aβ compared with vehicle-treated adult rats (140.00 ± 5.62; *$p<0.001$; ANOVA; Figure 3.15B). There was a significant

increase in the change in EPSP slope in adult rats treated with 20μM Aβ (116.79 ± 6.96) compared with adult rats treated with 60μM (95.18 ± 3.7) and 200μM (95.19 ± 3.81) Aβ (+$p<0.001$; ANOVA; Figure 3.16B).

In addition to analysis of the effects of Aβ on LTP, age-related changes in LTP were assessed. Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in vehicle-treated middle-aged (113.39 ± 2.20) and aged (116.33 ± 7.57) rats compared with vehicle-treated adult rats (132.96 ± 9.36; *$p<0.001$; ANOVA; Figure 3.18B).

Changes in EPSP slope in the first 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were decreased in middle-aged rats treated with 20μM Aβ (123.9 ± 3.54) compared with vehicle-treated middle-aged rats (153.54 ± 15.44; +$p<0.001$; ANOVA; n=6 in all cases; Figure 3.18A). Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in middle-aged rats treated with 20μM Aβ (94.68 ± 4.03) compared with vehicle-treated middle-aged rats (113.39 ± 2.20; +$p<0.001$; ANOVA; n=6 in all cases; Figure 3.18B)

Changes in EPSP slope in the first 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were decreased in aged rats treated with 20μM Aβ (120.89 ± 6.74) compared with vehicle-treated aged rats (133.35 ± 4.80; +$p<0.001$; ANOVA; Figure 3.18A). Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in aged rats treated with 20μM Aβ (103.21 ± 3.26) compared with vehicle-treated aged rats (116.33 ± 7.57; +$p<0.001$; ANOVA; n=6 in both cases; Figure 3.18B).

3.11 Aβ administration and age are associated with increases in the concentration of pro-inflammatory cytokines

The concentration of the proinflammatory cytokine IL-1β was assessed in hippocampal tissue prepared from vehicle-treated and Aβ-treated adult rats. Figure

3.19 shows that IL-1β concentration was significantly increased in hippocampal homogenate prepared from adult rats treated with 200μM Aβ (26.25pg/mg ± 3.06) compared with tissue prepared from vehicle-treated adult rats (11.33pg/mg ± 3.81; *p<0.05; ANOVA). There was no significant difference in IL-1β concentration in hippocampal homogenate prepared from adult rats treated with 20μM (12.60pg/mg ± 2.50) and 60μM (22.00pg/mg ± 7.26) Aβ and vehicle-treated adult rats (11.33 pg/mg ± 3.81).

IL-1β concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated middle-aged rats (27.00pg/mg ± 2.00) compared with vehicle-treated adult rats (11.33pg/mg ± 3.81; +p<0.05; ANOVA; Figure 3.20).

IL-1β concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated aged rats (27.50pg/mg ± 2.40) compared with vehicle-treated adult rats (11.33pg/mg ± 3.81; +p<0.05; ANOVA; Figure 3.21). Additionally IL-1β concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated aged rats (55.67pg/mg ± 12.12) in comparison with vehicle-treated aged rats (27.50pg/mg ± 2.40; *p<0.05; ANOVA).

These data indicate Aβ induces an inflammatory response and to consolidate these findings the effect of Aβ on another pro-inflammatory cytokine was assessed. The concentration of the proinflammatory cytokine interferon-γ (IFNγ) was assessed in hippocampal homogenate prepared from vehicle-treated and Aβ-treated adult rats. Figure 3.21 shows that IFNγ concentration was significantly increased in hippocampal homogenate prepared from adult rats treated with 20μM (416.72pg/mg ± 44.89), 60μM (462.06pg/mg ± 68.52) and 200μM (518.34pg/mg ± 47.03) Aβ compared with tissue prepared from vehicle-treated adult rats (144.80 pg/mg ± 71.82; *p<0.05; ANOVA).

IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated middle-aged rats (443.89pg/mg ± 61.12) compared with vehicle-treated adult rats (144.80pg/mg ± 71.82; *p<0.05; ANOVA; Figure 3.22).

IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated aged rats (354.67pg/mg ± 39.25) compared with vehicle-treated adult rats (144.80pg/mg ± 71.82; *p<0.05; ANOVA; Figure 3.22). There

was no significant difference in IFNγ concentration in hippocampal homogenate prepared from Aβ-treated aged rats (373.77pg/mg ± 25.90) in comparison with vehicle-treated aged rats (354.67pg/mg ± 39.25; Figure 3.22).

3.12 IL-1β is released from Aβ-treated cultured cortical neurons and glia

To address the question of the source of IL-1β, the concentration of IL-1β was assessed in supernatant from control- and Aβ-treated hippocampal neurons and cortical glia. Figure 3.23A shows that IL-1β release was significantly increased from cortical neurons treated with Aβ (7.13pg/mg ± 0.54) compared with IL-1β release from control-treated cortical neurons (3.99pg/mg ± 0.72; *$p<0.05$; Student's *t* test for independent means; n=5 in both cases). Figure 3.23B shows that IL-1β release was significantly increased from cortical glia treated with Aβ (65.56pg/mg ± 10.39) compared with IL-1β release from control-treated cortical glia (35.92pg/mg ± 7.17; *$p<0.05$; Student's *t* test for independent means; n=5 in both cases). The data show increased release of IL-1β from cultured glia compared with cultured neurons, however this occurs as a result of greater numbers of cells in primary cultures of glia compared with primary cultures of neurons. The purity of primary cultured neurons was determined by staining fixed cells with GFAP - Figure 3.23C indicates abundant GFAP immunofluorescence in primary glial cultures and no GFAP immunofluorescence in primary neuronal cultures.

3.13 Aβ administration and age are associated with an increase in JNK phosphorylation

Figure 3.24A shows one sample immunoblot indicating increased JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from 20μM (lane 2), 60μM (lane 3) and 200μM (lane 4) Aβ-treated adult rats compared with vehicle-treated adult rats (lane 1). Figure 3.24C shows mean data obtained from densitometric analysis; this revealed a significant increase in JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 20μM (6.95 arbitrary units ±0.44), 60μM (7.01 arbitrary units ± 0.22) and 200μM (7.41

arbitrary units ± 0.22) Aβ compared with vehicle-treated adult rats (5.59 arbitrary units ± 0.25; *p<0.05; **p<0.01; ANOVA).

Figure 3.25A shows one sample immunoblot indicating increased JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from 20μM (lane 2) Aβ-treated adult rats, vehicle-treated middle-aged (lane 3) and aged (lane 5) rats compared with vehicle-treated adult rats (lane 1). There was no significant difference between middle-aged (lane 4) and aged (lane 6) rats treated with 20μM Aβ and vehicle-treated middle-aged (lane 3) and aged (lane 5) rats. Figure 3.25C shows mean data obtained from densitometric analysis; this revealed a significant increase in JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 20μM (6.95 arbitrary units ± 0.44), vehicle-treated middle-aged (7.81 arbitrary units ± 0.35) and aged (6.67 arbitrary units ± 0.28) rats compared with vehicle-treated adult rats (5.59 arbitrary units ± 0.25; *p<0.05; ANOVA). There was no significant difference in JNK phosphorylation in whole-cell lysate of hippocampal tissue prepared from middle-aged (7.76 arbitrary units ± 0.45) and aged (7.04 arbitrary units ± 0.89) rats treated with 20μM Aβ and vehicle-treated middle-aged (7.81 arbitrary units ± 0.35) and aged (6.67 arbitrary units ± 0.28).

3.14 Aβ administration and age are associated with an increase in RAGE expression

Figure 3.26A shows one sample immunoblot indicating increased RAGE expression in whole-cell lysate of hippocampal tissue prepared from 20μM (lane 2), 60μM (lane 3) and 200μM (lane 4) Aβ-treated adult rats compared with vehicle-treated adult rats (lane 1). Figure 3.26C shows mean data obtained from densitometric analysis; this revealed a significant increase in RAGE expression in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 20μM (14.7 arbitrary units ± 1.6), 60μM (16.7 arbitrary units ± 1.5) and 200μM (13.7 arbitrary units ± 1.1) Aβ compared with vehicle-treated adult rats (9.3 arbitrary units ± 0.2; *p<0.05; ANOVA).

Figure 3.27A shows one sample immunoblot indicating increased RAGE expression in whole-cell lysate of hippocampal tissue prepared from 20μM (lane 2)

Aβ-treated adult rats, vehicle-treated middle-aged (lane 3) and aged (lane 5) rats compared with vehicle-treated adult rats (lane 1). There was no significant difference between middle-aged (lane 4) and aged (lane 6) rats treated with 20μM Aβ and vehicle-treated middle-aged (lane 3) and aged (lane 5) rats. Figure 3.27C shows mean data obtained from densitometric analysis; this revealed a significant increase in RAGE expression in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 20μM (14.6 arbitrary units ± 1.6), vehicle-treated middle-aged (14.7 arbitrary units ± 0.4) and aged (13.3 arbitrary units ± 0.8) rats compared with vehicle-treated adult rats (9.3 arbitrary units ± 0.2; *$p<0.05$; ANOVA). There was no significant difference in RAGE expression in whole-cell lysate of hippocampal tissue prepared from middle-aged (13.8 arbitrary units ± 2.0) and aged (16.1 arbitrary units ± 1.8) rats treated with 20μM Aβ and vehicle-treated middle-aged (14.7 arbitrary units ± 0.4) and aged (13.3 arbitrary units ± 0.8) rats.

3.15 Aβ administration and age are associated with an increase in CD40 expression

Figure 3.28A shows one sample immunoblot indicating increased CD40 expression in whole-cell lysate of hippocampal tissue prepared from 20μM (lane 2), 60μM (lane 3) and 200μM (lane 4) Aβ-treated adult rats compared with vehicle-treated adult rats (lane 1). Figure 3.28C shows mean data obtained from densitometric analysis; this revealed a significant increase in CD40 expression in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 60μM (21.5 arbitrary units ± 1.7) and 200μM (23.3 arbitrary units ± 2.4) Aβ compared with vehicle-treated adult rats (14 arbitrary units ± 0.7; *$p<0.05$; ANOVA). There was no significant difference in CD40 expression in whole-cell lysate of hippocampal tissue prepared from adult rats treated with 20μM Aβ (17.5 arbitrary units ± 1.5) compared with vehicle-treated adult rats (14 arbitrary units ± 0.7).

Figure 3.29A shows one sample immunoblot indicating increased CD40 expression in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (lane 3) and aged (lane 5) rats compared with vehicle-treated adult rats (lane 1). There was no significant difference between middle-aged (lane 4) and aged (lane 6) rats treated with 20μM Aβ and vehicle-treated middle-aged (lane 3) and aged

(lane 5) rats. Figure 3.29C shows mean data obtained from densitometric analysis; this revealed a significant increase in CD40 expression in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (23 arbitrary units ± 2.4) and aged (20.5 arbitrary units ± 2.0) rats compared with vehicle-treated adult rats (14 arbitrary units ± 0.7; *$p<0.05$; ANOVA). There was no significant difference in CD40 expression in whole-cell lysate of hippocampal tissue prepared from middle-aged (22 arbitrary units ± 2.2) and aged (17.6 arbitrary units ± 1.0) rats treated with 20μM Aβ and vehicle-treated middle-aged (23.0 arbitrary units ± 2.4) and aged (20.5 arbitrary units ± 2.0) rats.

3.16 Aβ treatment is associated with an increase in release of IL-1β *in vitro;* attenuation with minocycline hydrochloride

Since increased expression of RAGE and CD40 are indicative of microglial activation, the concentration of IL-1β was assessed in supernatant from cultured cortical glia treated with Aβ in the presence of minocycline hydrochloride – an inhibitor of microglial activation. Figure 3.30 shows that IL-1β release was significantly increased from cortical glia treated with Aβ (308.66pg/mg ± 42.47) compared with control-treated cortical glia (105.05pg/mg ± 26.51; *$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in IL-1β release between control-treated cortical glia pre-treated with vehicle (105.05pg/mg ± 26.51) and Aβ-treated cortical glia pre-treated with minocycline hydrochloride(216.14pg/mg ± 32.19).

3.17 Aβ administration, EPA treatment and age do not affect stimulus strength or baseline synaptic transmission

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups of adult (3 months) rats (1.98g/kg ± 0.16, 1.78g/kg ± 0.13 and 1.97g/kg ± 0.21 for vehicle-, 60μM and 200μM Aβ-treated control adult rats respectively and 1.88g/kg ± 0.15, 1.80g/kg ± 0.10 and 1.98g/kg ± 0.09 for vehicle-, 60μM and 200μM Aβ-treated adult rats that received EPA respectively; data not shown).

The mean stimulus strength needed to induce a spike was similar in all groups of adult rats (4.8V ± 1.8, 6.0V ± 1.1 and 7.0V ± 1.3 for vehicle-, 60μM and 200μM Aβ-treated control adult rats respectively and 5.6V ± 1.1, 4.8V ± 1.2 and 6.5V ± 1.0 for vehicle-, 60μM and 200μM Aβ-treated adult rats that received EPA respectively; data not shown).

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups for middle-aged (14 months) rats (2.68g/kg ± 0.13 and 2.53g/kg ± 0.06 for vehicle- and 20 μM Aβ-treated control rats respectively and 2.62g/kg ± 0.08 and 2.76g/kg ± 0.13 for vehicle- and 20 μM Aβ-treated rats that received EPA respectively; data not shown). The mean stimulus strength needed to induce a spike was similar in all groups of middle-aged rats (5.38V ± 0.67 and 4.8V ± 1.07 for vehicle- and 20 μM Aβ-treated control rats respectively and 4.16V ± 0.72 and 5.84V ± 0.62 for vehicle- and 20 μM Aβ-treated rats that received EPA respectively; data not shown).

There was no significant difference in the amount of urethane required to induce deep anaesthesia in the different treatment groups for aged (24 months) rats (2.62g/kg ± 0.17 and 2.40g/kg ± 0.07 for vehicle- and 20 μM Aβ-treated control rats respectively and 2.58g/kg ± 0.11 and 2.50g/kg ± 0.20 for vehicle- and 20 μM Aβ-treated rats that received EPA respectively; data not shown). The mean stimulus strength needed to induce a spike was similar in all groups of middle-aged rats (4.30V ± 0.93 and 6.11V ± 1.21 for vehicle- and 20 μM Aβ-treated control rats respectively and 5.30V ± 0.96 and 5.25V ± 0.95 for vehicle- and 20 μM Aβ-treated rats that received EPA respectively; data not shown).

3.18 Body weights

Mean body weights of adult (3 month-old) rats were similar in all treatment groups and, in all cases, were significantly greater at the end of the experimental treatment period compared with the start (**$p<0.01$; **$p<0.001$; ANOVA; Figure 3.31A). The mean body weights increased from 311.2g ± 6.7 to 354.5g ± 7.9 in adult rats receiving control diet (hereafter referred to as control adult rats) and from 314.9g ± 8.7 to 359.2g ± 9.1 in adult rats that received EPA.

Mean body weights of middle-aged (14 month-old) rats were similar in all treatment groups and in all cases were similar at the end of the experimental treatment period compared with the start (Figure 3.31B). The mean body weights of middle-aged rats receiving control diet (hereafter referred to as control middle-aged rats) were similar before (534.6g ± 18.0) and after (556.3g ± 18.5) the experimental period. The mean body weights of middle-aged rats receiving EPA were similar before (534.9g ± 17.7) and after (546.6g ± 17.3) the experimental period.

Mean body weights of aged (24 month-old) rats were similar in all treatment groups and in all cases were similar at the end of the experimental treatment period compared with the start (Figure 3.31C). The mean body weights of aged rats receiving control diet (hereafter referred to as control aged rats) were similar before (520.4g ± 19.2) and after (524.6.3g ± 18.2) the experimental period. The mean body weights of aged rats receiving EPA were similar before (536.6g ± 11.6) and after (539.7g ± 11.9) the experimental period.

3.19 Aβ administration is associated with impairment in LTP in dentate gyrus; abrogation with EPA

Figure 3.32A and B show that delivery of a high frequency train of stimuli to the perforant path (at time point 0) resulted in an immediate increase in the mean population EPSP slope in all groups of adult rats. Changes in EPSP slope in the first 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were decreased in control adult rats treated with 200μM (115.09 ± 10.59) Aβ compared with vehicle-treated control adult rats (129.04 ± 3.12; ***$p<0.01$; ANOVA; Figure 3.33A). Changes in EPSP slope in adult rats treated with 200μM Aβ that received EPA (140.01 ± 2.03) were significantly increased compared with control adult rats treated with 200μM Aβ (115.09 ± 10.59; +$p<0.05$; ANOVA; Figure 3.33A).

Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in 60μM (109.19 ± 11.02) and 200μM (107.21 ± 5.57) Aβ-treated control adult rats compared with vehicle-treated control adult rats (137.01 ± 13.19;

***p<0.001; ANOVA; Figure 3.33B). There was a significant increase in the change in EPSP slope in 60μM Aβ-treated adult rats that received EPA (131.28 ± 4.83) compared with 60μM Aβ-treated control adult rats (109.19 ± 11.02; +p<0.001; ANOVA; Figure 3.33B).

Figure 3.34A and B show that delivery of a high frequency train of stimuli to the perforant path (at time point 0) resulted in an immediate increase in the mean population EPSP slope in all groups of middle-aged rats. Changes in EPSP slope in the first 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were similar in vehicle- (139.70 ± 5.64) and Aβ-treated (142.33 ± 6.93) control middle-aged rats and vehicle- (138.61 ± 7.51) and Aβ-treated (143.71 ± 6.17) middle-aged rats that received EPA (Figure 3.35A). Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in 20μM (113.17 ± 8.39) Aβ-treated control middle-aged rats compared with vehicle-treated control middle-aged rats (122.83 ± 10.52; ***p<0.001; ANOVA; Figure 3.35B). There was a significant increase in the change in EPSP slope in 20μM Aβ-treated middle-aged rats that received EPA (125.72 ± 5.61) compared with 20μM Aβ-treated control middle-aged rats (113.17 ± 8.39; +++p<0.001; ANOVA; Figure 3.35B).

Figure 3.36A and B show that delivery of a high frequency train of stimuli to the perforant path (at time point 0) resulted in an immediate increase in the mean population EPSP slope in all groups of aged rats. Changes in EPSP slope in the first 2 min immediately following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were decreased in vehicle-treated control aged rats (129.42 ± 5.93) compared with vehicle-treated aged rats that received EPA (141.89 ± 4.34; ***p<0.001; ANOVA, n=6 in both cases). Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in 20μM Aβ-treated control aged rats (105.23 ± 7.65) compared with vehicle-treated control aged rats (118.11 ± 11.22; ***p<0.001; ANOVA; Figure 3.37B). There was a significant increase in the change in EPSP slope in 20μM Aβ-treated (128.16 ± 5.76) and vehicle-treated aged rats that received EPA (122.96 ± 3.78) compared with 20μM Aβ-treated control aged rats

(105.23 ± 7.65) and vehicle-treated control aged rats (113.66 ± 8.52; +++p<0.001; ANOVA; Figure 3.37B).

Changes in EPSP slope in the last 5 min of recording following tetanic stimulation (compared with the mean value in the 5 min prior to tetanus) were significantly decreased in vehicle-treated control aged rats (118.11 ± 11.22) compared with vehicle-treated control adult rats (137.01 ± 13.19; ***p<0.001; ANOVA; Figure 3.37C). This age-related impairment was partially abrogated by treatment with EPA (122.96 ± 3.78; ++p<0.01; ANOVA; Figure 3.37C).

3.20 Aβ administration is associated with an increase in the concentration of pro-inflammatory cytokines; abrogation by EPA

IL-1β concentration was significantly increased in hippocampal homogenate prepared from 60μM (485.23pg/mg ± 73.88) and 200μM (445.68pg/mg ± 39.84) Aβ-treated control adult rats compared with vehicle-treated control adult rats (264.53pg/mg ± 4.76) (*p<0.05; ANOVA; Figure 3.38). IL-1β concentration was similar in hippocampal homogenate prepared from vehicle-treated control adult rats (264.53pg/mg ± 4.76) and vehicle-treated adult rats that received EPA (283.58pg/mg ± 24.03) and 60μM (308.33pg/mg ± 33.31) and 200μM (339.73pg/mg ± 31.59) Aβ-treated adult rats that received EPA. This data suggests that EPA was capable of preventing inflammatory changes induced by Aβ.

IL-1β concentration was similar in hippocampal homogenate prepared from vehicle- (374.16pg/mg ± 26.20) and 20μM (355.39pg/mg ± 14.44) Aβ-treated middle-aged control rats and vehicle- (315.90pg/mg ± 13.62) and Aβ-treated (336.58pg/mg ± 30.42) middle-aged rats that received EPA (Figure 3.39).

Figure 3.40A indicates that IL-1β concentration was significantly increased in hippocampal homogenate prepared from 20μM Aβ-treated control aged rats (575.44pg/mg ± 38.74) compared with homogenate prepared from vehicle-treated control aged rats (393.49pg/mg ± 29.27). IL-1β concentration was significantly increased in hippocampal homogenate prepared from 20μM Aβ-treated control aged rats (573.44pg/mg ± 38.74) compared with 20μM Aβ-treated aged rats that received EPA (415.51pg/mg ± 57.00). Figure 3.40B indicates that IL-1β concentration was

significantly increased in hippocampal homogenate prepared from vehicle-treated control aged rats (393.49pg/mg ± 29.27) compared with vehicle-treated control adult rats (264.53pg/mg ± 4.76; *$p<0.05$; ANOVA). There was no significant difference in IL-1β concentration in hippocampal homogenate prepared from vehicle-treated control adult rats (264.53pg/mg ± 4.76), vehicle-treated adult rats that received EPA (283.58pg/mg ± 24.03) and vehicle-treated aged rats that received EPA (331.86pg/mg ± 12.83).

Interferon-γ (IFNγ) concentration was significantly increased in hippocampal homogenate prepared from 60μM (963.75pg/mg ± 262.25) and 200μM (770.25pg/mg ± 213.63) Aβ-treated control adult rats compared with vehicle-treated control adult rats (352.00pg/mg ± 51.72) (*$p<0.05$; ANOVA; Figure 3.41). IFNγ concentration was similar in hippocampal homogenate prepared from vehicle-treated control adult rats (352.00pg/mg ± 51.72), vehicle-treated adult rats that received EPA (436.79pg/mg ± 28.67) and 60μM (496.93pg/mg ± 109.09) and 200μM (1525.96pg/mg ± 140.08) Aβ-treated rats that received EPA.

IFNγ concentration was similar in hippocampal homogenate prepared from vehicle- (438.91pg/mg ± 33.82) and 20μM (495.60pg/mg ± 71.19) Aβ-treated control middle-aged rats and vehicle- (512.94pg/mg ± 35.02) and Aβ-treated (549.94pg/mg ± 75.88) middle-aged rats that received EPA (Figure 3.42).

Figure 3.43A indicates that IFNγ concentration was increased in hippocampal homogenate prepared from vehicle-treated (600.14pg/mg ± 56.28) and 20μM Aβ-treated control aged rats (621.58pg/mg ± 127.83) compared with 20μM Aβ-treated aged rats (519.74pg/mg ± 22.01) and vehicle-treated rats (436.62pg/mg ± 41.15) fed that received EPA. Figure 3.43B indicates that IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated (600.14pg/mg ± 56.28) control aged rats compared with vehicle-treated control adult rats (352.00pg/mg ± 51.72; *$p<0.05$; ANOVA). There was no significant difference in IFNγ concentration in hippocampal homogenate prepared from vehicle-treated control adult rats (352.00pg/mg ± 51.72) and vehicle-treated adult (436.79pg/mg ± 28.67) and aged rats (436.62pg/mg ± 41.15) that received EPA.

3.21 Aβ administration is associated with an increase in cytosolic expression of phosphorylated JNK; abrogation with EPA

To determine whether EPA may prevent some of the downstream consequences of cytokine production, JNK phosphorylation was measured in nuclear and cytosolic fractions of hippocampal tissue prepared from adult rats. Figure 3.44A indicates that JNK phosphorylation was similar in nuclear fraction of hippocampal tissue prepared from vehicle- (lane 1), 60μM (lane 2) and 200μM (lane 3) Aβ-treated control rats and vehicle- (lane 4), 60μM (lane 5) and 200μM (lane 6) Aβ-treated rats that received EPA. Analysis of the mean data revealed no difference between vehicle- (1.40 arbitrary units ± 0.02), 60 (1.58 arbitrary units ± 0.09) and 200μM (1.85 arbitrary units ± 0.16) Aβ-treated control rats and vehicle- (1.64 arbitrary units ± 0.27), 60 (1.73 arbitrary units ± 0.29) and 200μM (1.82 arbitrary units ± 0.38) Aβ-treated rats that received EPA.

Figure 3.45A shows one sample immunoblot indicating increased JNK phosphorylation in cytosolic fraction of hippocampal tissue prepared from 60μM (lane 2) and 200μM (lane 3) Aβ-treated control rats compared with vehicle-treated control rats (lane 1). JNK phosphorylation was similar in cytosolic fraction of hippocampal tissue prepared from 60μM (lane 5) and 200μM (lane 6) Aβ-treated rats that received EPA and vehicle-treated rats that received EPA (lane 4) and control rats (lane 1). Figure 3.45C shows mean data obtained from densitometric analysis; this revealed a significant increase in JNK phosphorylation in cytosolic fraction of hippocampal tissue prepared from control rats treated with 60μM (26.68 arbitrary units ± 2.33) and 200μM (26.67 arbitrary units ± 3.90) Aβ compared with vehicle-treated control rats (14.84 arbitrary units ± 2.33; *$p<0.05$; ANOVA). There was no significanr difference in JNK phosphorylation in a cytosolic fraction of hippocampal tissue prepared from vehicle-treated control adult rats (14.84 arbitrary units ± 2.33) and hippocampal tissue prepared from vehicle- (20.05 arbitrary units ± 0.54) and 60 (18.12 arbitrary units ± 2.04) and 200μM (6.83 arbitrary units ± 0.34) Aβ-treated adult rats that received EPA.

3.22 IL-4 concentration is altered in hippocampus of aged rats; abrogation with EPA Interleukin-4 (IL-4) concentration was similar in vehicle- (26.74pg/mg ± 4.29), 60 (26.19pg/mg ± 3.86) and 200μM (23.29pg/mg ± 4.65) Aβ-treated control adult rats and vehicle- (23.88pg/mg ± 2.58), 60 (24.13pg/mg ± 3.65) and 200μM (24.93pg/mg ± 4.07) Aβ-treated rats that received EPA (Figure 3.46). IL-4 concentration was increased in hippocampal homogenate prepared from vehicle-treated aged rats that received EPA (28.99pg/mg ± 4.66) compared with homogenate prepared from vehicle-treated control aged rats (19.69pg/mg ± 2.49; Figure 3.47). IL-4 concentration was similar in hippocampal homogenate prepared from vehicle-treated control aged rats (19.69pg/mg ± 2.49), Aβ-treated control aged rats (20.97pg/mg ± 3.84) and Aβ-treated aged rats that received EPA (21.38pg/mg ± 2.36). Samples were not available to assess IL-4 concentration in hippocampus of middle-aged rats.

3.23 Aβ administration and age are associated with a decrease in PPARγ expression; abrogation with EPA

Several studies have reported that EPA is an endogenous ligand for the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPARγ) and that it may exert its anti-inflammatory effects through activation of this receptor. Figure 3.48A shows one sample immunoblot indicating that PPARγ expression (50kDa) was decreased in a nuclear fraction of hippocampal tissue prepared from 60 (lane2) and 200μM (lane 3) Aβ-treated control adult rats compared with tissue prepared from vehicle-treated control adult rats (lane 1). PPARγ expression was similar in a nuclear fraction of hippocampal tissue prepared from vehicle-treated control adult rats (lane 1) and hippocampal tissue prepared from vehicle- and 60 and 200μM Aβ-treated adult rats that received EPA (lanes 4, 5 and 6 respectively).

PPARγ expression was decreased in a nuclear fraction of hippocampal tissue prepared from 60μM (1.89 arbitrary units ± 0.30) and 200μM (1.51 arbitrary units ± 0.34) Aβ-treated control adult rats compared with vehicle-treated control adult rats (2.60 arbitrary units ± 0.12; Figure 3.48B). PPARγ expression was similar in a nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats (2.60 arbitrary units ± 0.12) and vehicle-treated control adult rats that received EPA (2.54

arbitrary units ± 0.53) and 60μM (2.41 arbitrary units ± 0.48) and 200μM (2.65 arbitrary units ± 0.43) Aβ-treated rats that received EPA.

Figure 3.49A shows one sample immunoblot indicating that PPARγ expression (50kDa) was decreased in a nuclear fraction of hippocampal tissue prepared from vehicle-treated control aged rats (lane 7) compared with tissue prepared from vehicle-treated control adult rats (lane 1), vehicle-treated adult rats that received EPA (lane 4) and vehicle-treated aged rats that received EPA (lane 9).

Figure 3.49B shows mean data indicating that PPARγ expression was decreased in a nuclear fraction of hippocampal tissue prepared from vehicle-treated control aged rats (1.85 arbitrary units ± 0.62) compared with vehicle-treated control adult rats (2.60 arbitrary units ± 0.12). There was no difference between vehicle-treated control adult rats (2.60 arbitrary units ± 0.12), vehicle-treated adult (2.54 arbitrary units ± 0.53) and aged rats (3.22 arbitrary units ± 0.86) that received EPA.

Figure 3.49C shows one sample immunoblot indicating that PPARγ expression (50kDa) was decreased in a nuclear fraction of hippocampal tissue prepared from vehicle- (lane 1) and 20μM (lane 2) Aβ-treated control aged rats compared with tissue prepared from vehicle- (lane 3) and 20μM Aβ-treated aged rats (lane 4) that received EPA.

Figure 3.49D shows mean data indicating that PPARγ expression was decreased in a nuclear fraction of hippocampal tissue prepared from vehicle- (1.85 arbitrary units ± 0.62) and Aβ-treated (1.86 arbitrary units ± 0.70) control aged rats compared with vehicle- (3.22 arbitrary units ± 0.86) and Aβ-treated (3.63 arbitrary units ± 0.93) aged rats that received EPA.

3.24 PPARγ agonist abrogates Aβ-induced activation of glia *in vitro*

The data reported above indicate increased expression of PPARγ with EPA treatment. To assess whether PPARγ exerts an anti-inflammatory effect the concentration of IL-1β was assessed in supernatant from cultured cortical glia trated with Aβ in the presence of a PPARγ agonist troglitazone. Figure 3.50 shows that IL-1β release from cortical glia was significantly increased by treatment with Aβ (283.57pg/mg ± 27.38) compared with control-treatment (126.82pg/mg ± 20.58;

*$p<0.05$; ANOVA; n=11 in both cases). There was no significant difference in IL-1β release from control-treated cortical glia pre-treated with vehicle (126.82pg/mg ± 20.58) and Aβ-treated cortical glia pre-treated with troglitazone (122.89pg/mg ± 18.88). These data indicate a neuroprotective role for EPA that may be mediated by PPARγ.

Figure 3.1 Aβ administration is associated with an increase in the concentration of interleukin-1β in hippocampus

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (n=5; *$p<0.001$; Student's *t* test for independent means; n=5 in both cases). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

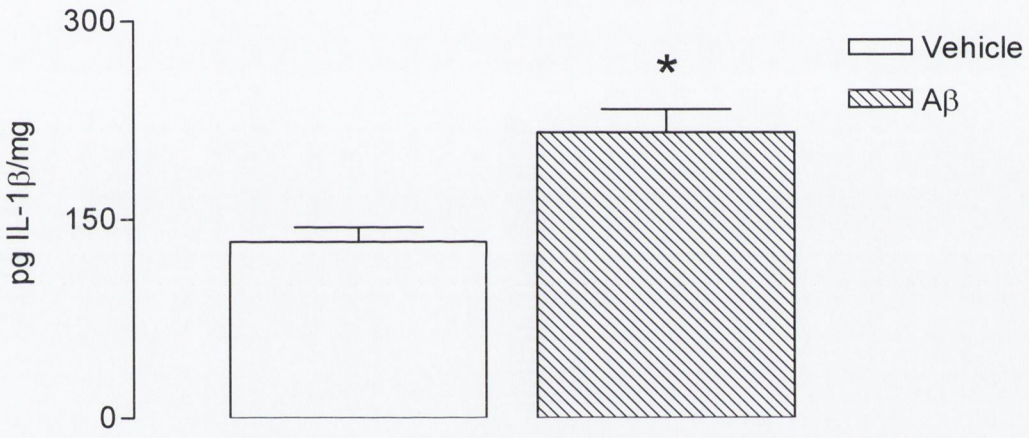

300
150
0
pg IL-1β/mg
*
Vehicle
Aβ

Figure 3.2 JNK phosphorylation is upregulated by Aβ administration

A. One sample immunoblot shows that JNK phosphorylation (46kDa) was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

B. Data from densitometric analysis revealed that mean JNK phosphorylation was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.001$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of JNK phosphorylation and are means ± standard error of the means.

C. A sample immunoblot shows no change in the expression of total JNK (46kDa) in whole-cell lysate of hippocampal tissue prepared from vehicle-treated rats (lane 1) compared with tissue prepared from Aβ-treated rats (lane 2).

D. Data from densitometric analysis confirmed similar mean total JNK expression in whole-cell lysate of hippocampal tissue prepared from both groups (n=5 in both cases). Values are expressed as arbitrary units of JNK expression and are means ± standard error of the means.

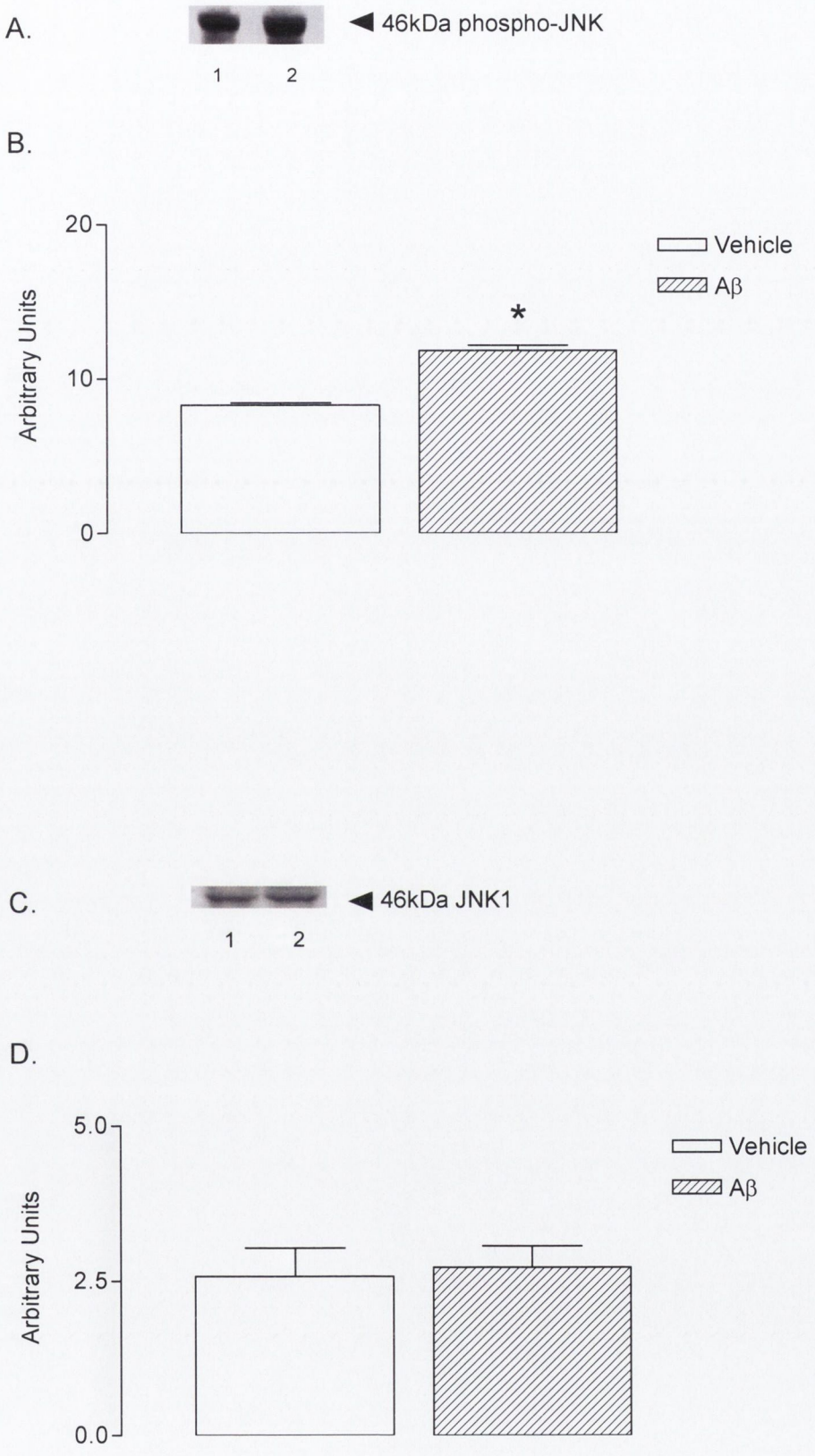

A.
46kDa phospho-JNK
1
2
B.
20
10
0
Arbitrary Units
Vehicle
Aβ
*
C.
46kDa JNK1
1
2
D.
5.0
2.5
0.0
Arbitrary Units
Vehicle
Aβ

Figure 3.3 Aβ administration is associated with an increase in c-Jun phosphorylation

A. One sample immunoblot shows that c-Jun phosphorylation (48kDa) was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample phospho-c-Jun immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate of hippocampal tissue prepared from vehicle-treated (lane 1) and Aβ-treated rats (lane 2).

C. Data from densitometric analysis revealed that mean c-Jun phosphorylation was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of c-Jun phosphorylation and are means ± standard error of the means.

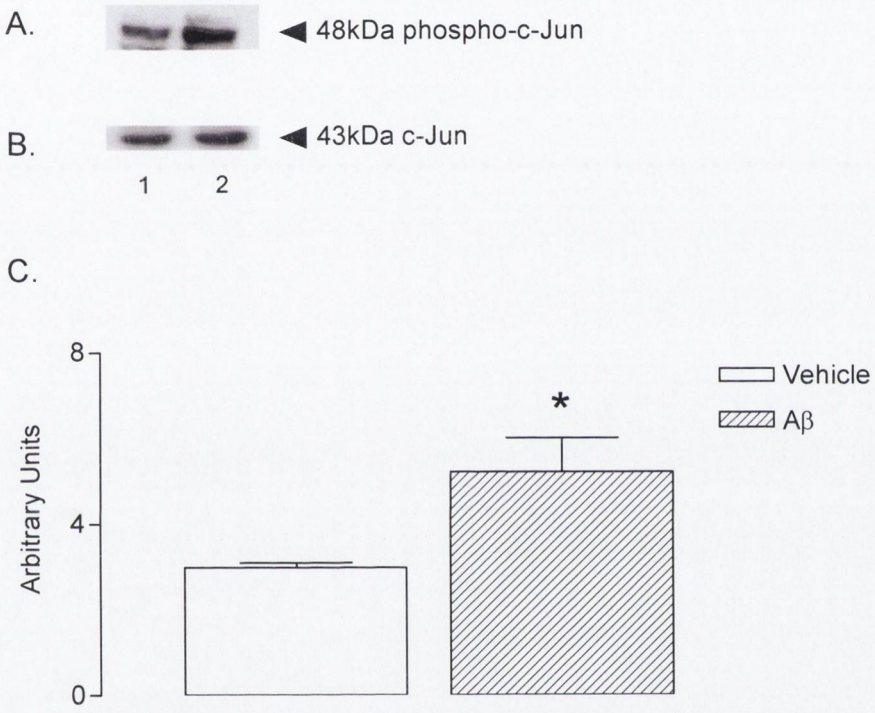

A.
48kDa phospho-c-Jun
B.
43kDa c-Jun
1
2
C.
8
4
0
Arbitrary Units
*
Vehicle
Aβ

Figure 3.4 Aβ administration is associated with an increase in mitochondrial Bax expression

A. One sample immunoblot shows that Bax expression (23kDa) was increased in mitochondrial fraction of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample Bax immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in mitochondrial fraction of hippocampal tissue prepared from vehicle-treated (lane 1) and Aβ-treated rats (lane 2).

C. Data from densitometric analysis revealed that mean Bax expression was increased in mitochondrial fraction of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of Bax expression and are means ± standard error of the means.

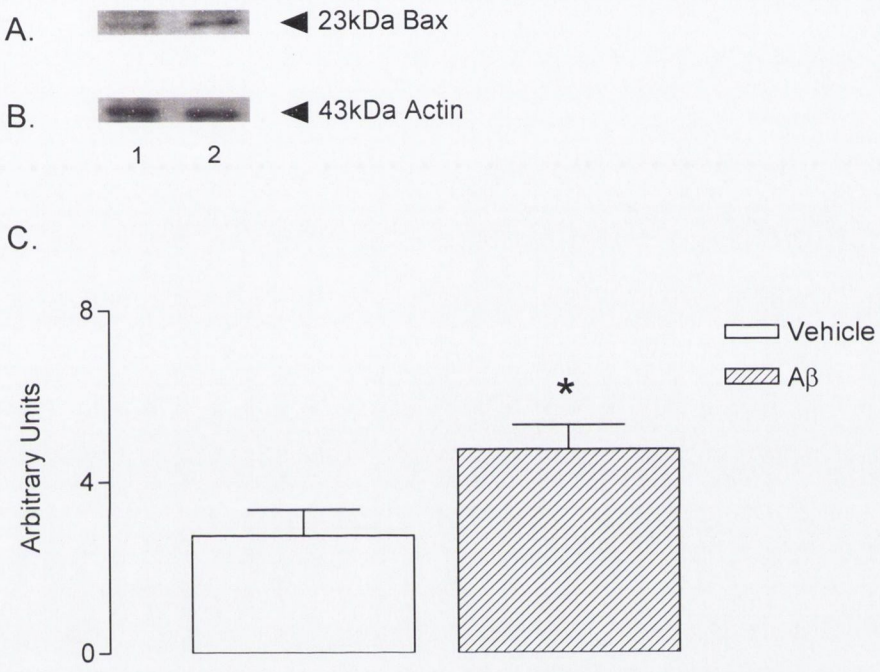

A.
23kDa Bax
B.
43kDa Actin
1
2
C.
Arbitrary Units
8
4
0
*
Vehicle
Aβ

Figure 3.5 Aβ administration is associated with an increase in cytosolic cytochrome *c* expression

A. One sample immunoblot shows increased cytochrome *c* (17kDa) expression in cytosolic fraction of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample cytochrome *c* immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in cytosolic fracton of hippocampal tissue prepared from vehicle-treated (lane 1) and Aβ-treated rats (lane 2).

C. Data from densitometric analysis revealed increased mean cytochrome *c* expression in cytosolic fraction of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of cytochrome *c* expression and are means ± standard error of the means.

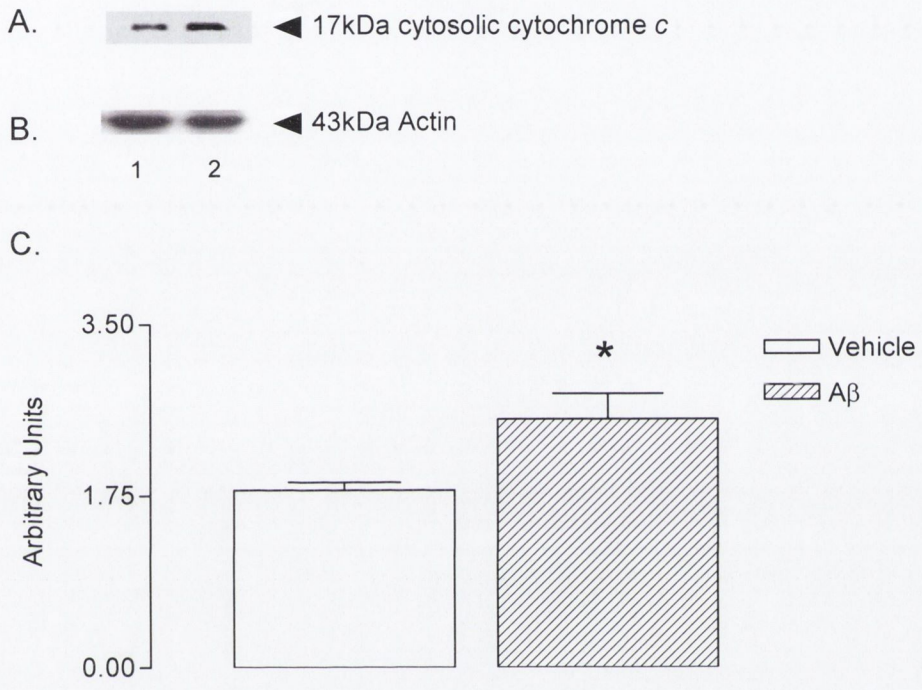

A.
17kDa cytosolic cytochrome c
B.
43kDa Actin
1
2
C.
3.50
1.75
0.00
Arbitrary Units
*
Vehicle
Aβ

Figure 3.6 Aβ administration is associated with a decrease in intact PARP expression (116kDa)

A. One sample immunoblot shows the intact fragment (116kDa) of poly(ADP-ribose) polymerase (PARP) decreased in whole-cell lysate of hippocampal tissue of Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

C. A sample PARP immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate of hippocampal tissue prepared from vehicle-treated (lane 1) and Aβ-treated rats (lane 2).

C. Data from densitometric analysis revealed decreased mean expression of the intact fragment of PARP in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of PARP expression and are means ± standard error of the means.

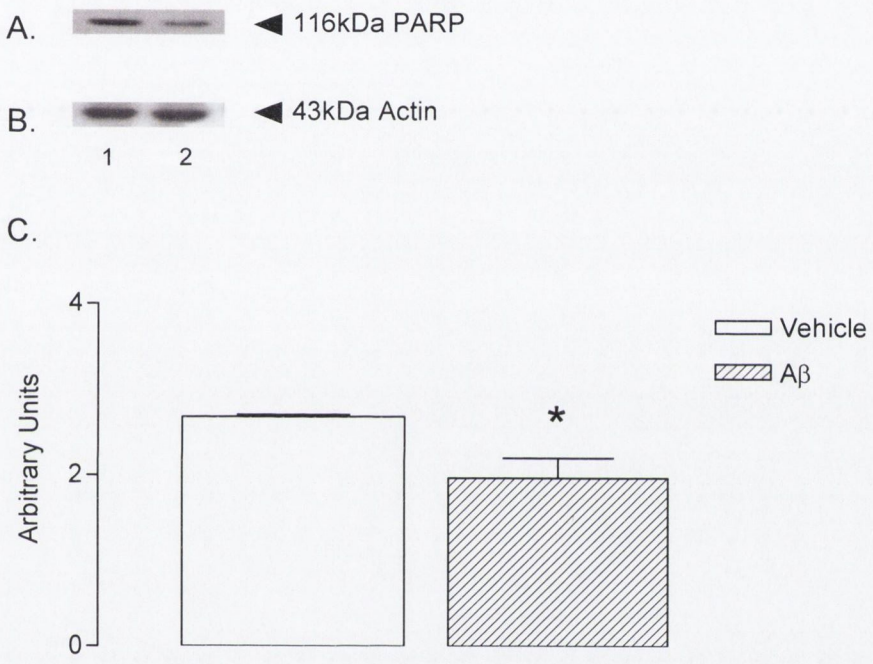

A.
116kDa PARP
B.
43kDa Actin
1
2
C.
Arbitrary Units
4
2
0
*
Vehicle
Aβ

Figure 3.7 Aβ administration is associated with an increase in FasL expression

A. One sample immunoblot shows that Fas Ligand (FasL; 38kDa) expression was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats (lane 2) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample FasL immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate of hippocampal tissue prepared from vehicle-treated (lane 1) and Aβ-treated rats (lane 2).

C. Data from densitometric analysis revealed that mean FasL expression was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.01$; Student's *t* test for independent means; n=5 in both groups). Values are expressed as arbitrary units of FasL expression and are means ± standard error of the means.

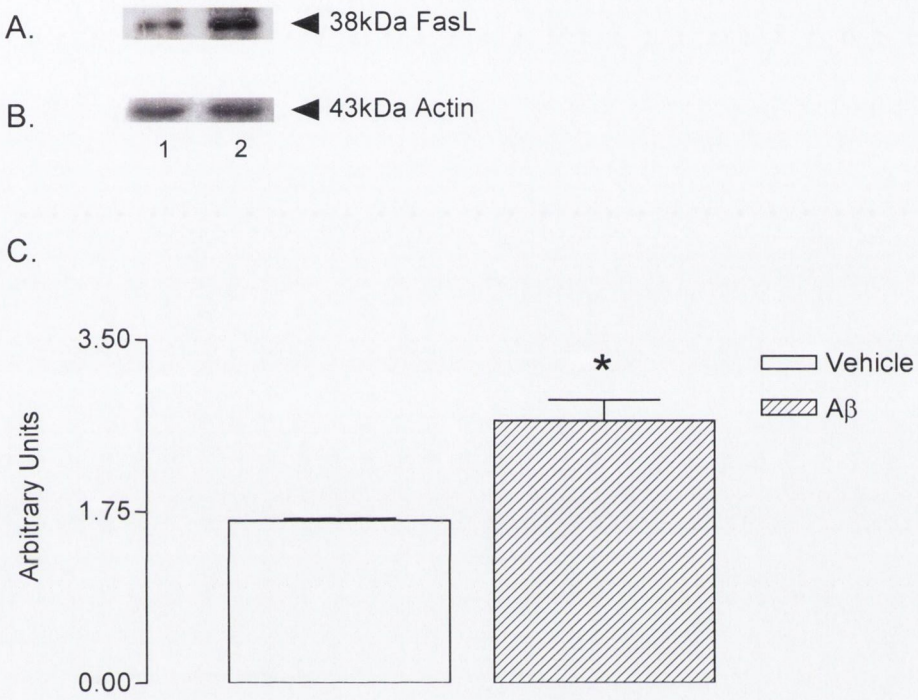

A.
38kDa FasL
B.
43kDa Actin
1
2
C.
3.50
1.75
0.00
Arbitrary Units
*
Vehicle
Aβ

Figure 3.8 Aβ treatment is associated with an increase in IL-1β release *in vitro*

Release of IL-1β was significantly increased from cortical neurons treated with Aβ compared with control-treated cortical neurons (*$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in IL-1β release between control-treated and Aβ-treated cortical neurons pre-treated with the caspase-1 inhibitor (Ac-YVAD-CMK; n=5 in both cases). Values are expressed as pg IL-1β/ml and are means ± standard error of the means.

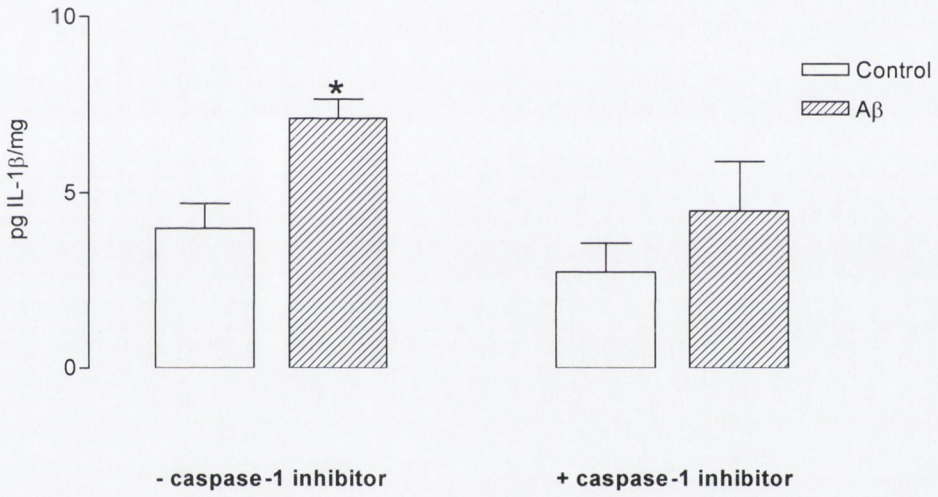

10
5
0
pg IL-1β/mg
*
Control
Aβ
- caspase-1 inhibitor
+ caspase-1 inhibitor

Figure 3.9 Aβ treatment is associated with an increase in JNK phosphorylation *in vitro*

JNK phosphorylation was increased in cortical neurons treated with Aβ (A) compared with control-treated cortical neurons (B). There was no difference in JNK phosphorylation between control-treated and Aβ-treated neurons pre-treated with the caspase-1 inhibitor (Ac-YVAD-CMK; C and D respectively).

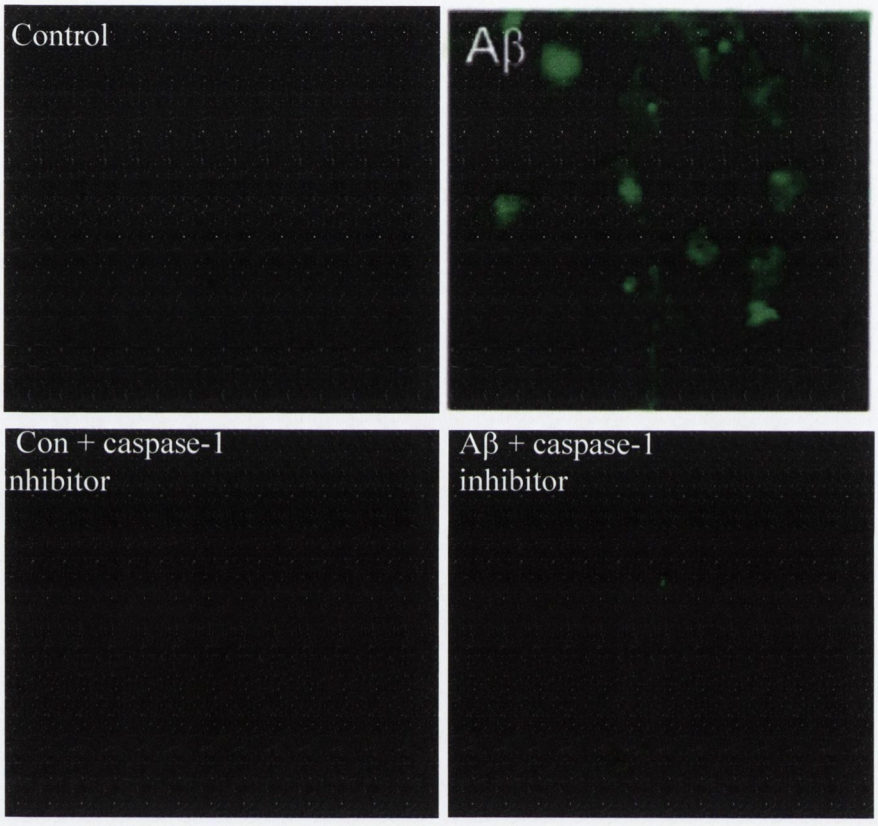

Control
Aβ
Con + caspase-1 inhibitor
Aβ + caspase-1 inhibitor

Figure 3.10 Aβ treatment is associated with an increase in c-Jun phosphorylation *in vitro*

A. One sample immunoblot shows that c-Jun phosphorylation was increased in whole-cell lysate prepared from cortical neurons treated with IL-1β (lane 2) compared with whole-cell lysate prepared from control-treated cortical neurons pre-treated with vehicle (lane 1) or JNK1 inhibitor DJNK1I (lane 3) and Aβ-treated cortical neurons pre-treated with DJNK1I (lane 4).

B. A sample phospho-c-Jun immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate prepared from cortical neurons treated with vehicle (lane 1), IL-1β (lane 2), IL-1β/DJNK1I (lane 3) and DJNK1I (lane 4).

C. Data from densitometric analysis revealed a significant increase in mean c-Jun phosphorylation in whole-cell lysate prepared from cortical neurons treated with IL-1β compared with control-treated cortical neurons (*$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in mean c-Jun phosphorylation in whole-cell lysate prepared from control-treated neurons pre-treated with vehicle or DJNK1I and Aβ-treated neurons pre-treated with DJNK1I.

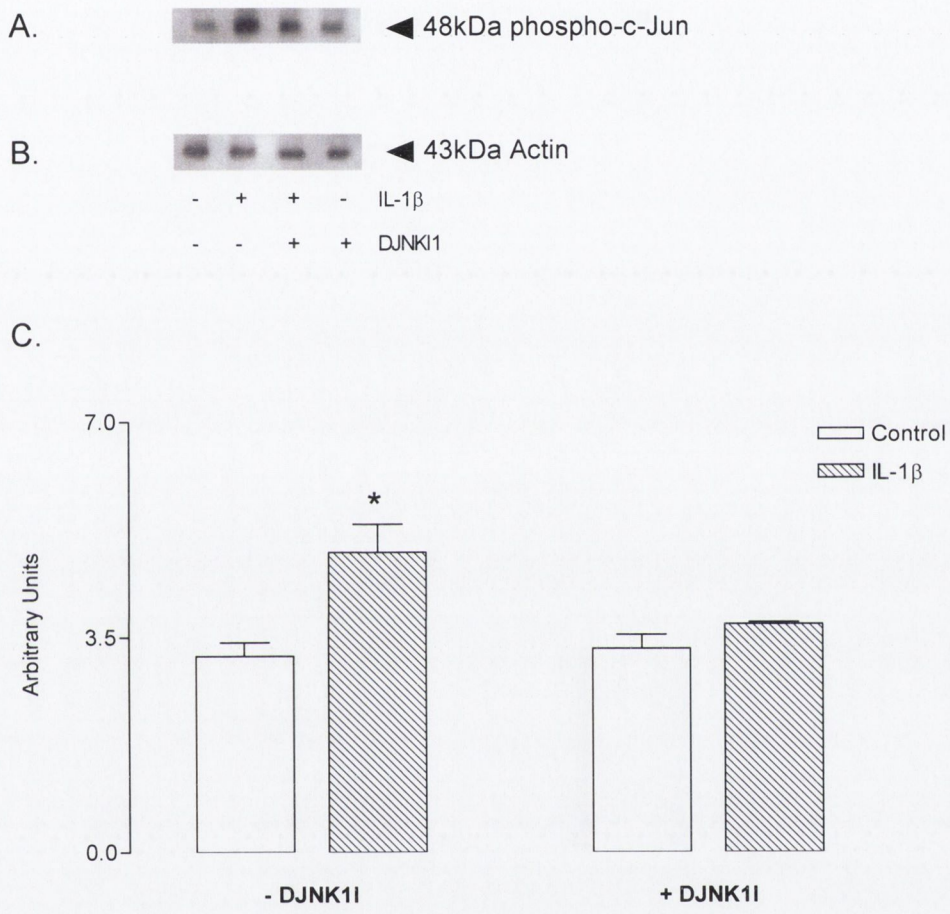

A.
48kDa phospho-c-Jun
B.
43kDa Actin
- + + - IL-1β
- - + + DJNKI1
C.
7.0
3.5
0.0
Arbitrary Units
*
Control
IL-1β
- DJNK1I
+ DJNK1I

Figure 3.11 IL-1β treatment is associated with an increase in FasL expression *in vitro*

A. One sample immunoblot shows increased FasL expression in whole-cell lysate prepared from cortical neurons treated with IL-1β (lane 2) compared with control-treated cortical neurons (lane 1), Aβ-treated cortical neurons pre-treated with JNK1 inhibitor, DJNK1I (lane 4) and control-treated cortical neurons pre-treated with DJNK1I (lane 3).

B. A sample FasL immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate prepared from cortical neurons treated with vehicle (lane 1), IL-1β (lane 2), IL-1β/DJNK1I (lane 3) and DJNK1I (lane 4).

C. Data from densitometric analysis revealed a significant increase in mean FasL expression in whole-cell lysate prepared from cortical neurons treated with IL-1β compared with control-treated cortical neurons (*$p<0.05$; ANOVA; n=5 in both cases). There was no significant difference in mean FasL expression in whole-cell lysate prepared from control-treated cortical neurons, Aβ-treated cortical neurons pre-treated with DJNKI1 or control-treated cortical neurons pre-treated with DJNK1I.

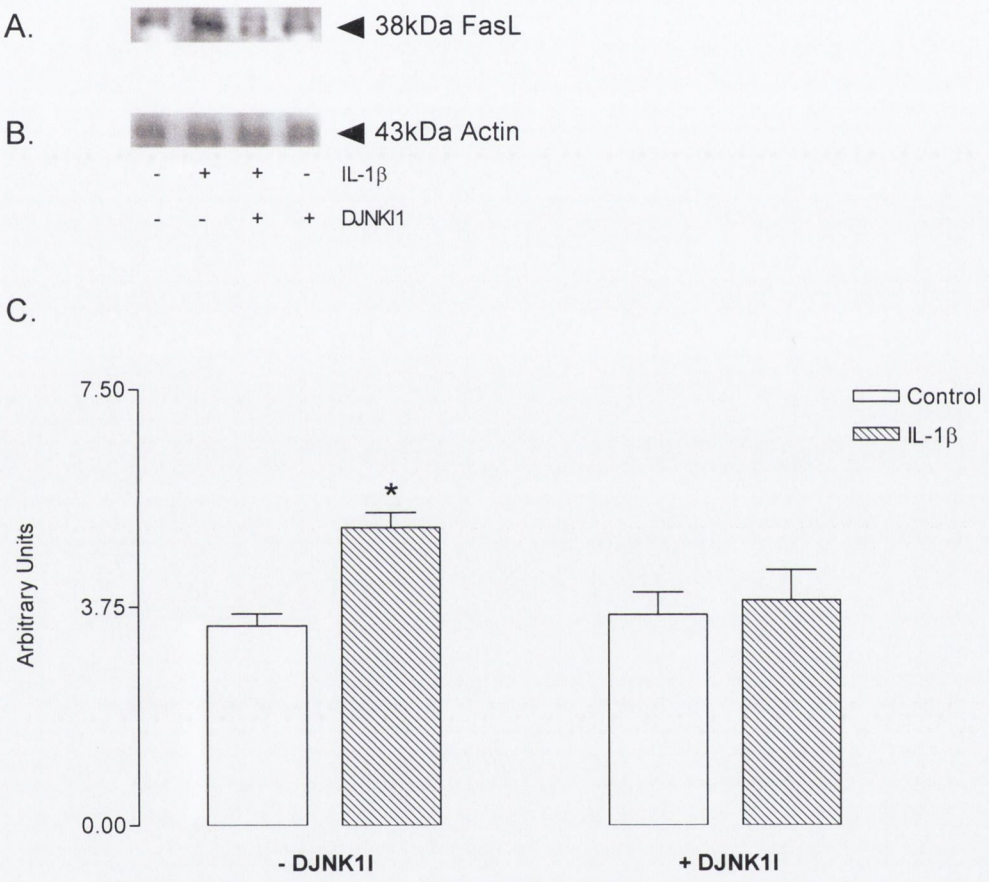

A.
38kDa FasL
B.
43kDa Actin
- + + - IL-1β
- - + + DJNKI1
C.
7.50
3.75
0.00
Arbitrary Units
*
Control
IL-1β
- DJNK1I
+ DJNK1I

Figure 3.12 Aβ treatment is associated with an increase in caspase-3 activity *in vitro*

Caspase-3 activity was increased in cortical neurons treated with Aβ (B) compared with control-treated cortical neurons (A). There was no difference in caspase-3 activity between control-treated and Aβ-treated neurons pre-treated with the caspase-1 inhibitor (Ac-YVAD-CMK; C) or JNK1 inhibitor DJNK1I (D).

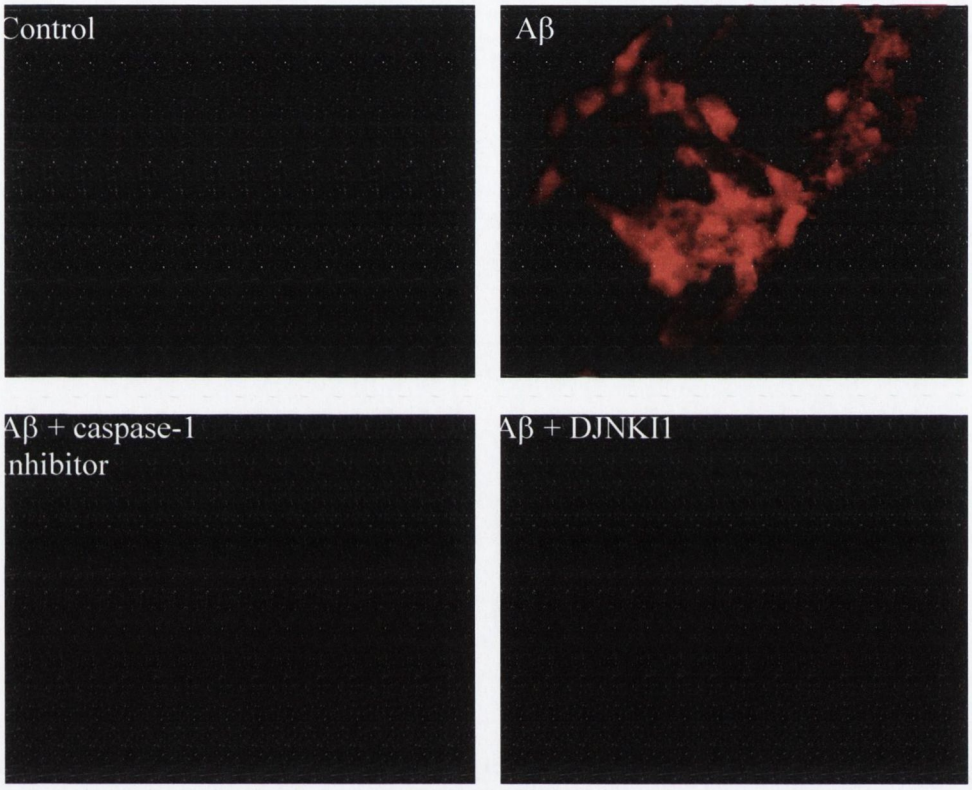

Control
Aβ
Aβ + caspase-1
nhibitor
Aβ + DJNKI1

Figure 3.13 Aβ treatment is associated with an increase in DNA fragmentation *in vitro*

DNA fragmentation was increased in cortical neurons treated with Aβ (B) compared with control-treated neurons (A). There was no difference in DNA fragmentation between control-treated neurons (A) and Aβ-treated neurons pre-treated with the caspase-1 inhibitor (Ac-YVAD-CMK; C)or JNK1 inhibitorDJNK1I (D).

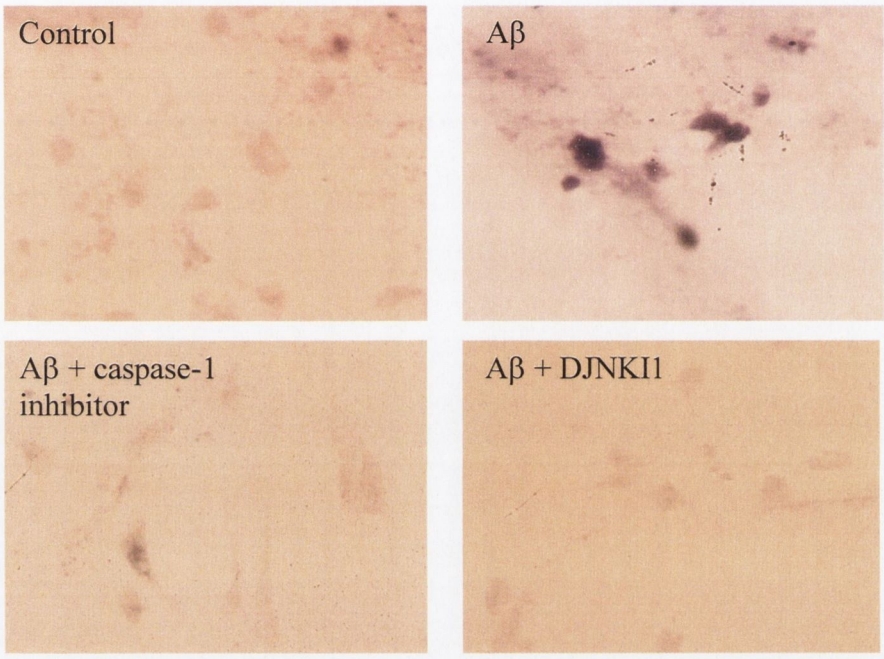

Control
Aβ
Aβ + caspase-1 inhibitor
Aβ + DJNKI1

Figure 3.14 Aβ administration is associated with impairment in LTP in dentate gyrus of adult rats

Long-term potentiation (LTP) in dentate gyrus was inhibited in 60μM (◎) and 200μM (♦) Aβ-treated rats compared with vehicle-treated (Δ) rats. LTP is similar in vehicle-treated (Δ) rats and in 20μM (◎) Aβ-treated rats.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 5 observations. Standard errors are included for every 10^{th} response.

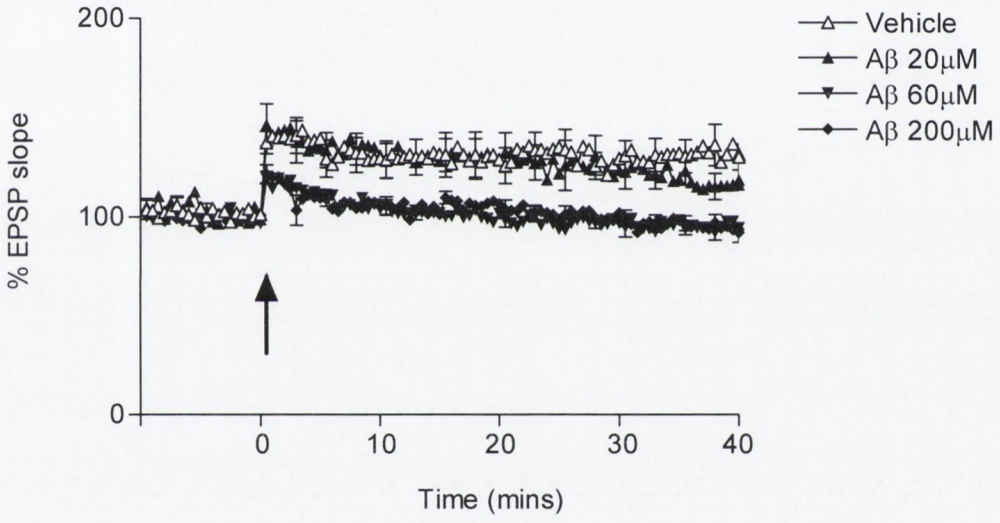
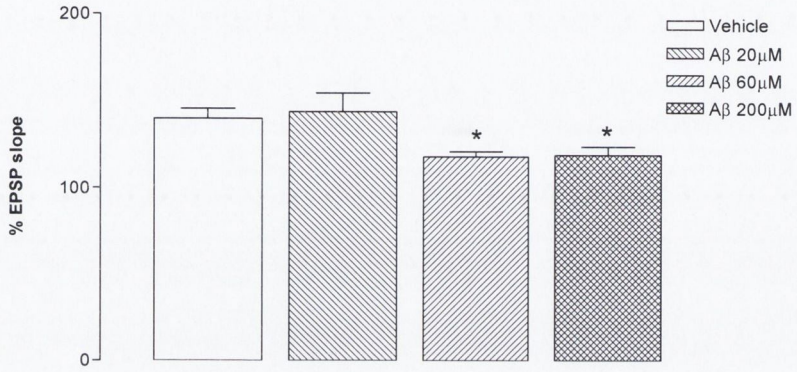

200
100
0
% EPSP slope
0
10
20
30
40
Time (mins)
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM

Figure 3.15 Aβ administration affects percentage change in EPSP slope in adult rats

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in Aβ-treated rats compared with vehicle-treated rats (***$p<0.001$; ANOVA; n=5 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

B. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in Aβ-treated rats compared with vehicle-treated rats (n=5 in all cases). There was a significant increase observed in the percentage change in EPSP slope in 20μM Aβ-treated rats compared with rats treated with 60 or 200μM Aβ (***$p<0.001$; ANOVA; n=5 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.


200
100
0
% EPSP slope
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
*
*

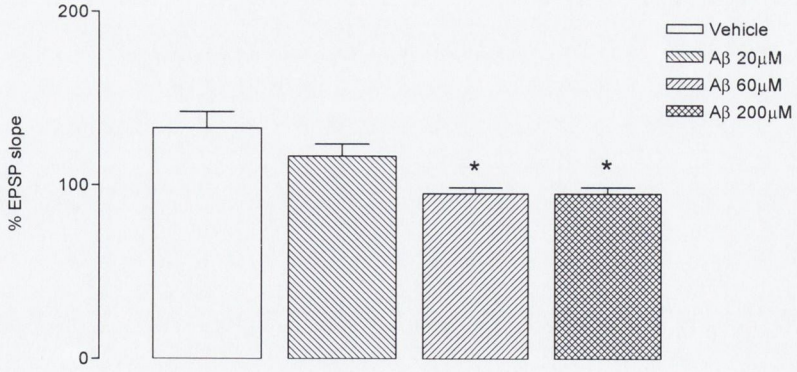

200
100
0
% EPSP slope
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
*
*

Figure 3.16 Aβ administration is associated with impairment in LTP in dentate gyrus of middle-aged rats

LTP in dentate gyrus was significantly impaired in 20μM (◉) Aβ-treated rats compared with vehicle-treated (Δ) rats.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 6 observations. Standard errors are included for every 10^{th} response.

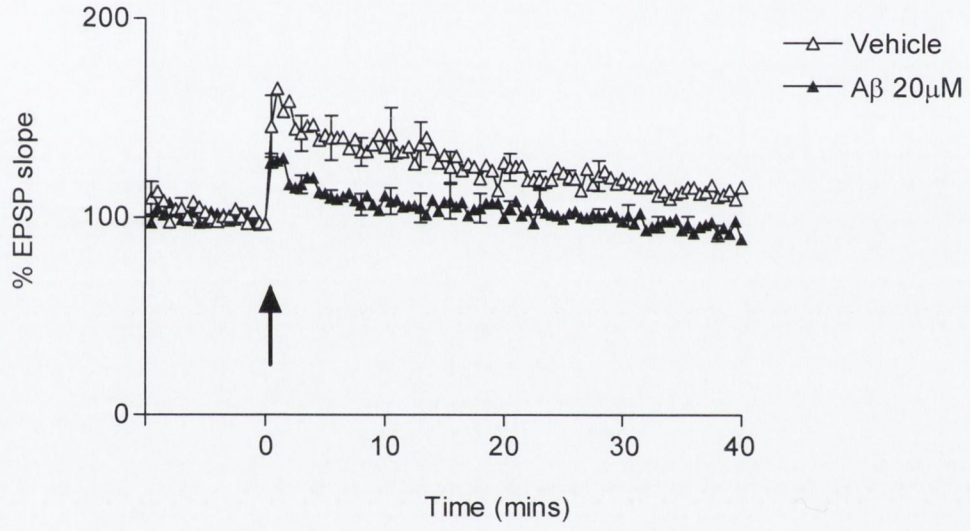

200
100
0
% EPSP slope
0
10
20
30
40
Time (mins)
Vehicle
Aβ 20μM

Figure 3.17 Aβ administration is associated with impairment in LTP in dentate gyrus of aged rats

LTP in dentate gyrus was significantly impaired in 20μM (◎) Aβ-treated rats compared with vehicle-treated (Δ) rats.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 7 observations. Standard errors are included for every 10^{th} response.

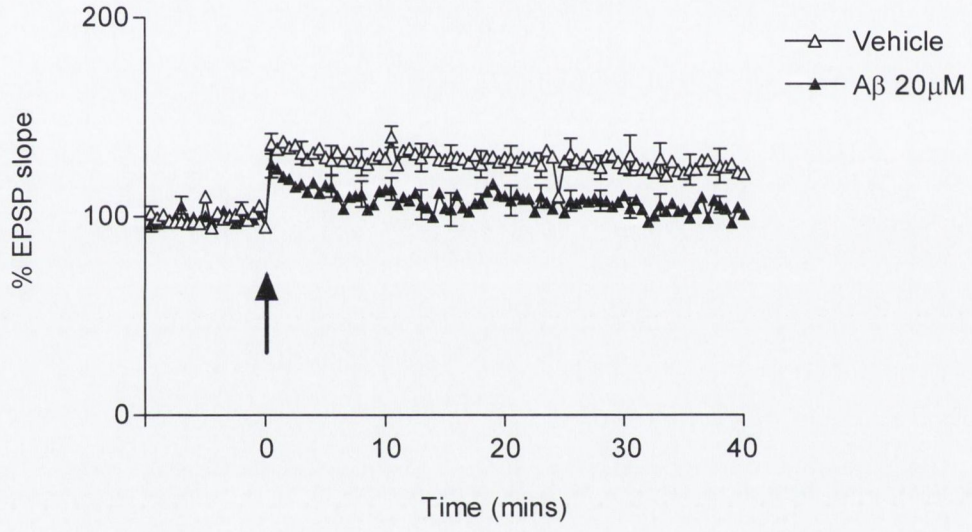
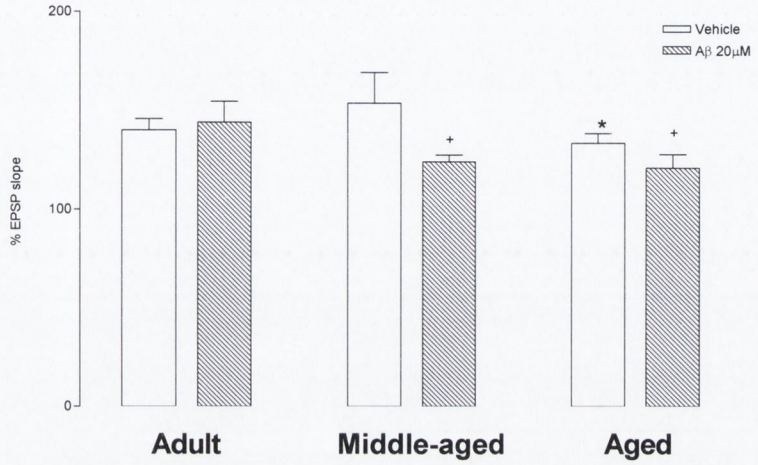

200
100
0
% EPSP slope
0
10
20
30
40
Time (mins)
Vehicle
Aβ 20μM

Figure 3.18 Aβ administration and age affect percentage change in EPSP slope

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in vehicle-treated aged rats (n=6) compared with vehicle-treated adult rats (***$p<0.001$; ANOVA; n=5). There was a significant decrease observed in the percentage change in EPSP slope in 20μM Aβ-treated middle-aged (n=6) and aged rats (n=6) compared with 20 μM Aβ-treated adult rats (+++$p<0.001$; ANOVA; n=5). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

B. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with vehicle-treated adult rats (***$p<0.001$; ANOVA; n=5). There was a significant decrease observed in the percentage change in EPSP slope in 20 μM Aβ-treated middle-aged (n=6) and aged rats (n=6) compared with 20 μM Aβ-treated adult rats (+++$p<0.001$; ANOVA; n=5). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

A.


200
100
0
% EPSP slope
Vehicle
Aβ 20μM
*
+
+
Adult
Middle-aged
Aged

B.

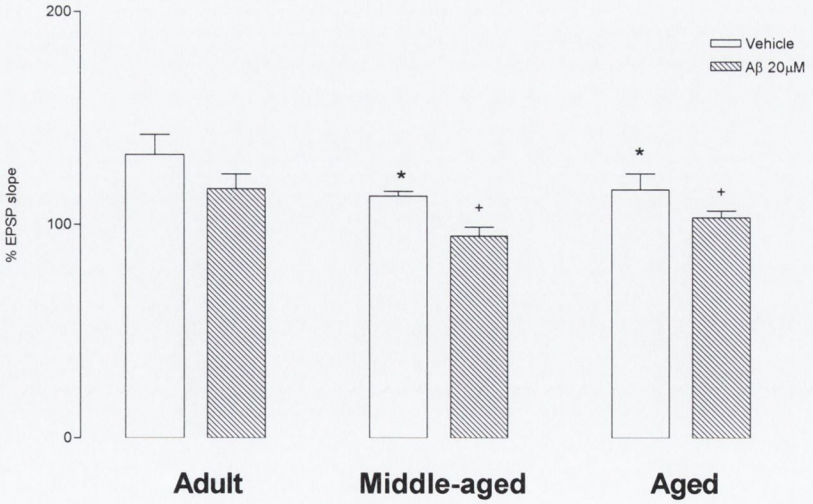

200
100
0
% EPSP slope
Vehicle
Aβ 20μM
*
+
*
+
Adult
Middle-aged
Aged

Figure 3.19 Aβ administration is associated with an increase in the concentration of IL-1β in hippocampus of adult rats

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal homogenate prepared from 60 and 200μM Aβ-treated rats compared with homogenate prepared from vehicle-treated rats (n=5; *$p<0.05$; ANOVA). IL-1β concentration was similar in 20μM Aβ-treated rats and vehicle-treated rats (n=5; *$p<0.05$; ANOVA). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

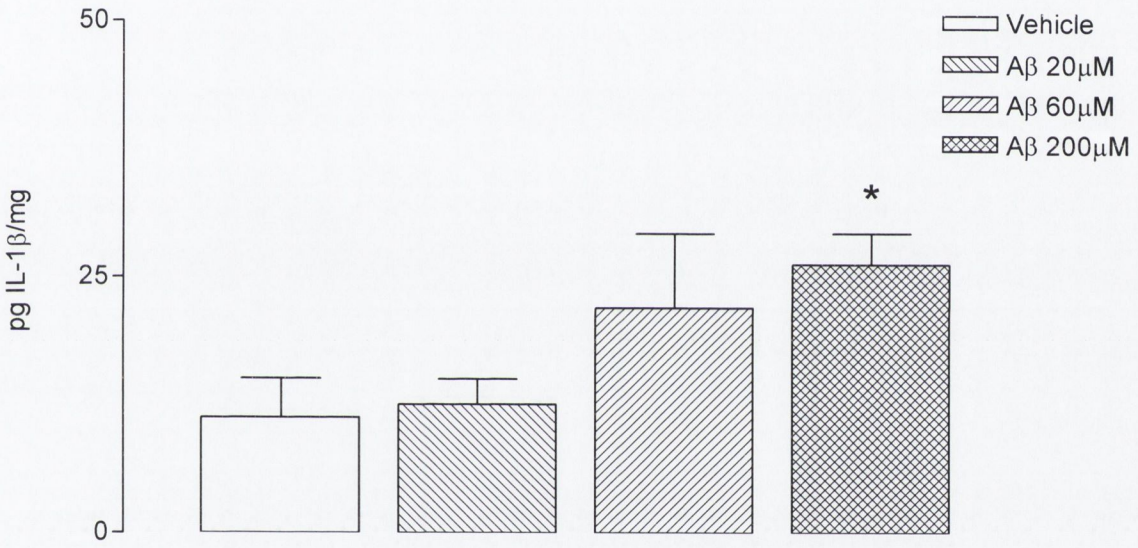

50
25
0
pg IL-1β/mg
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
*

Figure 3.20 Aβ administration and age are associated with an increase in the concentration of IL-1β

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with homogenate prepared from vehicle-treated adult rats (n=5; *$p<0.05$; ANOVA). IL-1β concentration was significantly increased in 20μM Aβ-treated aged rats (n=6) compared with 20μM Aβ-treated adult rats (n=5; *$p<0.05$; ANOVA). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.


Vehicle
Aβ 20μM
75.0
37.5
0.0
pg IL-1β/mg
*
*
+
Adult
Middle-aged
Aged

Figure 3.21 Aβ administration is associated with an increase in the concentration of IFN-γ in hippocampus of adult rats

Interferon-γ (IFNγ) concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated rats compared with tissue prepared from vehicle-treated rats (n=5; *$p<0.05$; ANOVA). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

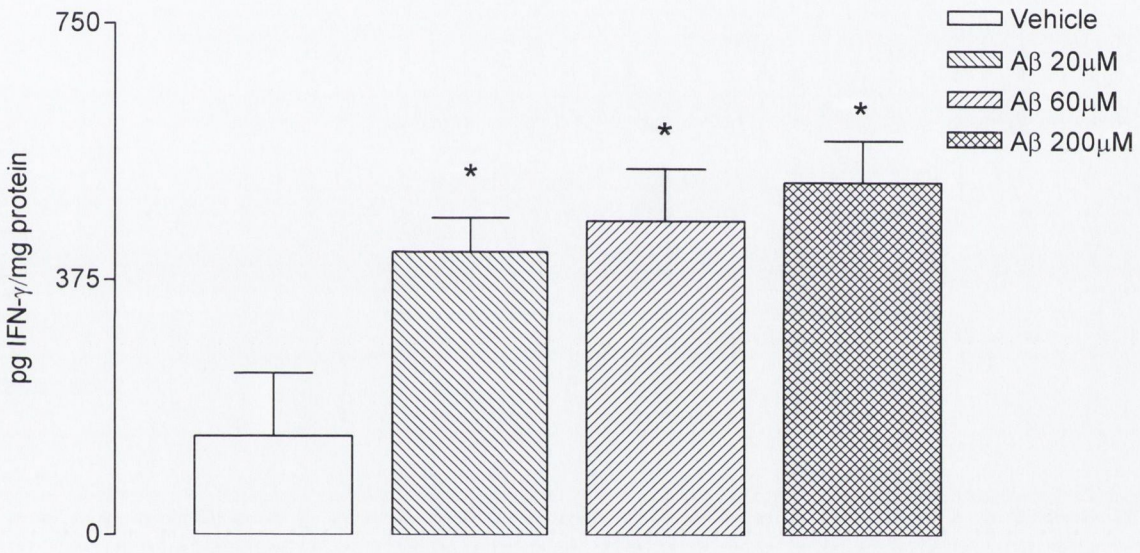

750
375
0
pg IFN-γ/mg protein
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
*
*
*

Figure 3.22 Aβ administration and age are associated with an increase in the concentration of IFNγ

IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with homogenate prepared from vehicle-treated adult rats (n=5; *$p<0.05$; ANOVA). IFNγ concentration was similar in hippocampal homogenate prepared from Aβ-treated adult, middle-aged and aged rats (n=5, 6 and 6 respectively). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

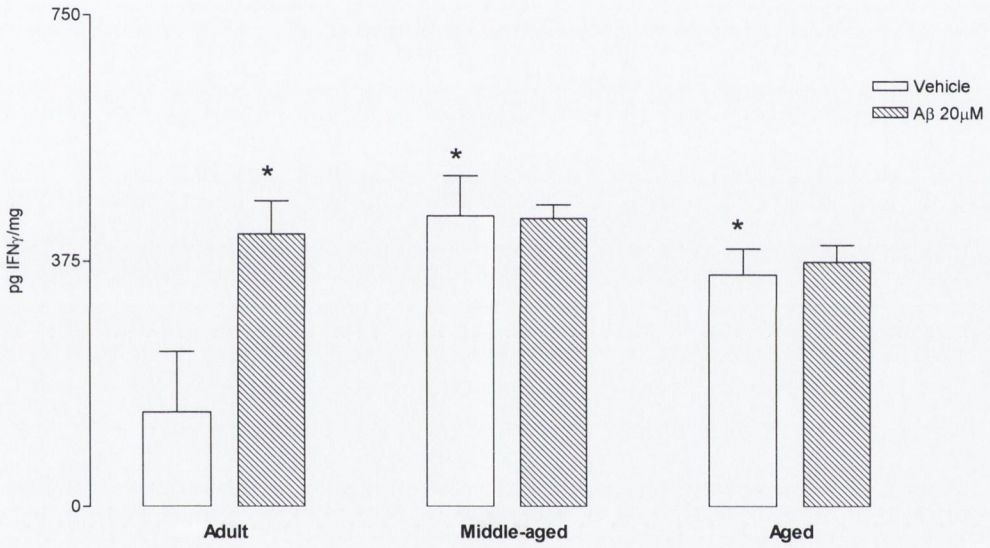

750
375
0
pg IFNγ/mg
Vehicle
Aβ 20μM
*
*
*
Adult
Middle-aged
Aged

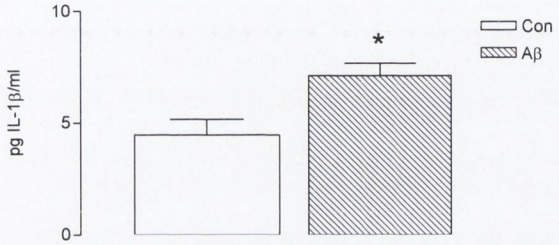
Figure 3.23 IL-1β is released from Aβ-treated cultured neurons and glia *in vitro*

A. Release of IL-1β was significantly increased from cortical neurons treated with Aβ compared with control-treated cortical neurons (*$p<0.05$; Student's *t* test for independent means; n=5 in both cases). Values are expressed as pg IL-1β/ml and are means ± standard error of the means.

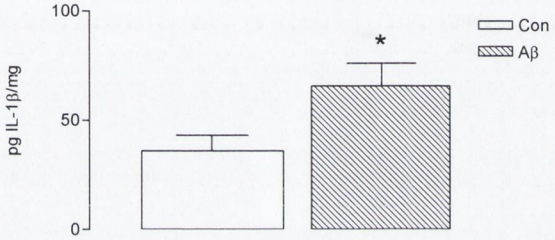
B. Release of IL-1β was significantly increased from cortical glia treated with Aβ compared with control-treated cortical glia (*$p<0.05$; Student's *t* test for independent means; n=5 in both cases). Values are expressed as pg IL-1β/ml and are means ± standard error of the means.

C. Cultured cortical neurons and glia were stained with GFAP to ensure purity of cultures. Cultured cortical neurons displayed no GFAP immunofluorescence while cultured cortical glia showed abundant GFAP immunofluorescence.

A.



B.



C.

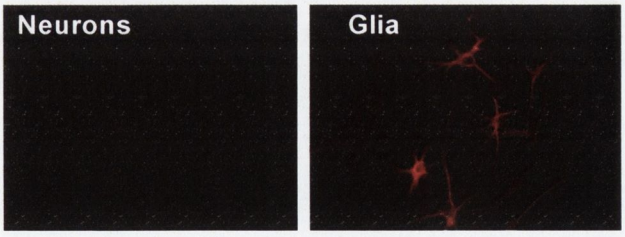


Figure 3.24 Aβ administration is associated with an increase in JNK phosphorylation in hippocampus of adult rats

A. One sample immunoblot shows that JNK phosphorylation (46kDa) was increased in whole-cell lysate of hippocampal tissue prepared from 20, 60 and 200μM Aβ-treated rats (lanes 2, 3 and 4 respectively) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample phospho-JNK immunoblot stripped and reprobed for JNK1 expression to confirm equal loading of proteins. No change was observed in the expression of JNK1 (46kDa) in whole-cell lysate prepared from vehicle-treated rats (lane 1) and 20, 60 and 200μM Aβ-treated rats (lanes 2, 3 and 4 respectively).

C. Data from densitometric analysis revealed that mean JNK phosphorylation was increased in whole-cell lysate of hippocampal tissue prepared from 20, 60 and 200μM Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; **$p<0.01$; ANOVA; n=5 in all groups). Values are expressed as arbitrary units of JNK phosphorylation and are means ± standard error of the means.

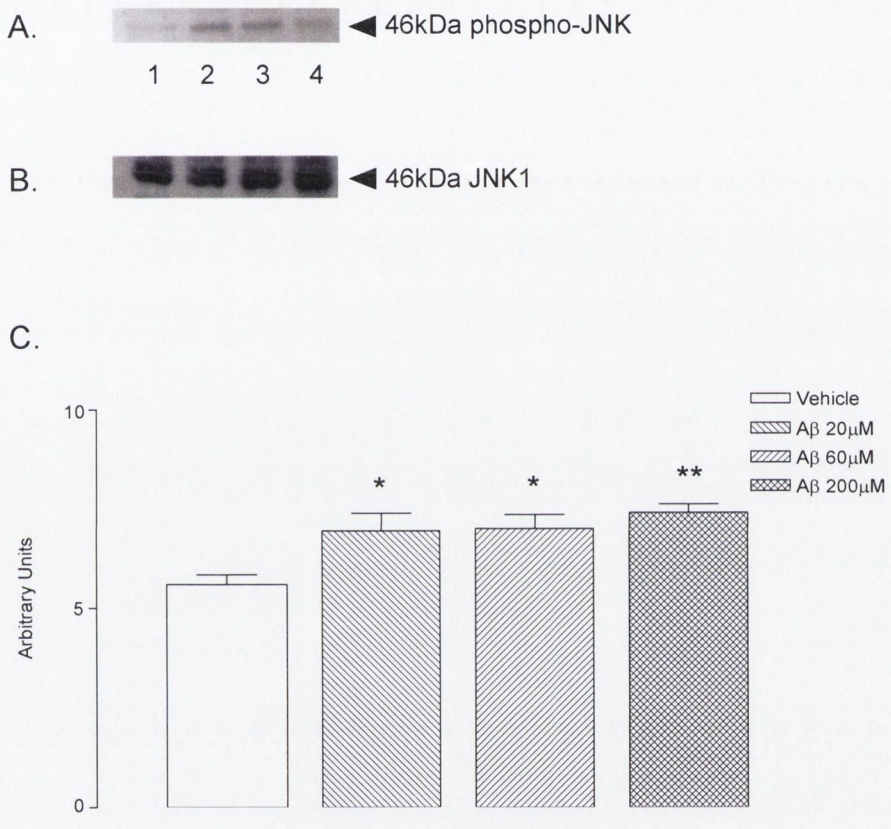

A.
46kDa phospho-JNK
1 2 3 4
B.
46kDa JNK1
C.
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
Arbitrary Units
10
5
0
*
*
**

Figure 3.25 Aβ administration and age are associated with an increase in JNK phosphorylation

A. One sample immunoblot shows that JNK phosphorylation (46kDa) was increased in whole-cell lysate of hippocampal tissue prepared from Aβ-treated adult rats (lane 2) compared with tissue prepared from vehicle-treated adult rats (lane 1). JNK phosphorylation was increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (lane 3) and aged rats (lane 5) compared with vehicle-treated adult rats (lane 1). JNK phosphorylation was similar in whole-cell lysate of hippocampal tissue prepared from all Aβ-treated rats (lanes 2, 4 and 6).

B. A sample phospho-JNK immunoblot stripped and reprobed for JNK1 expresson to confirm equal loading of proteins. No change was observed in the expression of JNK1 (46kDa) in whole-cell lysate prepared from vehicle-treated adult rats (lane 1), 20μM Aβ-treated adult rats (lane 2), vehicle- (lane 3) and 20μM Aβ-treated (lane 4) middle-aged rats and vehicle- (lane 5) and 20μM Aβ-treated (lane 6) aged rats.

C. Data from densitometric analysis confirmed that mean JNK phosphorylation was significantly increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with tissue prepared from vehicle-treated adult rats (n=5; *$p<0.05$; ANOVA). JNK phosphorylation was similar in whole-cell lysate of hippocampal tissue prepared from Aβ-treated adult, middle-aged and aged rats (n=5, 6 and 6 respectively). Values are expressed as arbitrary units of JNK phosphorylation and are means ± standard error of the means.

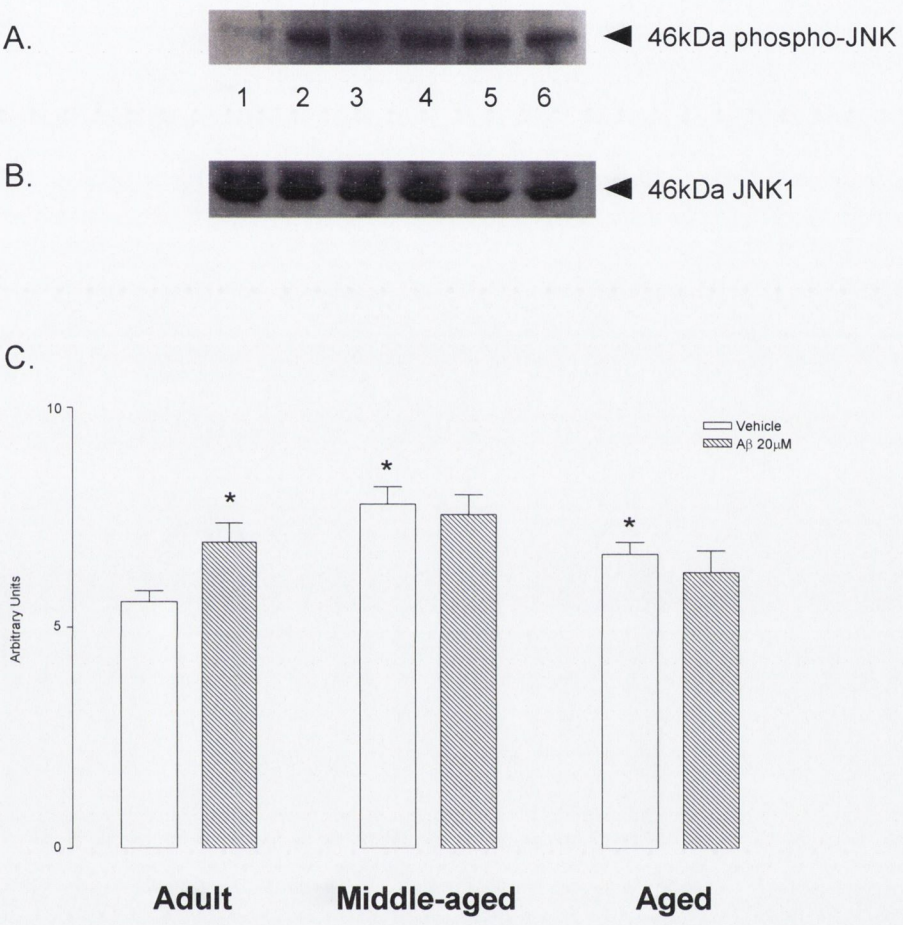

A.
46kDa phospho-JNK
1 2 3 4 5 6
B.
46kDa JNK1
C.
Vehicle
Aβ 20μM
Arbitrary Units
10
5
0
*
*
*
Adult
Middle-aged
Aged

Figure 3.26 Aβ administration is associated with an increase in RAGE expression in hippocampus of adult rats

A. One sample immunoblot shows that receptor for advanced glycation end-products (RAGE) expression was increased in whole-cell lysate of hippocampal tissue prepared from 20, 60 and 200μM Aβ-treated rats (lanes 2, 3 and 4 respectively) compared with tissue prepared from vehicle-treated rats (lane 1).

B. A sample RAGE immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate prepared from vehicle-treated rats (lane 1) and 20, 60 and 200μM Aβ-treated rats (lanes 2, 3 and 4 respectively).

C. Data from densitometric analysis confirmed that mean RAGE expression was increased in whole-cell lysate of hippocampal tissue prepared from 20, 60 and 200μM Aβ-treated rats compared with tissue prepared from vehicle-treated rats (*$p<0.05$; ANOVA; n=5 in all groups). Values are expressed as arbitrary units of RAGE expression and are means ± standard error of the means.

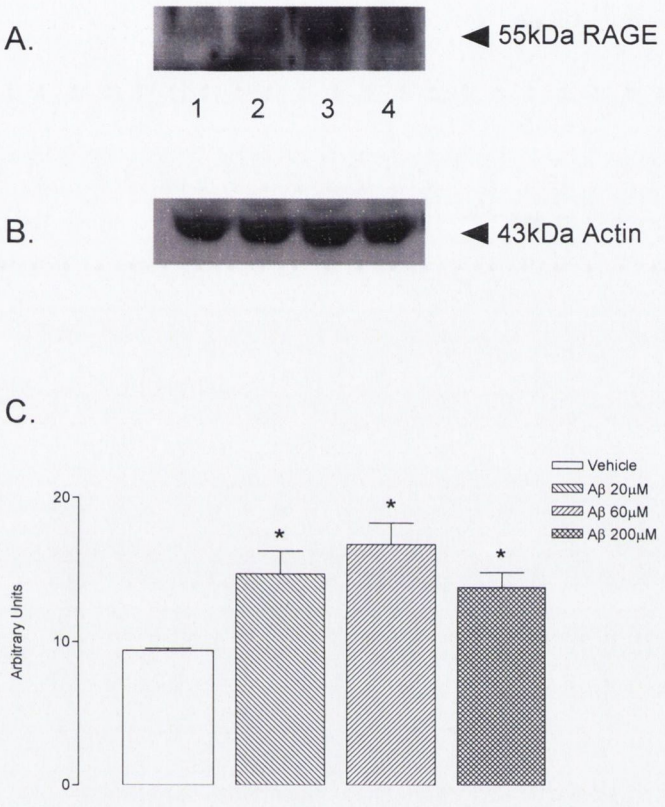

A.
55kDa RAGE
1 2 3 4
B.
43kDa Actin
C.
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
Arbitrary Units
20
10
0
*
*
*

Figure 3.27 Aβ administration and age are associated with an increase in RAGE expression

A. One sample immunoblot shows that RAGE expression was increased in whole-cell lysate of hippocampal tissue prepared from 20μM Aβ-treated adult rats (lane 2) compared with tissue prepared from vehicle-treated adult rats (lane 1). RAGE expression was increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (lane 3) and aged rats (lane 5) compared with vehicle-treated adult rats (lane 1). RAGE expression was similar in whole-cell lysate of hippocampal tissue prepared from all Aβ-treated rats (lanes 2, 4 and 6).

B. A sample RAGE immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate prepared from vehicle-treated adult rats (lane 1), 20μM Aβ-treated adult rats (lane 2), vehicle- (lane 3) and 20μM Aβ-treated (lane 4) middle-aged rats and vehicle- (lane 5) and 20μM Aβ-treated (lane 6) aged rats.

C. Data from densitometric analysis confirmed that mean RAGE expression was significantly increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with tissue prepared from vehicle-treated adult rats (n=5; *$p<0.05$; ANOVA). RAGE expression was similar in whole-cell lysate of hippocampal tissue prepared from Aβ-treated adult, middle-aged and aged rats (n=5, 6 and 6 rspectively). Values are expressed as arbitrary units of RAGE expression and are means ± standard error of the means.

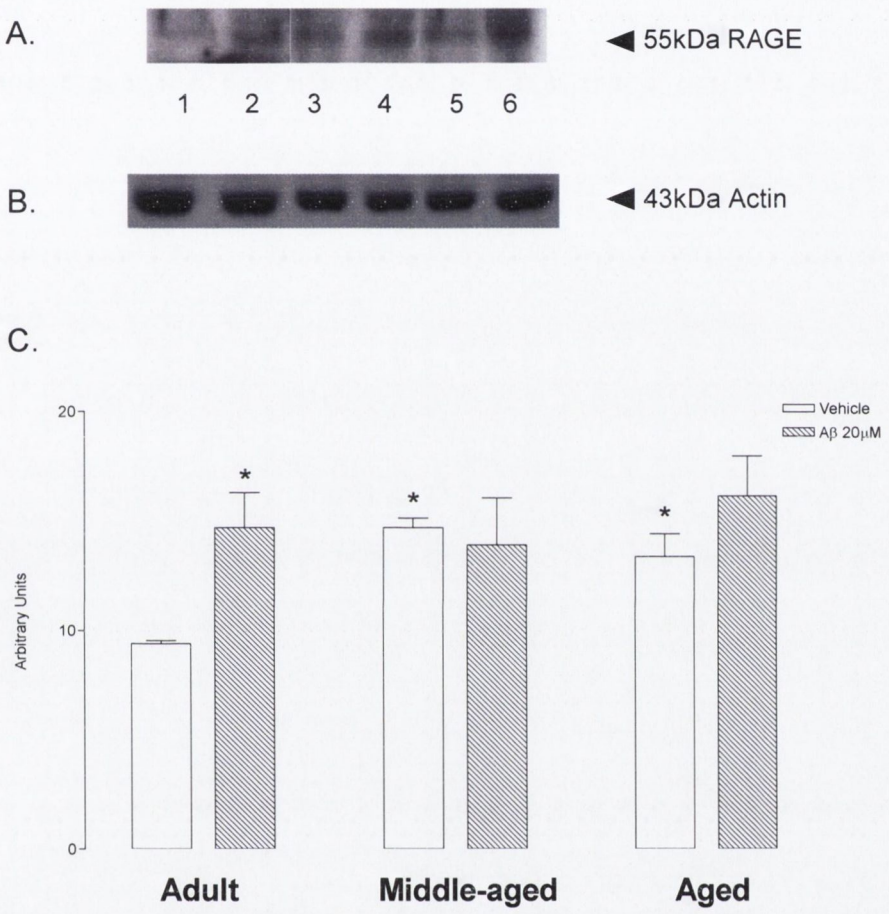

A.
55kDa RAGE
1 2 3 4 5 6
B.
43kDa Actin
C.
Vehicle
Aβ 20μM
Arbitrary Units
20
10
0
*
*
*
Adult
Middle-aged
Aged

Figure 3.28 Aβ administration is associated with an increase in CD40 expression in hippocampus of adult rats

A. One sample immunoblot shows that CD40 expression was increased in whole-cell lysate of hippocampal tissue prepared from 60 and 200μM Aβ-treated rats (lanes 3 and 4 respectively) compared with tissue prepared from vehicle-treated (lane 1) and 20μM Aβ-treated rats (lane 2).

B. A sample CD40 immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (43kDa) in whole-cell lysate prepared from vehicle-treated rats (lane 1) and 20, 60 and 200μM Aβ-treated rats (lanes 2, 3 and 4 respectively).

C. Data from densitometric analysis revealed that mean CD40 expression was increased in whole-cell lysate of hippocampal tissue prepared from 60 and 200μM Aβ-treated rats compared with tissue prepared from vehicle-treated and 20μM Aβ-treated rats (*$p<0.05$; ANOVA; n=5 in all groups). Values are expressed as arbitrary units of CD40 expression and are means ± standard error of the means.

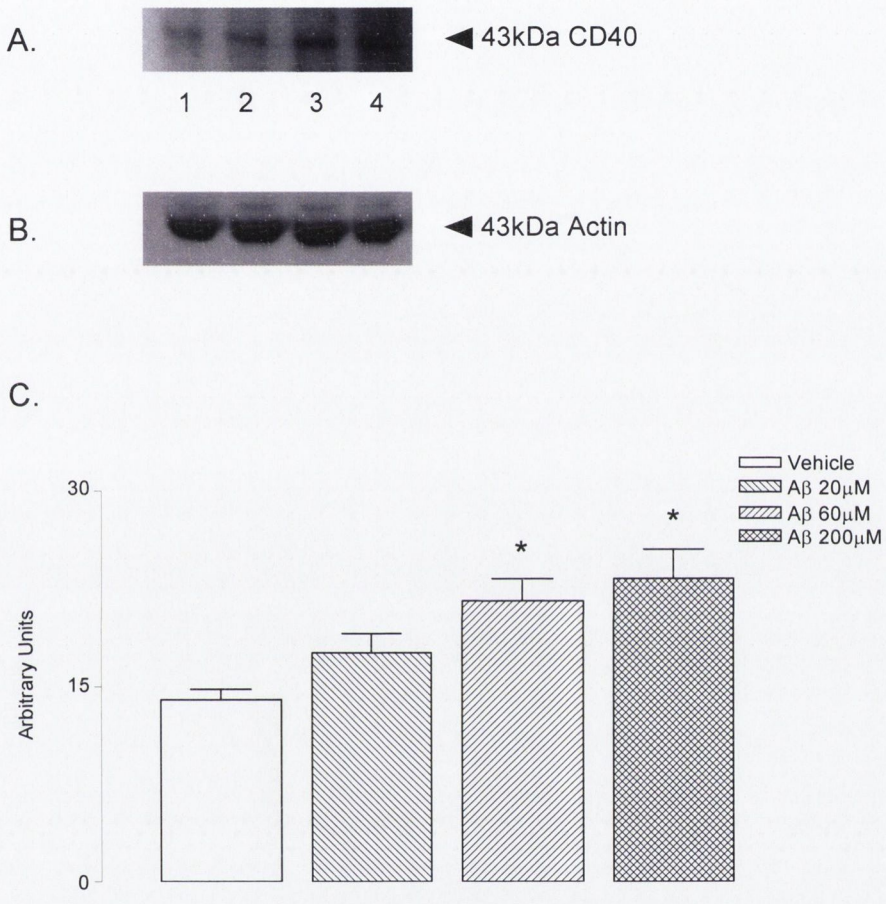

A.
43kDa CD40
1 2 3 4
B.
43kDa Actin
C.
Vehicle
Aβ 20μM
Aβ 60μM
Aβ 200μM
Arbitrary Units
30
15
0
*
*

Figure 3.29 Aβ administration and age are associated with an increase in CD40 expression

A. One sample immunoblot shows that CD40 expression was increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (lane 3) and aged rats (lane 5) compared with vehicle-treated adult rats (lane 1). CD40 expression was similar in whole-cell lysate of hippocampal tissue prepared from all Aβ-treated rats (lanes 2, 4 and 6).

B. A sample CD40 immunoblot stripped and reprobed for actin expression to confirm equal loading of proteins. No change was observed in the expression of actin (46kDa) in whole-cell lysate prepared from vehicle-treated adult rats (lane 1), 20μM Aβ-treated adult rats (lane 2), vehicle- (lane 3) and 20μM Aβ-treated (lane 4) middle-aged rats and vehicle- (lane 5) and 20μM Aβ-treated (lane 6) aged rats.

C. Data from densitometric analysis revealed that mean CD40 expression was significantly increased in whole-cell lysate of hippocampal tissue prepared from vehicle-treated middle-aged (n=5) and aged rats (n=6) compared with tissue prepared from vehicle-treated adult rats (n=5; *$p<0.05$; ANOVA). CD40 expression was similar in whole-cell lysate of hippocampal tissue prepared from Aβ-treated adult, middle-aged and aged rats (n=5, 6 and 6 rspectively). Values are expressed as arbitrary units of CD40 expression and are means ± standard error of the means.

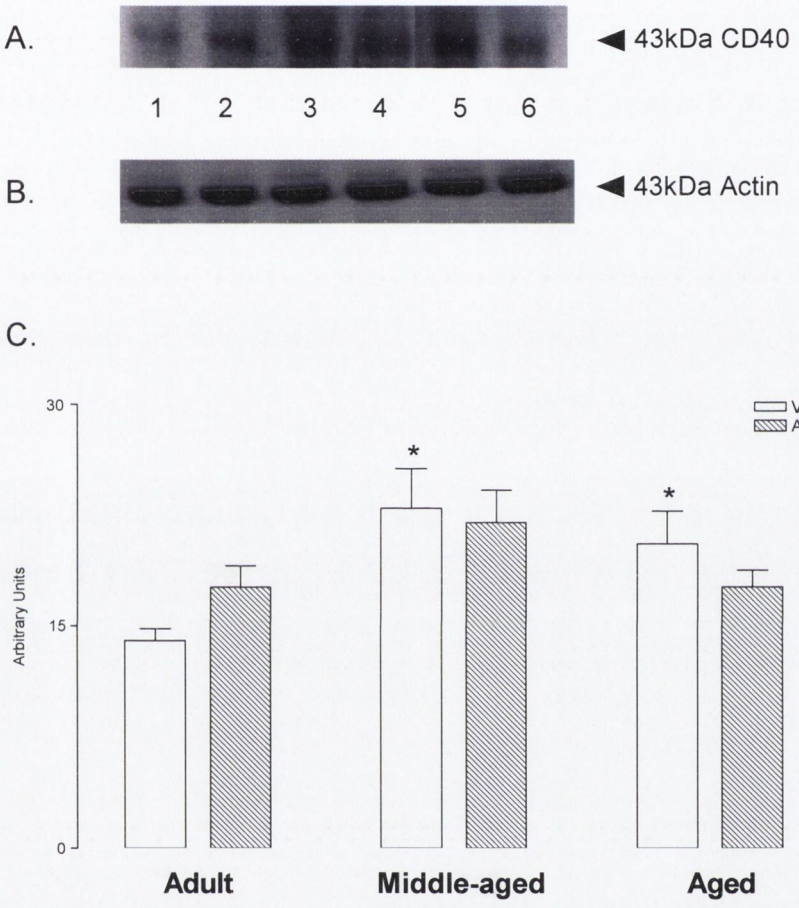

A.
43kDa CD40
1 2 3 4 5 6
B.
43kDa Actin
C.
Vehicle
Aβ 20μM
Arbitrary Units
30
15
0
*
*
Adult
Middle-aged
Aged

Figure 3.30 IL-1β is released from Aβ-treated glia *in vitro*; abrogation by minocycline hydrochloride

Release of IL-1β was significantly increased from cultured cortical glia treated with Aβ compared with control-treated cortical glia (**$p<0.01$; ANOVA; $n=6$ in both cases). IL-1β release was similar from control-treated and Aβ/minocycline-treated cortical glia. Values are expressed as pg IL-1β/ml and are means ± standard error of the means.

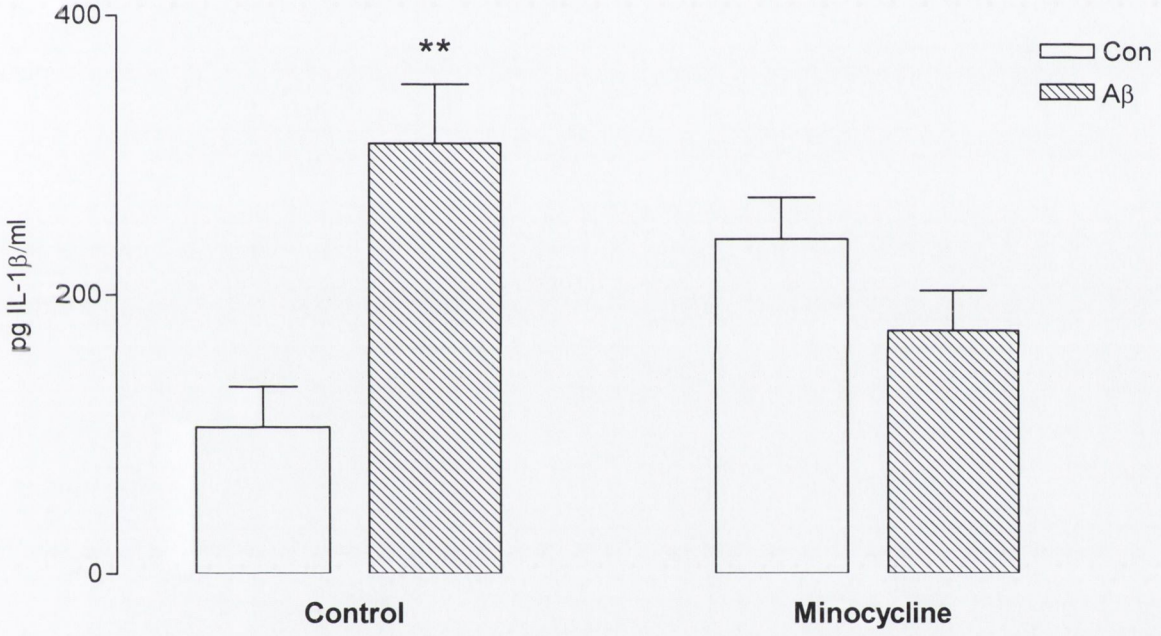

400
200
0
pg IL-1β/ml
**
Con
Aβ
Control
Minocycline

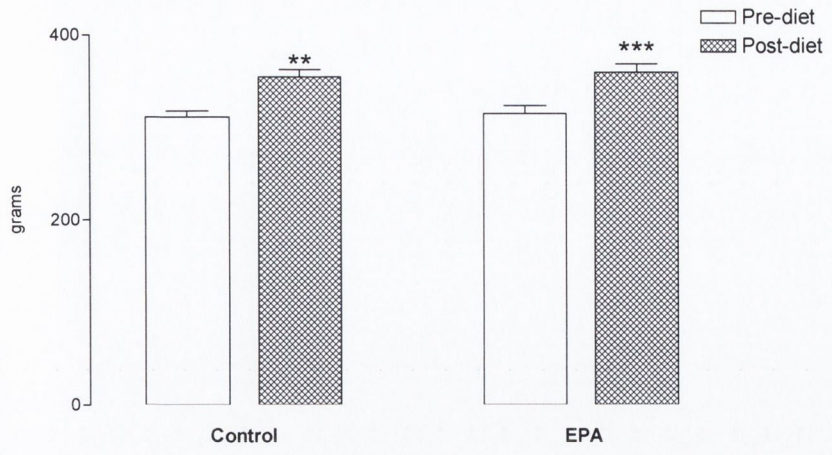
Figure 3.31 Body weights

A. Mean body masses of adult rats were similar in all treatment groups and, in all cases were significantly greater at the end of the experimental treatment period compared with the start (**$p<0.01$; ***$p<0.001$; ANOVA; n=5 in all groups).

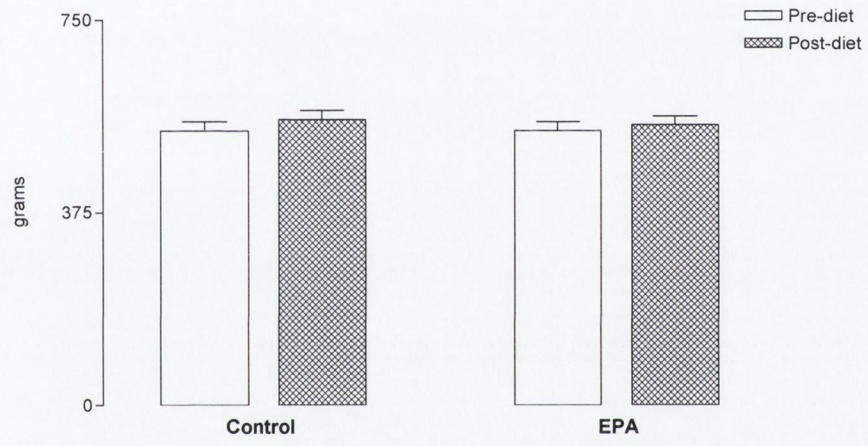
B. Mean body masses of middle-aged rats were similar in all treatment groups and in all cases were similar at the end of the experimental treatment period compared with the start.

C. Mean body masses of aged rats were similar in all treatment groups and in all cases were similar at the end of the experimental treatment period compared with the start.

A.


Pre-diet
Post-diet
400
200
0
grams
**
***
Control
EPA

B.


Pre-diet
Post-diet
750
375
0
grams
Control
EPA

C.

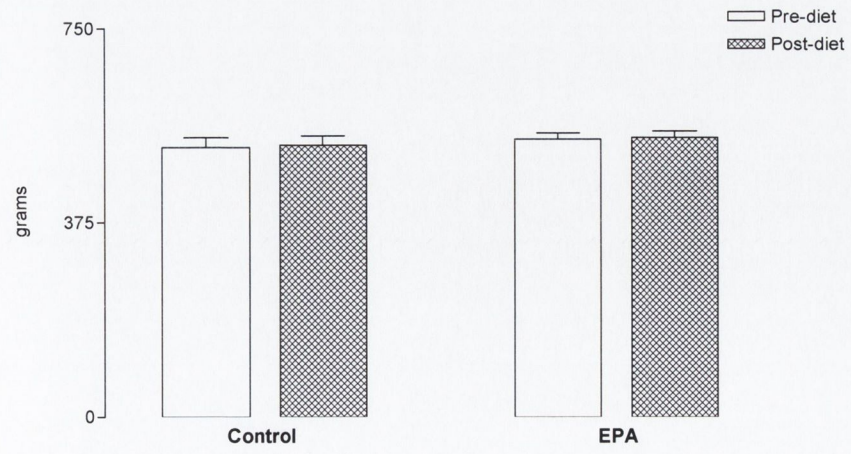

Pre-diet
Post-diet
750
375
0
grams
Control
EPA

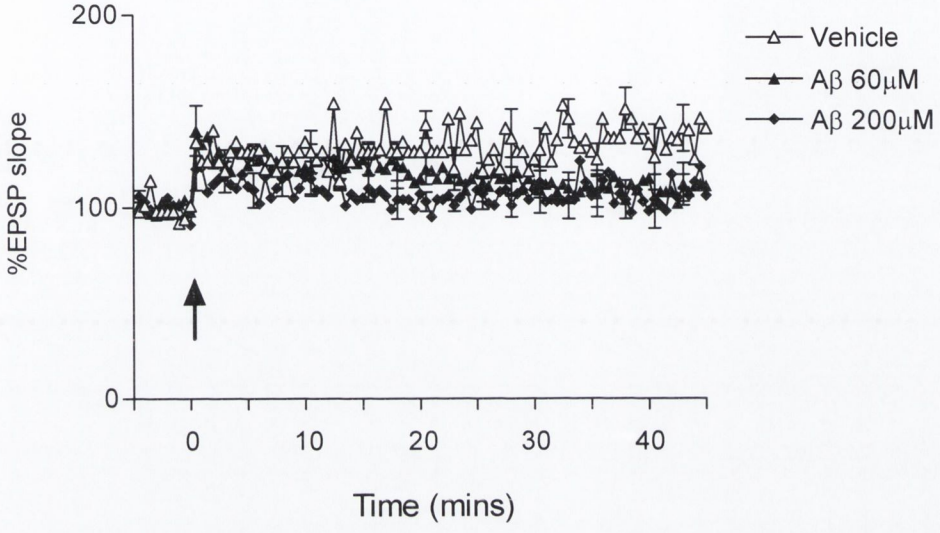
Figure 3.32 Aβ administration is associated with an impairment in LTP in dentate gyrus of adult rats; abrogation with EPA

A LTP in dentate gyrus was inhibited in 60μM (◎) and 200μM (♦) Aβ-treated rats compared with vehicle-treated (Δ) rats. LTP is similar in vehicle-treated (Δ) rats and in 20μM (◎) Aβ-treated rats.

B. LTP in dentate gyrus was similar in 60μM (◎) and 200μM (♦) Aβ-treated rats and vehicle-treated (Δ) rats that received EPA.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 5 observations. Standard errors are included for every 10^{th} response.

A. Control-treated adult rats



B. EPA-treated adult rats

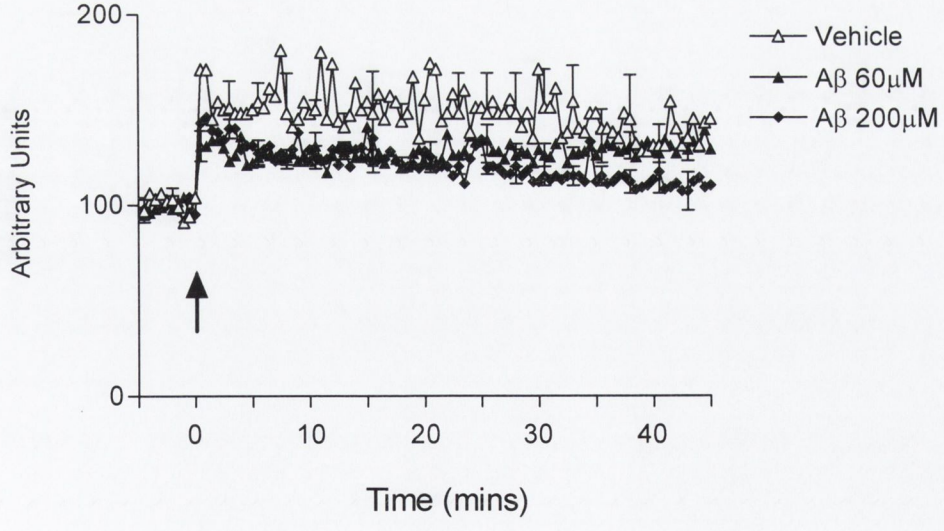
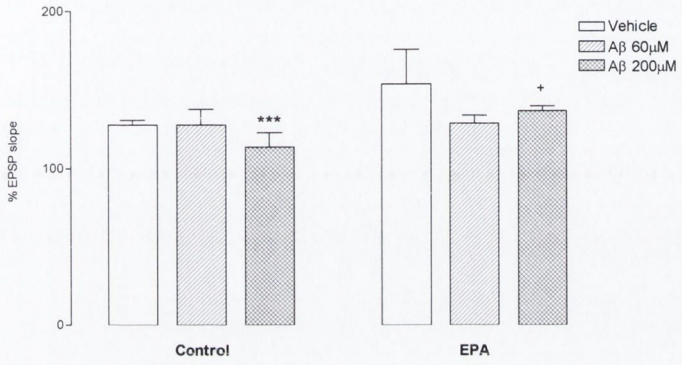


Figure 3.33 Aβ administration affects percentage change in EPSP slope in adult rats; attenuation with EPA

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in 200μM Aβ-treated rats compared with vehicle-treated control rats (***$p<0.001$; ANOVA; n=5 in all cases). Percentage change EPSP slope was significantly increased in 200μM Aβ-treated rats that received EPA compared with Aβ-treated control rats (+$p<0.01$; ANOVA; n=5 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

B. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in Aβ-treated rats compared with vehicle-treated control rats (***$p<0.001$; ANOVA; n=5 in all cases). There was a significant increase observed in percentage change in EPSP slope in Aβ-treated rats that received EPA compared with Aβ-treated control rats (+$p<0.05$; +++$p<0.001$; ANOVA; n=5 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.


200
100
0
% EPSP slope
***
+
Vehicle
Aβ 60μM
Aβ 200μM
Control
EPA

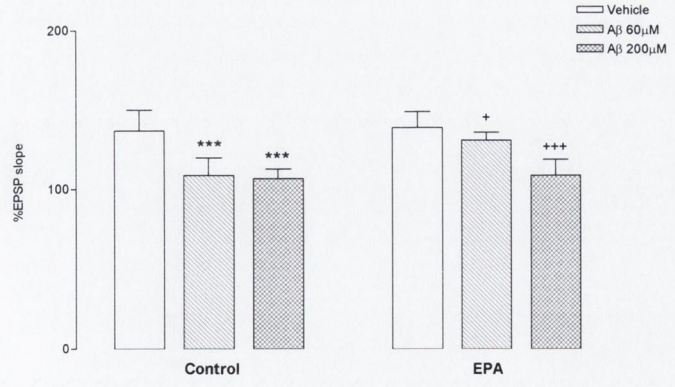

200
100
0
%EPSP slope
***
***
+
+++
Vehicle
Aβ 60μM
Aβ 200μM
Control
EPA

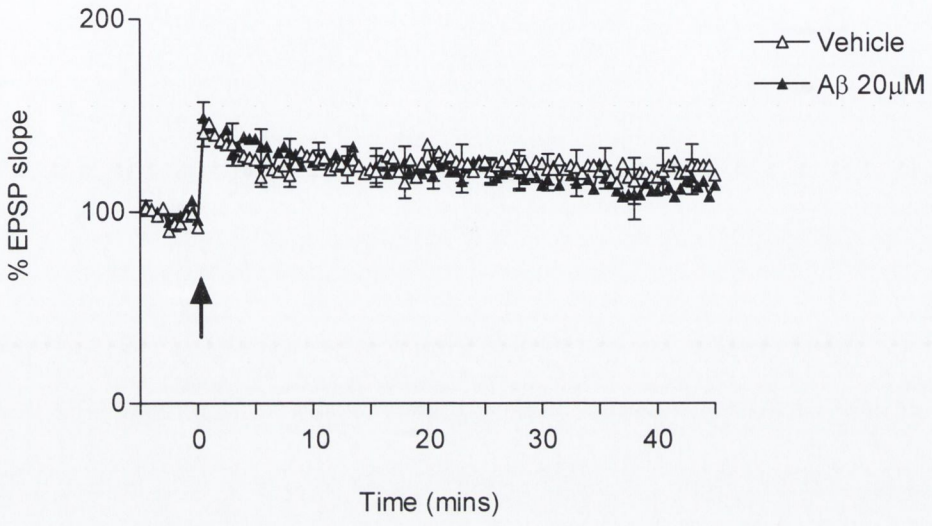
Figure 3.34 Aβ administration is associated with impairment in LTP in dentate gyrus of middle-aged rats; abrogation with EPA

A. LTP in dentate gyrus was significantly impaired in 20μM Aβ-treated (◎) control rats compared with vehicle-treated control (Δ) rats.

B. LTP in dentate gyrus was similar in 20μM Aβ-treated (◎) rats and vehicle-treated rats (Δ) that received EPA.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 6 observations. Standard errors are included for every 10^{th} response.

A. Control-treated middle-aged rats



B. EPA-treated middle-aged rats

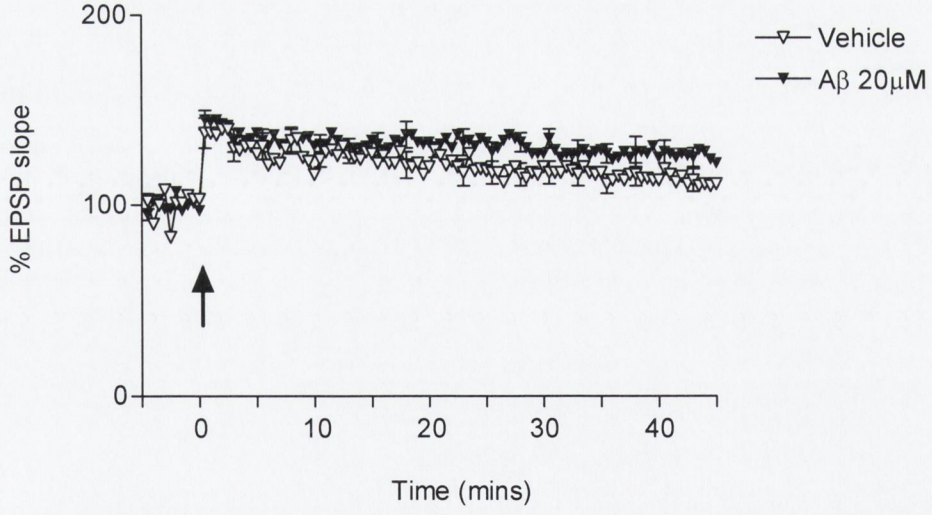
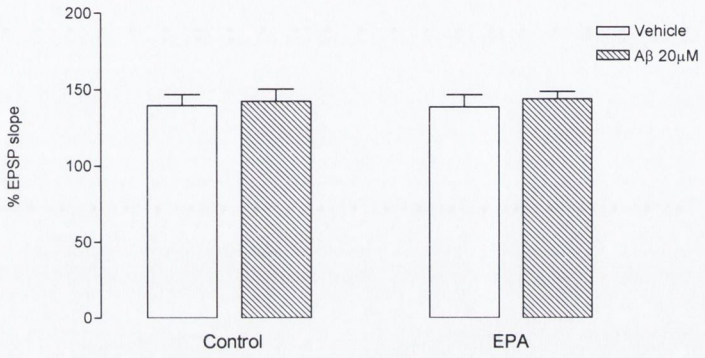


Figure 3.35 Aβ administration affects percentage change in EPSP slope in middle-aged rats; reversal by treatment with EPA

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was similar in all groups of rats (n=7 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

B. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in Aβ-treated control rats compared with vehicle-treated control rats (***$p<0.001$; ANOVA; n=6 in all cases). There was a significant increase observed in percentage change in EPSP slope in Aβ-treated rats that received EPA compared with Aβ-treated control rats (++$p<0.01$; ANOVA; n=7 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

A.


200
150
100
50
0
% EPSP slope
Vehicle
Aβ 20μM
Control
EPA

B.

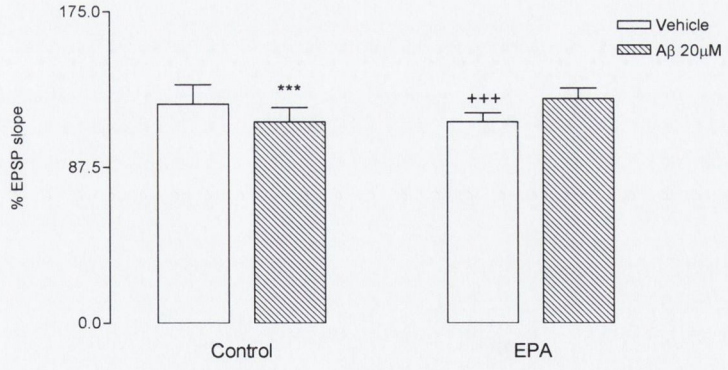

175.0
87.5
0.0
% EPSP slope
Vehicle
Aβ 20μM
***
+++
Control
EPA

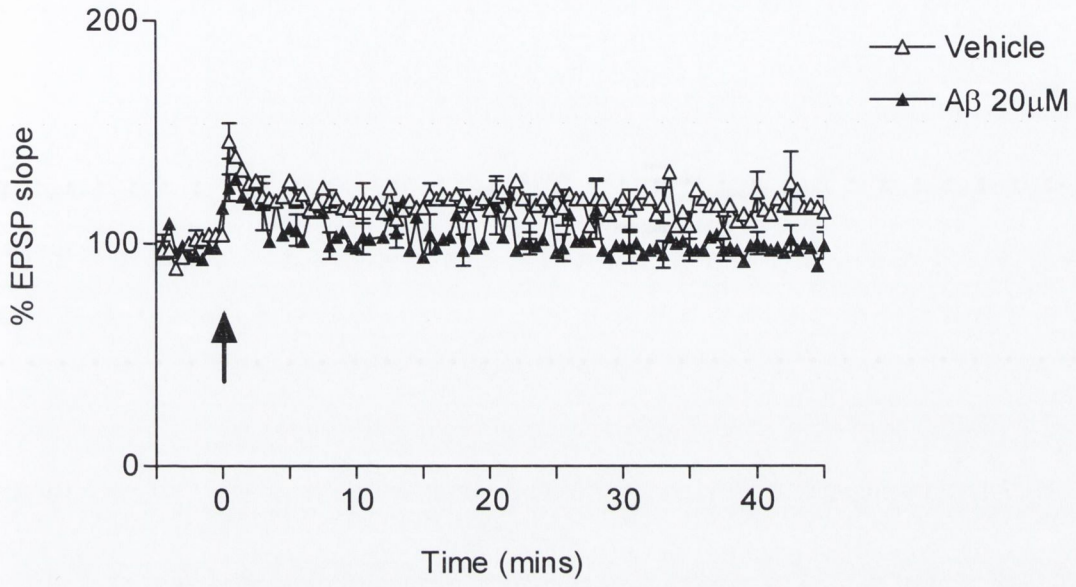
Figure 3.36 Aβ administration is associated with impairment in LTP in dentate gyrus of aged rats; abrogation with EPA

A. LTP in dentate gyrus was significantly impaired in 20μM Aβ-treated (◎) control rats compared with vehicle-treated control (Δ) rats.

B. LTP in dentate gyrus was similar in 20μM Aβ-treated (◎) rats and vehicle-treated rats (Δ)that received EPA.

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after tetanic stimulation is shown. Arrow indicates high-frequency stimulation. 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 ± standard error of the mean of 6 observations. Standard errors are included for every 10^{th} response.

A. Control-treated aged rats



B. EPA-treated aged rats

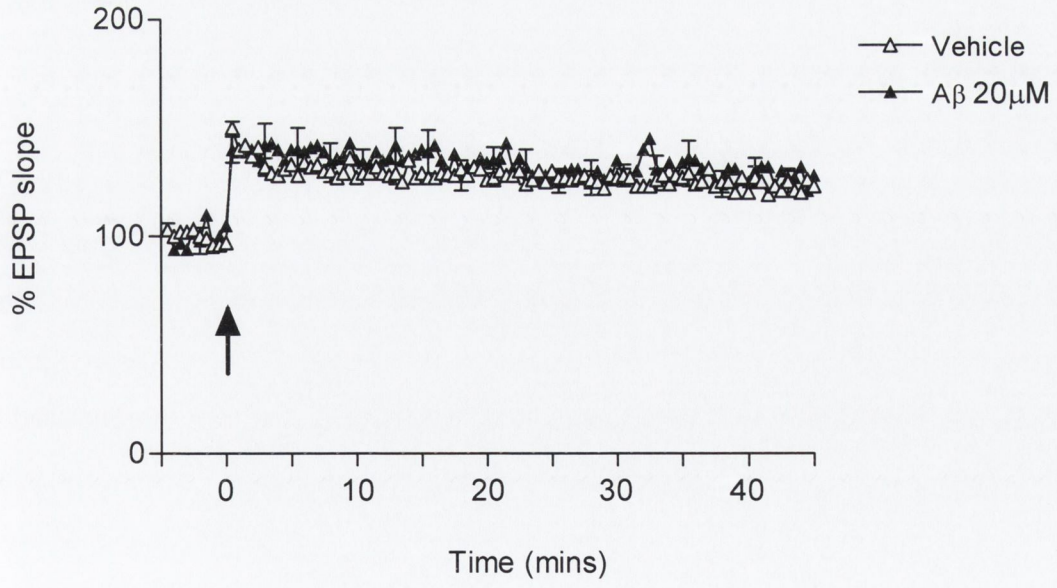


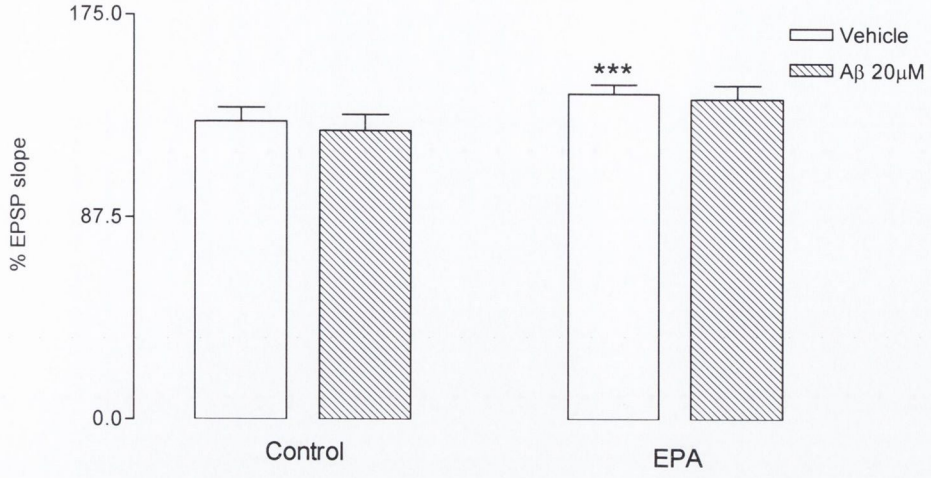
Figure 3.37 Aβ administration affects percentage change in EPSP slope in aged rats; attenuation with EPA

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was similar in all groups of rats (n=7 in all cases). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

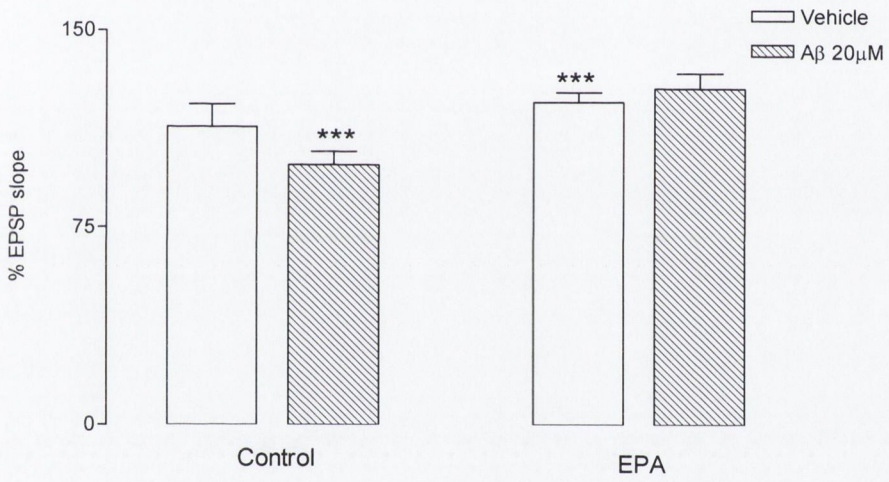
B. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in Aβ-treated control rats compared with vehicle-treated rats (***$p<0.001$; ANOVA; n=8 and 6 respectively). There was a significant increase observed in percentage change in EPSP slope in Aβ-treated rats that received EPA compared with Aβ-treated control rats (++$p<0.01$; ANOVA; n=8 and 6 respectively). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

C. The mean percentage change in EPSP slope in the last 5 min of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus) was significantly decreased in vehicle-treated aged control rats compared with adult control rats (***$p<0.001$; ANOVA; n=6 and 5 respectively). There was a significant increase observed in percentage change in EPSP slope in aged rats that received EPA compared with aged control rats (++$p<0.01$; ANOVA; n=8 and 6 respectively). Values are expressed as percentage change in EPSP slope and are means ± standard error of the means.

A.


175.0
87.5
0.0
% EPSP slope
Vehicle
Aβ 20μM
***
Control
EPA

B.


150
75
0
% EPSP slope
Vehicle
Aβ 20μM
***
***
Control
EPA

C.

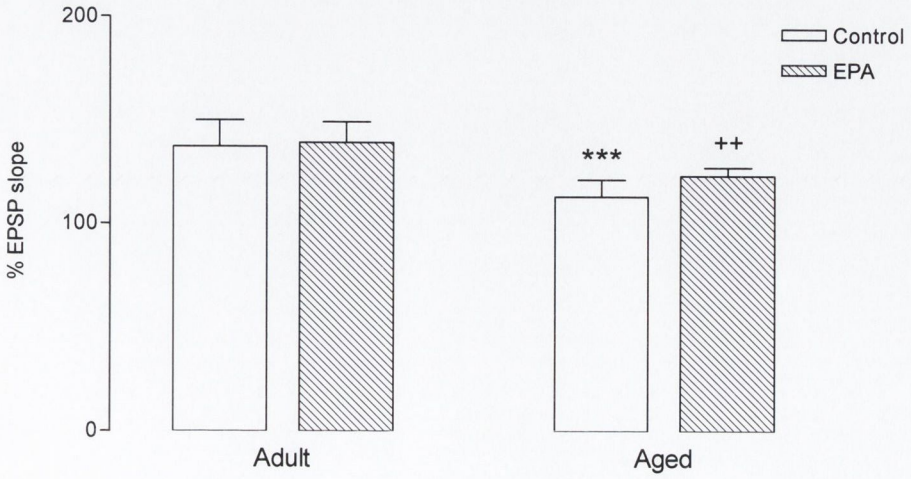

200
100
0
% EPSP slope
Control
EPA
***
++
Adult
Aged

Figure 3.38 Aβ administration is associated with an increase in the concentration of IL-1β in hippocampus of adult rats

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated control rats compared with homogenate from vehicle-treated control rats (*$p<0.05$; ANOVA; n=5 in all cases). IL-1β concentration was similar in hippocampal homogenate prepared from vehicle-treated control rats and vehicle-treated rats that received EPA and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

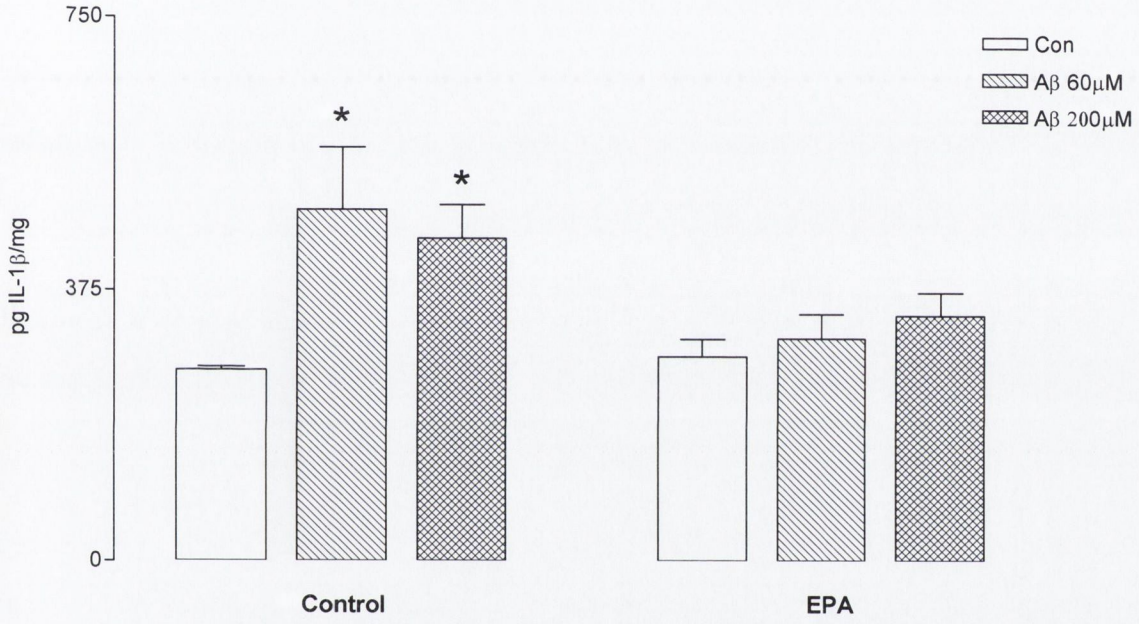

750
375
0
pg IL-1β/mg
Con
Aβ 60μM
Aβ 200μM
*
*
Control
EPA

Figure 3.39 IL-1β concentration is unaffected by Aβ in hippocampus of middle-aged rats

IL-1β concentration was similar in hippocampal homogenate prepared from all groups of middle-aged rats (n=7). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

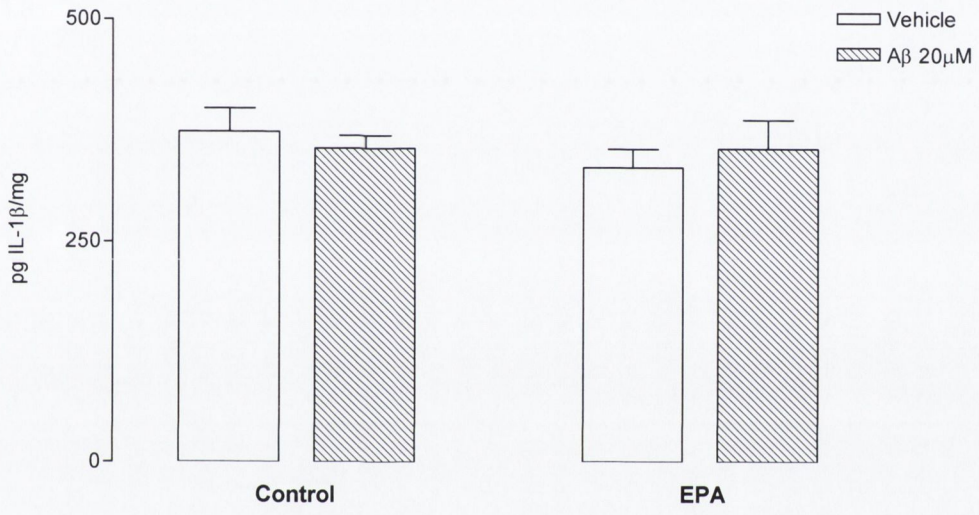
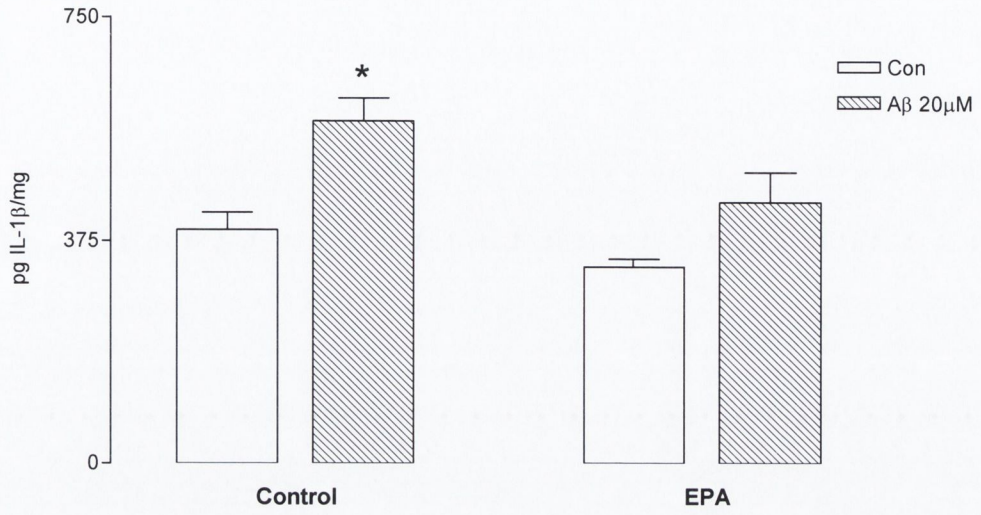

500
250
0
pg IL-1β/mg
Vehicle
Aβ 20μM
Control
EPA

Figure 3.40 Aβ administration is associated with an increase in the concentration of IL-1β in hippocampus of aged rats; abrogation with EPA

A. IL-1β concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated control aged rats compared with vehicle-treated control aged rats (*$p<0.05$; ANOVA; n=6 and 5 respectively). IL-1β concentration was similar in hippocampal homogenate prepared from vehicle-treated control aged rats (n=5) and homogenate prepared from Aβ-treated aged rats that received EPA (n=8). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

B. IL-1β concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated control aged rats (n=6) compared with tissue prepared from vehicle-treated control adult rats (*$p<0.05$; ANOVA; n=5). IL-1β concentration was similar in hippocampal homogenate prepared from vehicle-treated control adult rats (n=5) and homogenate prepared from vehicle- and Aβ-treated aged rats that received EPA (n=8). Values are expressed as pg IL-1β/mg tissue corrected for protein and are means ± standard error of the means.

A.


750
375
0
pg IL-1β/mg
*
Con
Aβ 20μM
Control
EPA

B.

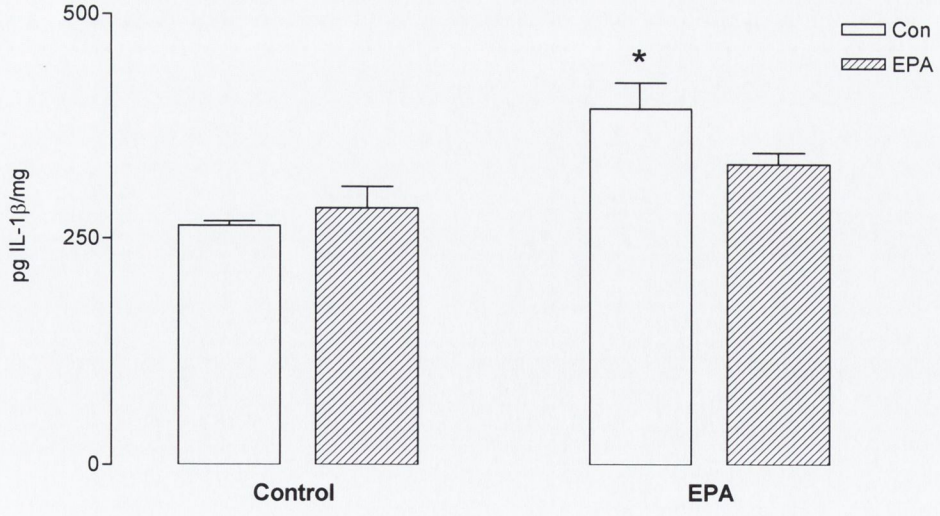

500
250
0
pg IL-1β/mg
*
Con
EPA
Control
EPA

Figure 3.41 Aβ administration is associated with an increase in the concentration of IFN-γ in hippocampus of adult rats

Interferon-γ (IFNγ) concentration was significantly increased in hippocampal homogenate prepared from Aβ-treated control rats compared with homogenate prepared from vehicle-treated control rats (*$p<0.05$; ANOVA; n=5 in all cases). IFNγ concentration was similar in hippocampal homogenate prepared from vehicle-treated control rats, vehicle-treated rats that received EPA and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

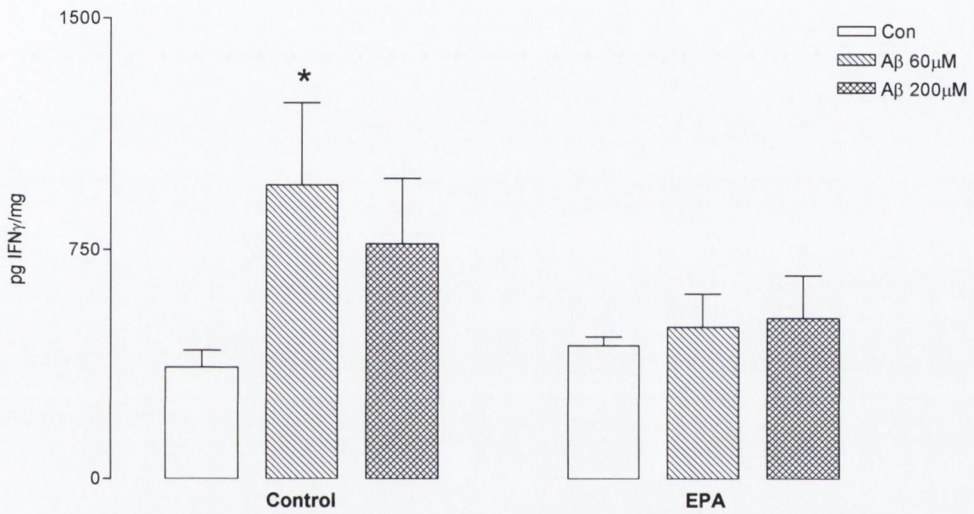

1500
750
0
pg IFNγ/mg
*
Con
Aβ 60μM
Aβ 200μM
Control
EPA

Figure 3.42 IFNγ concentration was unaffected by Aβ in hippocampus of middle-aged rats

IFNγ was similar in all groups of middle-aged rats (n=7). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

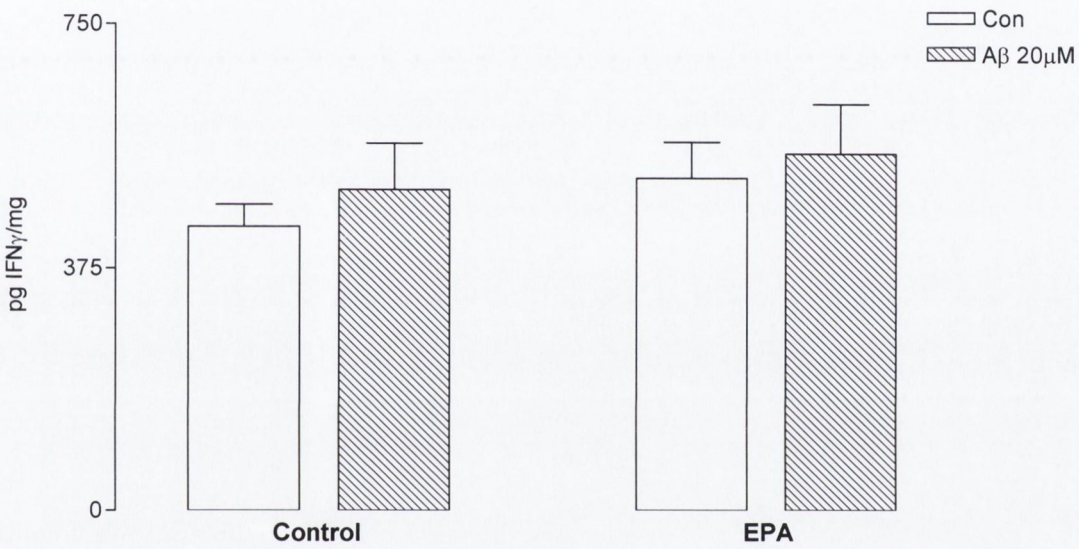
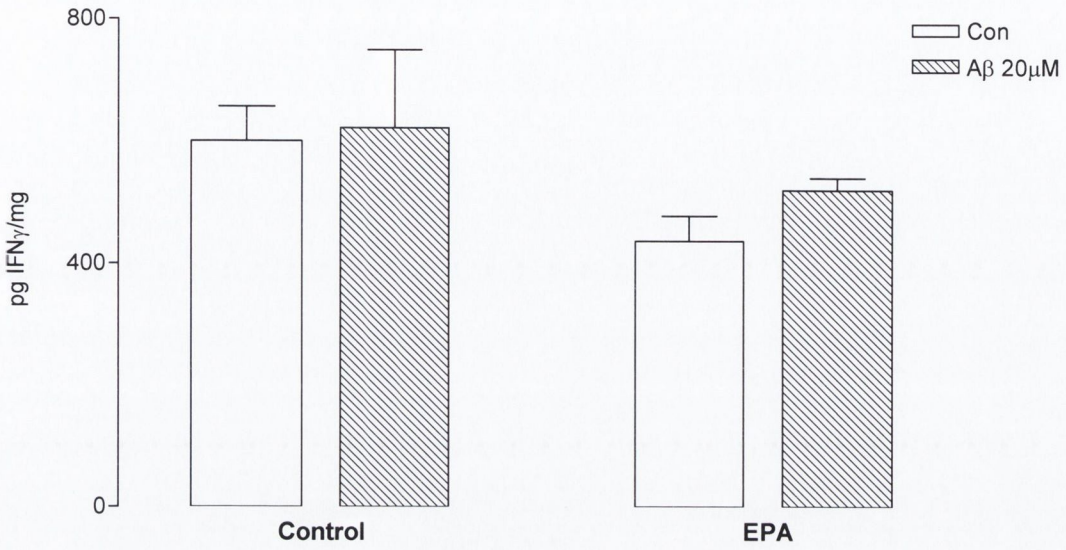

750
375
0
pg IFNγ/mg
Con
Aβ 20μM
Control
EPA

Figure 3.43 Aβ administration is associated with an increase in the concentration of IFNγ in hippocampus of aged rats; abrogation with EPA

A. IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle-treated control aged rats compared with vehicle-treated control adult rats (*$p<0.05$; ANOVA; n=6 and 5 respectively). IFNγ was similar in hippocampal homogenate prepared from vehicle-treated control adult rats (n=5), vehicle-treated adult rats that received EPA (n=5) and in vehicle-treated aged rats that received EPA (n=8). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

B. IFNγ concentration was significantly increased in hippocampal homogenate prepared from vehicle- and Aβ-treated control aged rats (n=6 in both cases) compared with tissue from vehicle- and Aβ-treated aged rats that received EPA (*$p<0.05$; ANOVA; n=8 in both cases). Values are expressed as pg IFNγ/mg tissue corrected for protein and are means ± standard error of the means.

A.


800
400
0
pg IFNγ/mg
Con
Aβ 20μM
Control
EPA

B.

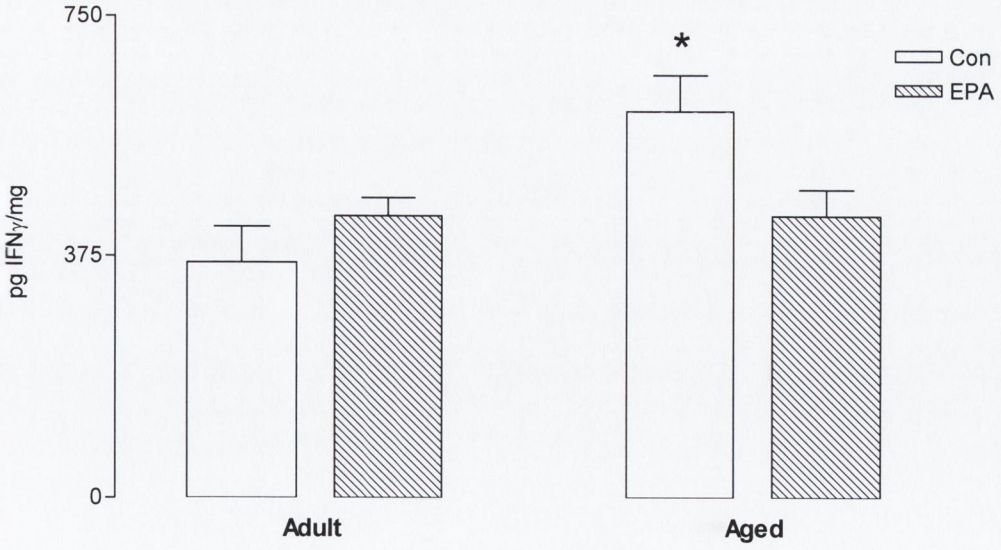

750
375
0
pg IFNγ/mg
*
Con
EPA
Adult
Aged

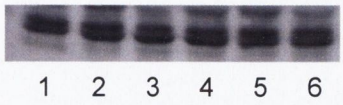
Figure 3.44 Nuclear expression of phosphorylated JNK is unaffected by Aβ administration in hippocampus of adult rats

A. One sample immunoblot shows that JNK phosphorylation (46kDa) was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats (lane 1), Aβ-treated control rats (lanes 2 and 3) and vehicle- and Aβ-treated rats that received EPA (lanes 4, 5 and 6 respectively).

B. A sample JNK immunoblot stripped and reprobed for total JNK1 expression to confirm equal loading of proteins. No change was observed in the expression of JNK1 (46kDa) in nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats (lane 1) and 60 (lane 2) and 200μM (lane 3) Aβ-treated control rats and vehicle-treated rats (lane 4) and 60 (lane 5) and 200μM (lane 6) Aβ-treated rats that received EPA.

C. Data from densitometric analysis revealed that mean JNK phosphorylation was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats, Aβ-treated control rats and vehicle- and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as arbitrary units of PPARγ expression and are means ± standard error of the means.

A.



◀ 46kDa phospho-JNK

B.

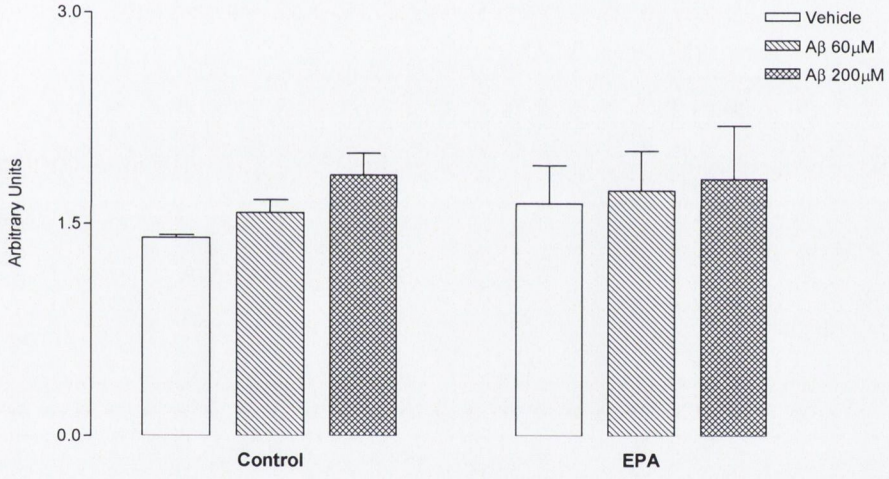


Figure 3.45 Cytosolic expression of phosphorylated JNK is increased by Aβ administration in hippocampus of adult rats; attenuation with EPA

A. One sample immunoblot shows that JNK phosphorylation (46kDa) was increased in cytosolic fraction of hippocampal tissue prepared from Aβ-treated control rats (lanes 2 and 3) compared with vehicle-treated control rats (lane 1). JNK phosphorylation was similar in cytosolic fraction of hippocampal tissue prepared from vehicle-treated control rats (lane 1) and vehicle- and Aβ-treated rats that received EPA (lanes 4, 5 and 6 respectively).

B. A sample JNK immunoblot stripped and reprobed for JNK1 expression to confirm equal loading of proteins. No change was observed in the expression of JNK1 (46kDa) in whole-cell lysate prepared from vehicle-treated control rats (lane 1) and 60 (lane 2) and 200μM (lane 3) Aβ-treated control rats and vehicle-treated rats (lane 4) and 60 (lane 5) and 200μM (lane 6) Aβ-treated rats that received EPA.

C. Data from densitometric analysis revealed that mean JNK phosphorylation was increased in cytosolic fraction of hippocampal tissue prepared from Aβ-treated control rats compared with vehicle-treated control rats (*$p<0.05$; ANOVA; n=5 in all groups). JNK phosphorylation was similar in cytosolic fraction of hippocampal tissue prepared from vehicle-treated control rats and vehicle- and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as arbitrary units of JNK phosphorylation and are means ± standard error of the means.

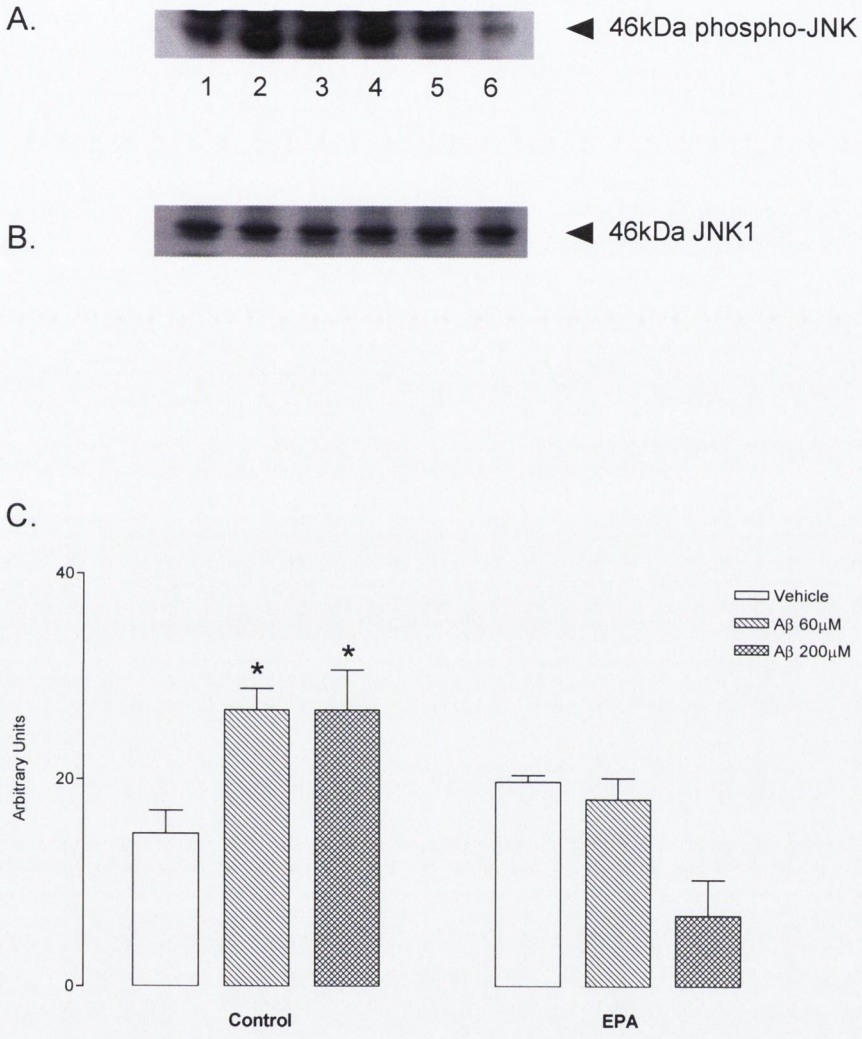

A.
46kDa phospho-JNK
1 2 3 4 5 6
B.
46kDa JNK1
C.
40
20
0
Arbitrary Units
*
*
Vehicle
Aβ 60μM
Aβ 200μM
Control
EPA

Figure 3.46 IL-4 concentration is not affected by Aβ or EPA treatment in hippocampus of adult rats

Interleukin-4 (IL-4) concentration was similar in hippocampal homogenate prepared from vehicle-treated control rats, vehicle-treated rats that received EPA and hippocampal homogenate prepared from Aβ-treated control rats and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as pg IL-4/mg tissue corrected for protein and are means ± standard error of the means.

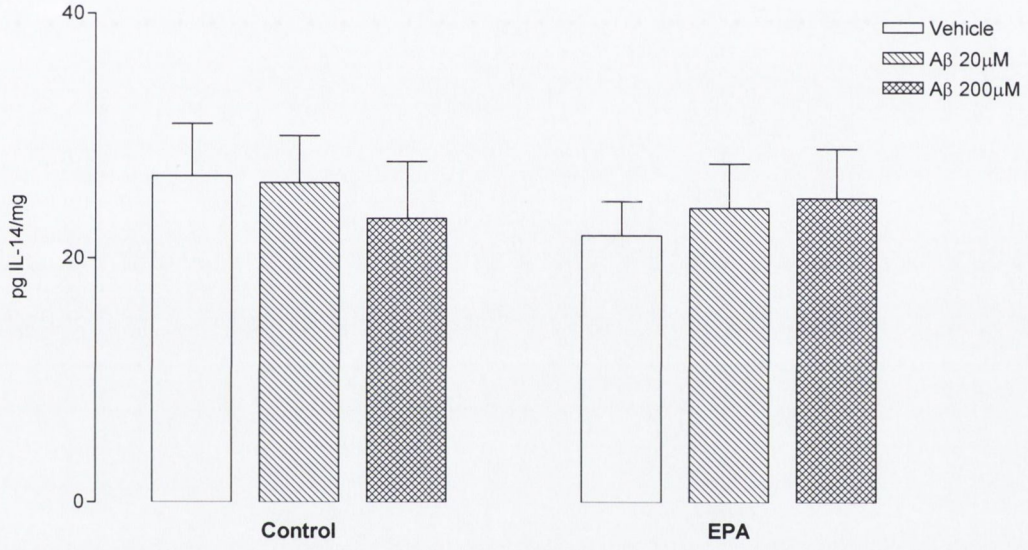

40
20
0
pg IL-14/mg
Vehicle
Aβ 20μM
Aβ 200μM
Control
EPA

Figure 3.47 IL-4 concentration is decreased in hippocampus of aged rats; abrogation with EPA

IL-4 concentration was increased in hippocampal homogenate prepared from vehicle-treated aged rats that received EPA (n=4) compared with homogenate prepared from vehicle-treated control aged rats (n=6). IL-4 concentration was similar in vehicle-treated control aged rats (n=6) and Aβ-treated control rats (n=6) and Aβ-treated rats that received EPA (n=6).

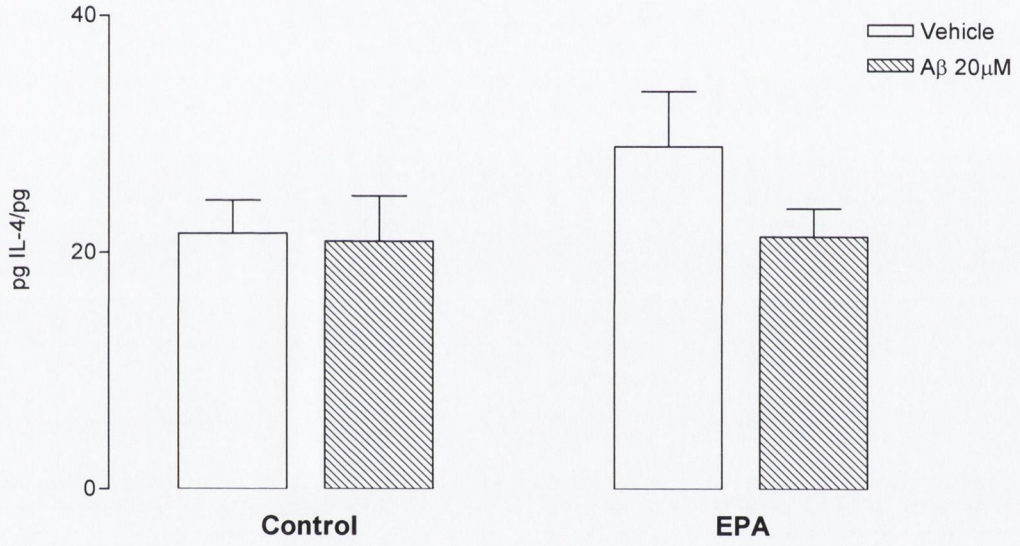
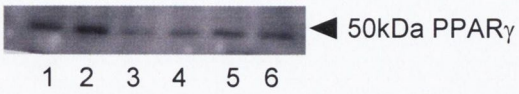

40
20
0
pg IL-4/pg
Vehicle
Aβ 20μM
Control
EPA

Figure 3.48 Aβ administration is associated with a decrease in PPARγ expresssion in hippocampus of adult rats; abrogation with EPA

A. One sample immunoblot shows that PPARγ expression (50kDa) was decreased in nuclear fraction of hippocampal tissue prepared from Aβ-treated control rats (lanes 2 and 3) compared with vehicle-treated control rats (lane 1). PPARγ expression was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats (lane 1) and vehicle- and Aβ-treated rats that received EPA (lanes 4, 5 and 6 respectively).

B. Data from densitometric analysis revealed that mean PPARγ expression was decreased in nuclear fraction of hippocampal tissue prepared from Aβ-treated control rats compared with vehicle-treated control rats (*$p<0.05$; ANOVA; n=5 in all groups). PPARγ expression was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control rats and vehicle- and Aβ-treated rats that received EPA (n=5 in all cases). Values are expressed as arbitrary units of PPARγ expression and are means ± standard error of the means.

A.


50kDa PPARγ
1 2 3 4 5 6

B.

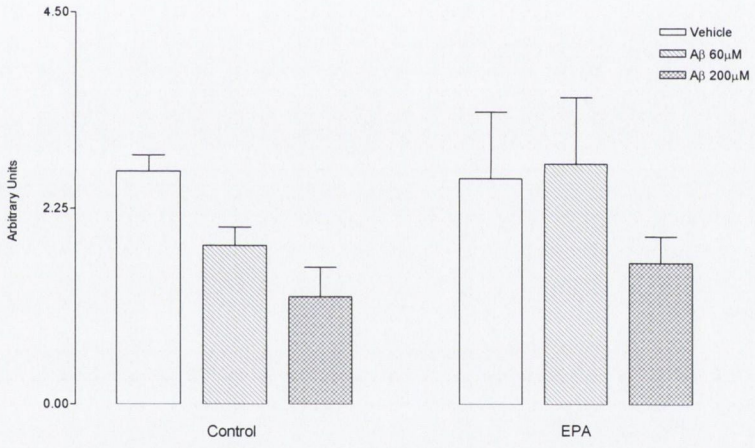

4.50
2.25
0.00
Arbitrary Units
Vehicle
Aβ 60μM
Aβ 200μM
Control
EPA

Figure 3.49 Aβ administration is associated with a decrease in PPARγ expression in hippocampus of aged rats; abrogation with EPA

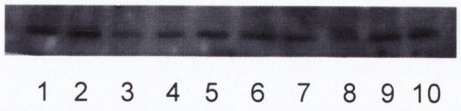
A. One sample immunoblot shows that PPARγ expression (50kDa) was decreased in nuclear fraction of hippocampal tissue prepared from vehicle-treated control aged rats (lane 7) compared with tissue prepared from vehicle-treated control adult rats (lane 1). PPARγ expression was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control adult rats (lane 1), vehicle-treated adult rats that received EPA (lane 5) and vehicle-treated aged rats that received EPA (lane 9).

B. Data from densitometric analysis revealed that mean PPARγ expression was decreased in nuclear fraction of hippocampal tissue prepared from vehicle-treated control aged rats (n=6) compared with tissue prepared from vehicle-treated control adult rats (*$p<0.05$; ANOVA; n=5). PPARγ expression was similar in nuclear fraction of hippocampal tissue prepared from vehicle-treated control adult rats (n=5), vehicle-treated adult rats that received EPA (n=5) and vehicle-treated aged rats that received EPA (n=8). Values are expressed as arbitrary units of PPARγ expression and are means ± standard error of the means.

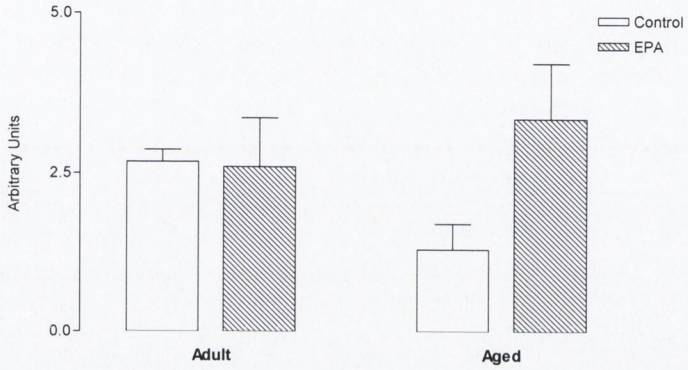
C. One sample immunoblot shows that PPARγ expression (50kDa) was decreased in nuclear fraction of hippocampal tissue prepared from vehicle- and Aβ-treated control aged rats (lanes 1 and 2) compared with tissue prepared from vehicle- and Aβ-treated aged rats that received EPA (lanes 3 and 4).

D. Data from densitometric analysis revealed that mean PPARγ expression was decreased in nuclear fraction of hippocampal tissue prepared from vehicle- and Aβ-treated control aged rats (n=6) compared with vehicle- and Aβ-treated aged rats that received EPA (*$p<0.05$; ANOVA; n=8 in both cases). Values are expressed as arbitrary units of PPARγ expression and are means ± standard error of the means.

A.


50kDa PPARγ
1 2 3 4 5 6 7 8 9 10

B.


5.0
2.5
0.0
Arbitrary Units
Control
EPA
Adult
Aged

C.


50kDa PPARγ
1 2 3 4

D.

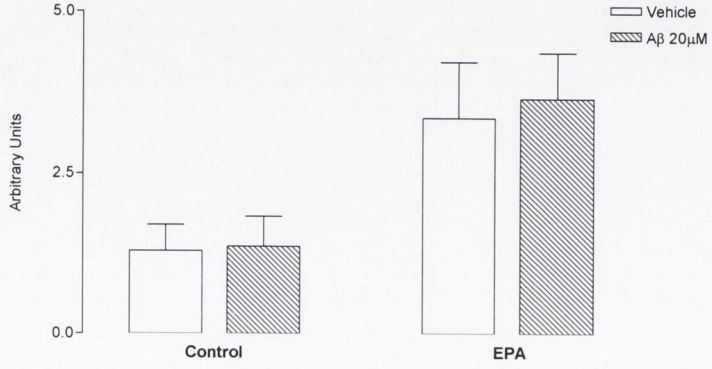
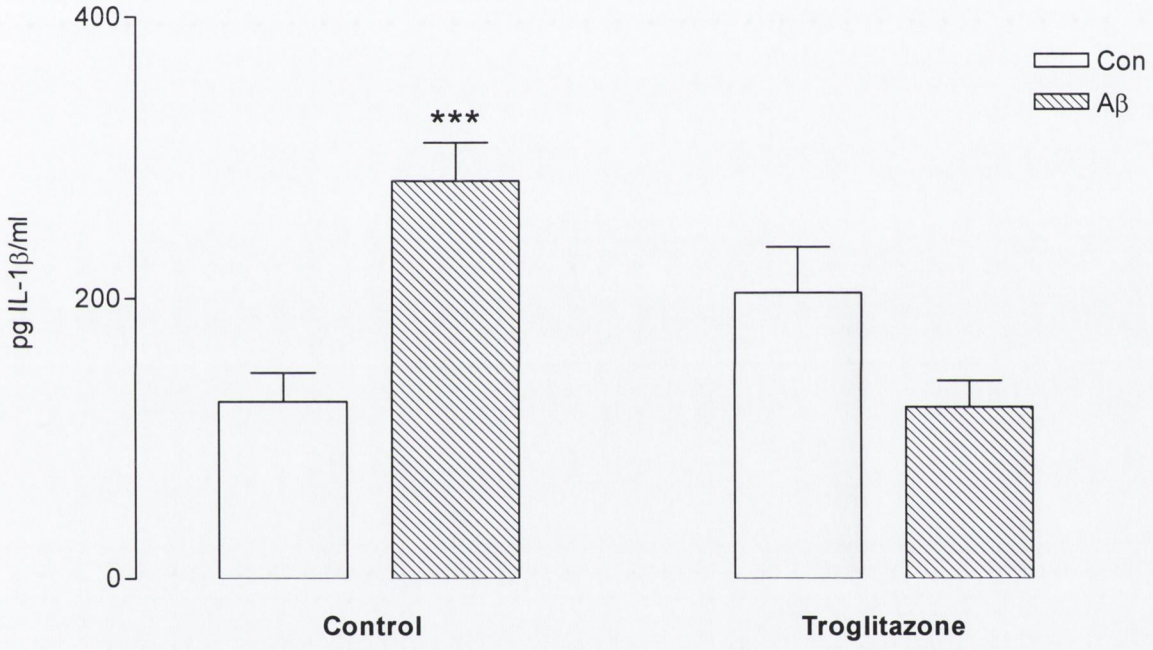

5.0
2.5
0.0
Arbitrary Units
Vehicle
Aβ 20μM
Control
EPA

Figure 3.50 IL-1β is released from Aβ-treated glia *in vitro;* abrogation by PPARγ agonist troglitazone

Release of IL-1β was significantly increased from cultured cortical glia treated with Aβ compared with control-treated cortical glia (***$p<0.001$; ANOVA; n=6 in both cases). IL-1β release was similar from control-treated and Aβ/troglitazone-treated cortical glia. Values are expressed as pg IL-1β/ml and are means ± standard error of the means.


400
200
0
pg IL-1β/ml
***
Con
Aβ
Control
Troglitazone

Chapter 4

Discussion

The objectives of this study were (a) to establish the effects of amyloid-β (Aβ) and age on LTP in perforant path-granule cell synapses *in vivo,* (b) to identify the cellular events stimulated by Aβ administration and that occur with age and (c) to assess the effectiveness of eicosapentaenoic acid (EPA) in abrogating Aβ-induced and age-related changes.

The pro-inflammatory cytokine IL-1β is a molecule involved in integration of neuronal responses with those of the endocrine system and is commonly upregulated by LPS (Vereker et al., 2000a), γ-irradiation (Lonergan *et al.,* 2002), oxidative stress (Kelly *et al.,* 2001) and age (Martin *et al.,* 2002). Compelling evidence for a central role for IL-1β in mediation of stress-induced responses is the finding that IL-1β is capable of impairing LTP in all major pathways of the hippocampus (Katsuki *et al.,* 1990; Bellinger *et al.,* 1993; Cunningham *et al.,* 1996; Murray and Lynch, 1998; Vereker *et al.,* 2000b). Consistent with its relatively high expression in hippocampus, IL-1β impairs contextual fear conditioning (Pugh *et al.,* 1999) – a form of learning that depends on the integrity of the hippocampus. Similarly, IL-1β inhibits learning in the Morris Water Maze (Gibertini, 1998) and mediates the *Legionella pneumophilia*-induced impairment in navigational learning (Gibertini *et al.,* 1995). These findings point to (a) a central role for IL-1β in stress-induced impairments of hippocampal-dependent learning and (b) a susceptibility of hippocampus to stress as a result of its relatively high physiological expression of IL-1β.

One of the significant findings in this study is that Aβ administration resulted in increased concentration of IL-1β in hippocampus of adult rats. Whether Aβ induced an increase in IL-1βmRNA is not clear. However it is evident that the observed increase in IL-1β concentration resulted from enhanced cleavage of pro-IL-1β into active IL-1β since inhibition of caspase-1 prevented the Aβ-stimulated release of IL-1β from neurons *in vitro.* Overexpression of IL-1 in brain of Alzheimer's disease (AD) patients has been documented both as an in increase in numbers of IL-1-immunoreactive microglia and as elevated tissue levels of IL-1 (Griffin *et al.,* 1989). These IL-1-expressing microglia are frequently found associated with Aβ plaques and the pattern of distribution of acitvated microglia across different brain regions correlates with the pattern of distribution of Aβ plaques (Sheng *et al.,* 1995). In AD,

early deposits of amyloid contain activated microglia overexpressing IL-1 (Griffin *et al.*, 1995) in contrast to a lack of microglia in similar diffuse plaques sometimes found in non-demented elderly individuals (MacKenzie *et al.*, 1995). These reports suggest a putative role for IL-1 and activated microglia in the initiation of plaque progression and neuritic pathology associated with AD (Mrak and Griffin, 2001). Epidemiological studies demonstrate a protective effect of anti-inflammatory medications on the occurrence of AD (Andersen *et al.*, 1995; Breitner *et al.*, 1994, 1995) suggesting a role for neuroinflammatory processes in the aetiology of AD. Indeed, oral administration of ibuprofen, a non-steroidal anti-inflammatory drug, suppresses plaque pathology and IL-1 expression in transgenic mouse models of AD (Lim *et al*, 2000). Furthermore, two studies have shown increased risk for AD associated with polymorphisms in IL-1 genes (Grimaldi *et al.*, 2000; Nicoll *et al.*, 2000).

Several studies *in vitro* have reported increases in cytokine expression in response to Aβ stimulation. Akama and Eldik (2000) demonstrated that Aβ induced IL-1β and TNFα production from cultured rat astrocytes prior to induction of iNOSmRNA and that this effect could be prevented by treatment with IL-1ra. Similarly, production of IL-1β, IL-6 and TNFα by neuronal-glial co-cultures has been reported in response to stimulation with Aβ (Haas *et al.*, 2002). Aβ induces release of IL-1β from human macrophages and microglia (Rampe *et al.*, 2004). Transcription of iNOS and overproduction of NO was also reported (Haas *et al.*, 2002). Some studies suggest that the reported increases in ROS implicate these species as signalling molecules for the induction of IL-1βmRNA since this effect was blocked by inhibitors of COX and LOX (Kang *et al.*, 2001).

Additionally, results from this study demonstrate that cleavage of IL-1β is enhanced by Aβ, at least in neurons *in vitro,* and inhibition of this effect by caspase-1 inhibitor attenuated the downstream signalling events triggered by Aβ. While these effects occur *in vitro,* it seems reasonable to propose that they may be mirrored in the *in vivo* situation since Aβ-induced upregulation of IL-1β occurs both *in vitro* and *in vivo*. Interestingly, inhibitors of caspase-1 have been shown to abrogate Aβ-induced apoptotic cell death (Egashira *et al.*, 2002) and potassium-induced apoptosis of rat cerebellar-granule neurons (Lynch *et al.*, 1997). Recently, intracerebroventricular

(i.c.v.) injection of Aβ has been shown to induce upregulation of IL-1β and IL-6mRNA in rat brain *in vivo* (Zhou *et al.,* 2004) and increased expression of iNOSmRNA within cortices of AD patients has been found (Haas *et al.,* 2002). Cytokine-stimulated astrocytes have been found to injure neurons via a NO-mediated mechanism (Chao *et al.,* 1996). While, this study indicates that caspase-1 activity may be upregulated by Aβ *in vitro,* it is possible that the observed increase in IL-1β concentration in hippocampus of adult rats induced by Aβ may occur as a consequence of upregulation of IL-1βmRNA and protein. These data point to a central role for IL-1β in the neurodegenerative processes associated with Aβ administration and in AD.

Since Aβ accumulation is a determining factor in AD, it is important to elucidate the mechanism by which Aβ induces neuronal cell death. Studies have indicated that the dying cells display characteristics of apoptotic cell death (Anderson *et al.,* 1996, Estus *et al.*; 1997; Stadelmann *et al.,* 1999). However, the specific intracellular signalling pathways by which Aβ triggers apoptosis are not yet defined. Several pieces of evidence implicate the JNK pathway in mediation of Aβ-induced apoptosis. Firstly, Aβ induces oxidative stress (Gibson *et al.,* 2004) and JNK is activated by oxidative stress in rat hippocampus (Kelly *et al.,* 2001) and in rat ventricular cells (Han *et al.,* 2004). Secondly activation of the JNK cascade results in induction of gene transcription (Anderson *et al.,* 1994) and Aβ-induced apoptosis requires protein synthesis (Imaizumi *et al.,* 1999).

In this study phosphorylation of JNK was shown to be upregulated in hippocampus *in vivo* following acute Aβ administration - a parallel increase occurred in cultured cortical neurons treated with Aβ. These data imply that JNK becomes activated, at least in neurons, in response to stimulation with Aβ. Interestingly, neurons susceptible to toxicity by Aβ and corresponding populations of neurons vulnerable in AD show increased expression of immediate early genes *c-jun* and *c-fos* (Anderson *et al.,* 1994; Ferrer *et al.,* 1996; Iwasaki *et al.,* 1996; Estus *et al.,* 1997) - genes that are activated by JNK. Similar results have been reported previously although the majority of these report increased activation of JNK in response to Aβ stimulation *in vitro.* JNK has been shown to become activated in response to Aβ stimulation in PC12

cells and sympathetic neurons (Troy *et al.*, 2001), rat cortical neurons (Bozyczko-Coyne *et al.*, 2001; Morishima *et al.*, 2001) and in mouse cortex (Savage *et al.*, 2002).

Of interest in this study was the finding that pharmacological inhibition of JNK with DJNKI1 *in vitro* abrogated the Aβ-associated apoptotic changes as measured by TUNEL reactivity. Inhibition of JNK by pharmacological or genetic means has been shown to provide protection in multiple paradigms of neuronal death including growth factor deprivation (Xia *et al.*, 1995), DNA damage and oxidative stress (Maroney *et al.*, 1999) *in vitro*, as well as ischaemia (Ferrer *et al.*, 1997) excitotoxicity (Yang *et al.*, 1997) and inflammation (Dong *et al.*, 1998; Ip and Davis; 1998). Cultures of neuronal PC12 cells show rapid activation of JNK and c-Jun in response to Aβ treatment. CEP-1347, a specific inhibitor of the JNK pathway protected PC12 neurons and sympathetic neurons from apoptosis evoked by Aβ (Troy *et al.*, 2001) and prevented activation of downstream effectors of JNK such as c-Jun phosphorylation and AP-1 activation (Bozyczko-Coyne *et al.*, 2001). Similarly, CEP-1347 has been reported to abrogate apoptosis induced by trophic factor deprivation, oxidative stress and DNA damage in the same cell types (Maroney *et al.*, 1999) emphasising the pivotal role of JNK in apoptosis. JNK activation alone may not be sufficient for death since JNK activity is present in normal brain (Carletti *et al.*, 1995) and during neuronal differentiation of PC12 cells (Eilers *et al.*, 1998) where it has been suggested to play a role in neuronal plasticity. Activation of elements in addition to JNK may be required for Aβ-stimulated cell death.

The present data show that i.c.v. administration of Aβ can induce an increase in JNK activation as early as 3 hours and that this effect appears to persist since a similar increase in JNK phosphorylation was observed 6 hours following Aβ administration. Activation of JNK isoforms has been reported to occur within 2 hours of exposure of neurons to Aβ *in vitro* while markers of cell death such as enhanced caspase-2 and -3 activity does not become apparent until 6 hours after Aβ treatment (Troy *et al.*, 2001) - these time points suggest that JNK acts as a trigger for subsequent events in the apoptotic mechanisms in response to Aβ. JNK1 deficient neurons are also resistant to Aβ toxicity (unpublished observation cited in Morishima *et al.*, 2001) and depletion of JNK1 following exposure to antisense oligonucleotide prevented the

deleterious effects of Aβ (Fogarty *et al.*, 2003). Likewise hippocampal neurons from JNK3 knockout mice are highly resistant to kainic acid-induced cell death (Yang *et al.*, 1997). Taken together these data implicate JNK as a key mediator of Aβ-induced cellular deterioration.

Among the substrates of JNK are c-Jun and FasL. Both of these have been causally implicated in various cell death paradigms. In this study c-Jun phosphorylation and FasL expression were increased in rat hippocampus 6 hours after i.c.v. administration of Aβ. c-Jun activation has been shown to occur after Aβ treatment *in vitro* (Iwasaki *et al.*, 1996) and c-Jun immunoreativity is co-localised with apoptotic neurons in affected brain areas of patients with AD (Anderson *et al.*, 1994). Similarly FasL transcription is activated on neurotrophic factor withdrawal; this has been identified to occur via a JNK-dependent mechanism (Le-Niculescu *et al.*, 1999).

Additionally, considerable protection against Aβ-induced toxicity is conferred by expression of dominant negative c-Jun (Troy *et al.*, 2001). Interestingly, sympathetic neurons from c-Jun null mice are resistant to Aβ-stimulated toxicity and kainic acid-induced cell death (Kihiko *et al.*, 1999; Yang *et al.*, 1997). Previous studies have indicated that survival factor withdrawal leads to induction of FasL mRNA and protein in cerebellar granule neurons and PC12 cells (Le-Niculescu *et al.*, 1999). Inhibition of interaction of FasL with Fas receptor abrogates apoptosis (Faris *et al.*, 1998; Le-Niculescu *et al.*, 1999). Consistent with the idea that FasL is a target for JNK-c-Jun signalling is the finding that the FasL gene has c-Jun binding sites within its promotor (Faris *et al.*, 1998a,b). Indeed, transcription of FasL protein in response to Aβ treatment of cortical neurons occurs subsequent to JNK-c-Jun activation (Morishima *et al.*, 2001). Neurons from JNK3 null mice showed no increase in FasL mRNA or protein indicating that induction of FasL by Aβ occurs in a JNK-dependent manner (Morishima *et al.*, 2001). Soluble Fas-Fc protein effectively competes with membrane bound Fas for FasL and thereby blocks the effects of Fas (Faris *et al.*, 1998; Ishiyama *et al.*, 1998; Le-Niculescu *et al.*, 1999). Incubation of cortical neurons with Fas-Fc or a neutralising anti-Fas antibody resulted in a significant reduction in Aβ-induced apoptosis (Morishima *et al.*, 2001). Although c-Jun phosphorylation is

decreased in JNK3 null mice significant phosphorylation remains suggesting the involvement of JNK1 and 2 in Aβ-induced toxicity.

Data from this study indicate that the Aβ-induced increase in IL-1β concentration *in vivo* is paralleled by activation of JNK – an effect that was mimicked *in vitro*. Indeed, inhibition of caspase-1 activity prevented the Aβ-induced activation of JNK indicating that the Aβ-induced change in JNK activation was mediated by IL-1β. Reports from several groups indicate that JNK activation occurs in response to a number of mitogenic stimuli including LPS administration (Vereker *et al.*, 2000a; Comalada *et al.*, 2003; Lonergan *et al.*, 2004), γ-irradiation (Lonergan *et al.*, 2002; Lynch *et al.*, 2003) and oxidative stress (Uciechowski *et al.*, 1996; Kelly *et al.*, 2001). Interestingly these are the same stimuli that induce increases in IL-1β concentration in hippocampus. Likewise, much evidence exists to indicate that IL-1β stimulates activation of JNK in human neuroma fibroblasts (Lu *et al.*, 1997) and in stromal cells (Rizzo and Carlo-Stella, 1996). This effect has been reported in several other tissues; for example, IL-1β-induced activation of JNK has been reported in human glomerular mesangial cells (Uciechowski *et al.*, 1996), HeLa cells (Raingeaud *et al.*, 1995) and in rat hepatocytes (Karakashian *et al.*, 2004). Central injection of IL-1β results in activation of JNK in rat hippocampus (Vereker *et al.*, 2000b; Kelly *et al.*, 2001). Indeed, IL-1β-induced inhibition of glutamate release is suppressed by the non-specific JNK inhibitor vasoactive intestinal peptide (E. Vereker and M.A. Lynch, unpublished observation). These data indicate that IL-1β and JNK are intimately involved in responses to cellular stress, that IL-1β may act as a trigger for JNK activation and that JNK may mediate IL-1β-induced effects on cell function.

Neuronal cell loss is one feature of AD and evidence from analysis of changes in cultured cells suggests that Aβ acts as the executioner. Neuronal cell cultures exposed to Aβ demonstrate signs of apoptosis (Anderson *et al.*, 1996; Estus *et al.*, 1997; Stadelmann *et al.*, 1999). Aβ-induced parallel increases in JNK activation and TUNEL reactivity have been reported in PC12 cells whereas activation of JNK was shown to be localised to amyloid deposits in 7 and 12 month old mice that overexpress APP (Savage *et al.*, 2002). There is accumulating evidence that mitochondria play an essential role in many forms of apoptosis (Green and Reed,

1998) by releasing apoptogenic factors (Kluck *et al.*, 1997; Yang *et al.*, 1997) from the intermembrane space that activate downstream effectors of apoptosis. Subcellular localisation studies have demonstrated that Bcl-2 and Bcl-X_L reside on the mitochondrial outer membrane while the pro-apoptotic family members may be present in the cytosol or on the mitochondrial membrane. Although they reside elsewhere, the main effects of the pro-apoptotic members are exerted on the mitochondrial membrane. During apoptosis pro-apoptotic members are activated, usually through conformational change (Desagher *et al.*, 1999) leading to exposure of their pro-apoptotic BH3 domains, and translocate to the mitochondria. Bax must undergo homo-oligomerisation to translocate to the nucleus (Gross *et al.*, 1998). Translocation of Bax and other pro-apoptotic family members result in release of proteins from the intermembrane space. One such protein is cytochrome *c*. Cytochrome *c* is encoded by a nuclear gene and, on entry to the mitochondria, a heme group is added which makes the enzyme active. It is only the active form that functions to induce caspase activation (Yang *et al.*, 1997). While the anti-apoptotic proteins serve to preclude release of cytochrome *c* and preserve cell survival, the pro-apoptotic members play a role in initiating cytochrome *c* release in certain settings of apoptosis. Bid is implicated in Fas-mediated apoptosis, Bax in DNA damage-induced apoptosis and neurotrophin deprivation-induced death, and Bad in lymphokine deprivation-induced cell death of certain neuronal cells (Zimmermann *et al.*, 2001).

Mitochondrial Bax expression was assessed in this study in an effort to address the question of the mechanisms induced by Aβ resulting in cellular deterioration in hippocampus. The findings indicate that Aβ induces translocation of Bax to mitochondria in hippocampus *in vivo*. This observation concurs with previous observations which were made *in vitro* for instance Aβ induces upregulation of Bax expression in murine cortical neurons *in vitro* (Yan *et al.*, 2000). This may imply that Bax is an important component of the IL-1β/JNK signalling cascade initiated by Aβ and resulting in apoptosis. Although increased Bax translocation to mitochondria has been identified as a key factor in triggering Aβ-induced changes (Gross *et al.*, 1998; Green and Reed, 1998; Kihiko *et al.*, 1999), the mechanism by which Aβ induces this effect is unclear; recent studies have pinpointed a role for JNK. A recent study has

shown that activated JNK can phosphorylate 14-3-3, a cytoplasmic anchor of Bax. Phosphorylation of this protein leads to its dissociation from Bax allowing Bax to translocate to the mitochondria. Expression of phosphorylation defective mutants of 14-3-3 prevented the JNK-induced translocation of Bax to mitochondria, subsequent release of cytochrome *c* and ensuing apoptosis (Tsuruta *et al.*, 2004).

The present data identify parallel Aβ-triggered changes in IL-1β and Bax and therefore it seems reasonable to propose that IL-1β may mediate the Aβ-induced change. A number of studies have addressed the possibility that IL-1β may affect translocation of Bax. Bax expression has been shown to be upregulated by IL-1β in rat cardiac fibroblasts *in vitro* (Tian *et al.*, 2002). Furthermore, IL-1ra protects rat myocardium from apoptosis induced by ischaemia-reperfusion injury thought to occur as a result of inflammatory changes (Suzuki *et al.*, 2001). IL-1ra prevented concomitant increases in Bax and caspase-3, indicating a role for IL-1 signalling in induction of Bax translocation to mitochondria and subsequent apoptosis (Suzuki *et al.*, 2001).

In an effort to assess potential downstream changes induced by the Aβ-triggered increase in Bax translocation, particularly focusing on the patency of the mitochondrial membrane, cytosolic expression of cytochrome *c* was assessed. Consistent with the reported increase in mitochondrial Bax expression, cytosolic cytochrome *c* expression was increased in hippocampus in response to Aβ administration. Time-lapse confocal microscopy using HeLa cells expressing cytochrome *c* green fluorescent protein revealed that release of this protein is an early event in apoptosis occurring hours before phosphatidylserine exposure and loss of plasma membrane integrity (Goldstein *et al.*, 2000). Aβ-stimulated release of cytochrome *c* into cytosol has been reported in neuronal cultures (Movsesyan *et al.*, 2004) in human teratocarcinoma cells expressing mitochondrial DNA from AD subjects (Cardoso *et al.*, 2004) and PC12 cells expressing the Swedish double mutation of APP (Marques *et al.*, 2003). Data from this laboratory has indicated that IL-1β may play a role in apoptosis via the IL-1 type I receptor (IL-1RI; Martin *et al.*, 2002). Specifically, cortical tissue incubated with IL-1β led to increased expression of cytochrome *c* in the cytosol – an effect that was blocked by IL-1ra (Martin *et al.*, 2002).

IL-1ra competes for binding sites on IL-1RI and therefore these data suggest that interaction of IL-1β with IL-1RI may result in cytochrome *c* release *in vitro.* Proteasome inhibitor Bortezomib-induced apoptosis is accompanied by activation of JNK and release of cytochrome *c* from mitochondria and activation of caspases in Jurkat lymphoblastic and U937 myelomonocytic leukemia cells (Yu *et al.*, 2004). SP600125, a specific inhibitor of JNK, prevents release of cytochrome *c* in response to Bortezomib and subsequent activation of caspases (Yu *et al.*, 2004). In addition to its role in activating caspases, cytochrome *c*, on its release from mitochondria following apoptotic signals, can accumulate in the nucleus and induce chromatin condensation (Nur-E-Kamal *et al.*, 2004). These data are consistent with the idea that Aβ-induced release of cytochrome *c* may be mediated by IL-1β and JNK. The results of the present study extend the findings to an *in vivo* situation and it seems reasonable to propose that Aβ administration leads to mitochondrial changes as a consequence of increased activation of JNK.

Poly(ADP-ribose) polymerase (PARP) is a DNA repair enzyme that plays a crucial role in determining cell survival. It is known to be a cellular substrate for caspase-3 and has a defined motif DXXD which is the key site for cleavage (Kannan and Jain, 2000). PARP cleavage is considered to be another reliable marker of apoptotic cell death and in order to consolidate the findings indicating that Aβ administration leads to cell death in hippocampus, cleavage of the intact fragment of PARP was assessed. The data reveals an Aβ-induced decrease in the 116kDa form of the protein, implying enhanced cleavage of PARP following Aβ administration. Similar reports of Aβ-induced effects *in vitro* have been made (Boland *et al.*, 2002; Fogarty *et al.*, 2003). Furthermore, evidence from this laboratory indicates that LPS administration (Lonergan *et al.*, 2004) and γ-irradiation (Lonergan *et al.*, 2002) induce cleavage of PARP – paradigms that also increase IL-1β concentration and JNK activation in hippocampus. Concomitant increases occur in IL-1βmRNA and PARP cleavage in pontine reticular formation following spinal cord transection in rat (Wu *et al.*, 2003). Similarly, inhibition of caspase-1 blocked LPS-induced increases in IL-1β concentration in parallel with a decrease in PARP cleavage (Vereker *et al.*, 2000a).

Increased mitochondrial Bax expression, release of cyotchrome *c* into the cytosol and enhanced cleavage of PARP constitute strong evidence that apoptosis is occurring. Therefore, it can be proposed that following Aβ administration there is evidence of cell death in hippocampus and that this may occur as a result of increased IL-1β concentration and JNK activation. In an effort to consolidate these findings the effects of Aβ and IL-1β in the presence of a caspase-1 inhibitor and a JNK1 inhibitor (DJNKI1) on cultured cortical neurons were assessed.

Initially, Aβ treatment of cultured cortical neurons resulted in production of IL-1β. However, caspase-1 inhibition prevented release of IL-1β from cultured neurons stimulated with Aβ - indicating that release of IL-1β from neurons occurs in a caspase-1 dependent manner. These findings concur with earlier reports which established release of IL-1β from Aβ-stimulated neuronal cultures (Akama and Eldik, 2000) as well as glial cultures (Haas *et al.*, 2002). Aβ has also been shown to stimulate production of pro-inflammatory cytokines from differentiated human monocytes and from a microglial cell line (Szczepanik *et al.*, 2001).

Similarly, Aβ induced phosphorylation of JNK in cortical neurons however, JNK activation by Aβ was abrogated by pre-treatment with caspase-1 inhibitor indicating that Aβ-stimulated phosphorylation of JNK occurs as a result of increased IL-1β release from neurons. Aβ-induced phosphorylation of JNK in cortical neurons has been consistently demonstrated (Morishima *et al.*, 2001; Troy *et al.*, 2001; Bozyczko-Coyne *et al.*, 2001; Boland *et al.*, 2002; Fogarty *et al.*, 2003). A direct role for IL-1β has not been defined previously however, caspase-1 inhibition has been shown to preclude JNK activation and subsequent signalling events in response to other stressful stimuli including LPS administration (Vereker *et al.*, 2000).

Of interest is the finding that treatment of cultured cortical neurons with IL-1β mimicked some of the effects induced by Aβ *in vivo.* FasL expression was upregulated by IL-1β. Interestingly, the IL-1β-induced effects on FasL expression in cortical neurons were reversed by pre-treatment with JNK1 inhibitor DJNKI1. This data indicates that IL-1β upregulates FasL protein through activation of JNK signalling cascades. FasL mRNA and protein have been reported to be upregulated in IL-1β-treated astrocytes of HIV-1 associated dementia and IL-1β upregulated FasL

promoter activity, an effect that was mediated by NFκB (Ghorpade *et al.*, 2003). Similarly, treatment of HK-2 cells, immortalized human proximal tubular cell lines, with IL-1β resulted in increased expression of FasL protein and the intracellular adaptor protein FADD (Jo *et al.*, 2003). These effects were accompanied by enhanced cleavage of PARP and caspase-3 activation. IL-1β treatment of RINm5F cells also resulted in increased FasL protein expression (Darville and Eizirik, 2001).

Stimulation of cultured cortical neurons with IL-1β resulted in phosphorylation of c-Jun. Similar to its effect on IL-1β-induced upregulation of FasL, JNK inhibition prevented the associated increases in c-Jun phosphorylation. These results which indicate that IL-1β increases c-Jun phosphorylation extend the earlier finding that IL-1β induces activation of the JNK signalling cascade (Vereker *et al.*, 2000b; Parker *et al.*, 2002).

Of particular significance are the observations that the inhibitory effect of a caspase-1 inhibitor on Aβ-induced changes in cortical neurons is closely paralleled by the effects of DJNKI1. For example, caspase-1 inhibition abrogated cell death induced by Aβ as evidenced by caspase-3 activation and TUNEL reactivity. Similarly DJNKI1 prevented the neurodegenerative effects induced by Aβ *in vitro* as evidenced by caspase-3 activity and TUNEL reactivity. Caspase-3 plays a key role in several models of apoptosis (Lonergan *et al.*, 2002; Martin *et al.*, 2002; Downer *et al.*, 2003). In particular analysis of caspase-3 deficient mice revealed a decrease in apoptosis in the developing brain. In AD brain, protein levels of caspase-3 are upregulated (Zhao *et al.*, 2003). Moreover, cleavage of actin, a substrate for caspase-3 was detected in plaque-associated neurons and microglia in AD (Ervin *et al.*, 2004). Caspase-3 activity is enhanced by Aβ stimulation of primary neuronal cultures (Fogarty *et al.*, 2003; Movsesyan *et al.*, 2004), primary mixed population cortical cultures (Harada and Sugimoto, 1999) and PC12 cells (Iuvone *et al.*, 2004).

Caspase-3 activation induced by cantharidin in U937 cells is attenuated by treatment with JNK inhibitor SP100625 (Huh *et al.*, 2004). Additionally, treatment of primary neuronal cultures with JNK1 antisense oligonucleotides abrogates Aβ-induced activation of caspase-3, cleavage of PARP and subsequent DNA fragmentation (Fogarty *et al.*, 2003). Inhibition of caspase-3 prevented DNA

fragmentation in mixed population primary cultures of neurons and glia, however, neurons in these cultures seemed to be more specifically affected by Aβ since Aβ induced downregulation of microtubule-associated protein-2 rather than the glial marker GFAP (Harada and Sugimoto, 1999). This finding indicates that while Aβ induces activation of glia it causes specific degeneration of neurons possibly through mediators produced by glia.

These data point to a central role for IL-1β and IL-1β-induced signalling cascades e.g. JNK activation in mediating Aβ-stimulated effects on neurons. IL-1β-induced phosphorylation of c-Jun and FasL expression provide further evidence in support of this observation since IL-1β is capable of mimicking cellular responses initiated by Aβ. Furthermore these data suggest that in hippocampus a causal relationship between all of these factors exists that has been shown previously in experimental ischaemia injury (Matsuyama *et al.*, 1995; Herdegen *et al.*, 1998; Martin-Villalba *et al.*, 1999), Parkinson's disease and Down's syndrome (de la Monte *et al.*, 1997, 1998; Seidl *et al.*, 1999). Data from this study indicates a functional coupling between Aβ-induced changes and enhanced IL-1β signalling since Aβ-induced activation of caspase-3 was prevented by inhibition of caspase-1 and also by inhibition of JNK.

The role of stressors in mediating synaptic disruption has been investigated by studying their direct effects on synaptic mechanisms, especially LTP. LTP is a neurophysiological model of activity dependent changes in synaptic strength that are believed to underlie information storage (Elgersma and Silva, 1999; Luscher *et al.*, 2000; Martin *et al.*, 2000). Aβ-induced impairments of LTP have been reported in hippocampus both *in vivo* (Kim *et al.*, 2001; Walsh *et al.*, 2002) and *in vitro* (Hartley *et al.*, 1999; Chen *et al.*, 2000). Specifically, acute injection of Aβ blocked LTP without affecting baseline synaptic transmission (Cullen *et al.*, 1997). However, the precise mechanism by which any of these stresses impairs LTP is not yet clear.

Evidence from several studies indicates that LTP is impaired by a wide variety of stressors; including age (de Toledo-Morrell and Morrell, 1985; Lynch and Voss, 1994; Murray and Lynch, 1998a, 1998b, Martin *et al.*, 2002), γ-irradiation (Tolliver and Pellmar, 1987; Pellmar *et al.*, 1990), oxidative stress (Pellmar *et al.*, 1991;

Kelly *et al.*, 2001) and LPS administration (Vereker *et al.*, 2000a). It is significant that LPS inhibits LTP in a JNK-dependent manner (Barry and Lynch, unpublished observation) and that the effect was dependent on LPS-induced increase in hippocampal IL-1β concentration. Similarly, IL-1β concentration in hippocampus is increased by γ-irradiation (Lynch *et al.*, 2003), oxidative stress (Kelly *et al.*, 2001) and LPS administration (Vereker *et al.*, 2000; Nolan *et al.*, 2002). Interestingly, the deficit in LTP observed in aged rats has been correlated with increased IL-1β concentration and is reversed when the age-related increase in IL-1β concentration is attenuated (Martin *et al.*, 2002). These findings indicate that the aged brain may be more susceptible to Aβ, which induces inflammatory changes, because of the underlying inflammation associated with age.

In an effort to determine whether hippocampus of aged rats was more susceptible to Aβ, the effects of a number of different doses of Aβ on LTP in young adult (3-4 months) rats was examined so as to establish a subthreshold dose with which to treat aged rats. Central administration of exogenous Aβ impaired LTP in adult rats with the impairment being more profound in the 60 and 200μM Aβ-treated groups in comparison with the 20μM Aβ-treated group. Previous reports have indicated that doses of Aβ in the range of 80-200μM can inhibit LTP (Cullen *et al.*, 1997; Freir *et al.*, 2001). Interestingly, while the 20μM Aβ-treated group showed slightly decreased LTP in comparison with vehicle-treated animals LTP was still maintained - albeit to a slightly lesser extent than in vehicle-treated adult rats. This points to a differential effect of the three doses on LTP in hippocampus of adult rats. Similarly, Aβ 200μM (Freir and Herron, 2003) and 80μM (Cullen *et al.*, 1997) that did not affect baseline transmission have been shown to inhibit LTP in Schaffer-collateral/commissural pathway *in vivo*. Recent studies suggest that rather than Aβ-containing plaques being solely responsible for synaptic dysfunction associated with AD, soluble diffusible Aβ may play a critical role (Lambert *et al.*, 1998; Hartley *et al.*, 1999; Lue *et al.*, 1999). Administration of Aβ *in vitro* produces neurodegeneration and neurite dystrophy similar to that seen in autopsy samples of AD patients (Pike *et al.*, 1991, 1992). Similarly, prolonged infusion of Aβ into brain can produce learning and memory deficits in rats (Nitta *et al.*, 1997; Sweeney *et al.*, 1997) and overexpression of

Aβ in aged transgenic mice is associated with cognitive decline (Chapman *et al.*, 1999; Nalbantoglu *et al.*, 1997).

Results from this study reveal an age-related impairment in the maintenance phase of LTP. Impairment of cognitive function (Barnes, 1979, 1988), accompanied by an impaired ability to sustain LTP is associated with age (Landfield *et al.*, 1978; Barnes, 1979; de Toledo-Morrell and Morrell, 1985; Lynch and Voss, 1994; McGahon *et al.*, 1997; Murray and Lynch, 1998a,b; O'Donnell *et al.*, 2000; Martin *et al.*, 2002). Significantly, 20μM Aβ, which had a relatively small effect on the later phase of LTP in hippocampus of adult rats, was capable of completely inhibiting LTP in hippocampus of middle-aged and aged rats and interfered with the early phase of LTP. The data indicate that, in terms of its ability to sustain LTP, the hippocampus of aged rats is indeed more susceptible to exogenous Aβ. Deficits in memory have been reported in aged transgenic mice undergoing isolation stress in comparison to age-matched controls (Dong *et al.*, 2004) and APP null mice show impaired LTP and deficits in learning and memory in comparison to age-matched wildtype mice (Seabrook *et al.*, 1999).

In an effort to establish the underlying cause of Aβ-induced and age-related impairments, the concentrations of two pro-inflammatory cytokines, IL-1β and IFNγ, were assessed in hippocampal tissue from the various treatment groups. In hippocampus of adult rats, IL-1β concentration was increased in groups treated with 60 and 200μM Aβ but not in 20μM Aβ-treated rats. Several studies report IL-1β-induced inhibition of LTP in CA1, CA3 and dentate gyrus *in vitro* (Katsuki *et al.*, 1990; Bellinger *et al.*, 1993; Cunningham *et al.*, 1996) and in dentate gyrus *in vivo* (Murray and Lynch, 1998a, 1998b; O'Donnell *et al.*, 2000). In addition LTP is inhibited in stressed rats (Murray and Lynch, 1998a) and LPS-injected rats (Vereker *et al.*, 2000; Nolan *et al.*, 2002; Lonergan *et al.*, 2004) in which increased hippocampal IL-1β concentrations were observed.

Data from this study demonstrate an age-related increase in IL-1β concentration in hippocampus – these results support earlier findings, which indicate that IL-1β concentration is increased in hippocampus of aged rats (Murray and Lynch, 1998a, 1998b; O'Donnell *et al.*, 2000; Martin *et al.*, 2002) and in hippocampus

and hypothalamus of senescence accelerated mice (Tha *et al.*, 2000). The mechanism leading to an increase in IL-1β in hippocampus of aged rats is not clear however, enhanced activity of caspase-1 has been reported to occur with age (Lynch and Lynch, 2002) – a finding of significant interest since data from this study indicate that Aβ induces IL-1β release through activation of caspase-1. Significantly, 20μM Aβ, which exerted no effect on IL-1β concentration in hippocampus of adult rats, enhanced IL-1β concentration in hippocampus of aged rats. IL-1 concentration is enhanced within 12 hours of head injury, a risk factor for later development of AD (Griffin *et al.*, 1994, 1995) a finding that indicates that underlying inflammation may enhance susceptibility to subsequent exposure to Aβ. Susceptibility of aged tissues to damage has been reported elsewhere – retinas of aged rats are more susceptible to ischaemic damage (Kim *et al.*, 2004). Taken together these data support evidence of a negative correlation between IL-1β concentration and LTP and are consistent with the view that the hippocampus of the aged rat is more susceptible to Aβ, which induces inflammatory changes, because of the underlying inflammation associated with age.

The concentration of IFNγ was increased in hippocampus of adult rats by all doses of Aβ. These data seem at odds with that for LTP – 20μM Aβ was not capable of inhibiting LTP in adult rats but induced an increase in IFNγ concentration in hippocampus of adult rats. These findings show a clearer correlation between IL-1β and LTP than IFNγ and LTP however, IFNγ may have a significant contributory role in the inflammatory response to Aβ. IFNγ (but not IL-1β) was increased in hippocampus of adult rats treated with 20μM Aβ and this group showed a small impairment of LTP – this finding indicates that IL-1β and IFNγ may work synergistically to impair LTP. Others have suggested that IFNγ may be involved in Aβ-induced degenerative changes since upregulation of IFNγ mRNA and protein occurs in cortical regions of brains of transgenic mice overexpressing human APP (Abbas *et al.*, 2002). Additionally, increased transcripts of IFNγ and IL-12 genes were found in reactive microglia and astrocytes surrounding Aβ deposits (Abbas *et al.*, 2002). Several cytokines and chemokines including IL-1β (Griffin *et al.*, 1995), IL-6 (Strauss *et al.*, 1992), TNFα (Tarkowski *et al.*, 1999), IL-18 (Xia and Hyman, 1999),

TGFβ (van der Wal *et al.*, 1993) and macrophage inflammatory protein (MIP-1α; Xia and Hyman., 1999) are upregulated with AD. IL-1, IL-6, TNFα, MIP1α and MCP-1 increase in a dose-dependent manner after cultured AD microglia are incubated with Aβ (Lue *et al.*, 2002). Human monocytes and mouse microglia show increased IL-1β, IL-1ra and MIP1α mRNA and protein expression when stimulated in vitro with Aβ (Meda *et al.*, 1999). Moreover, Aβ elevates IFNγ, IFNγ receptor and IL-1β in human endothelial cells (Suo *et al.*, 1998). Evidence indicates that IFNγ could act as an inflammatory amplifier aggravating the neurodegenerative process by priming microglia or monocytes/macrophages for the secretion of pro-inflammatory cytokines (Aloisi *et al.*, 1999) since microglia activated by Aβ exhibit increased IFNγ immunoreactivity (Kim *et al.*, 2004).

In parallel with the age-related increase in IL-1β concentration in hippocampus, this study indicates an increase in IFNγ concentration in hippocampus of aged rats. This is the first report of an age-related increase in IFNγ concentration in hippocampus *in vivo*. Increased GFAP, a marker for glia, is upregulated with age in corpus callosum, basal ganglia and hippocampus (Major *et al.*, 1997) indicating increased reactive gliosis exists with age. This effect of age may help to explain the underlying inflammation that occurs. In this study, Aβ did not stimulate any additional increase in the concentration of IFNγ in hippocampus of aged rats. Recent studies suggest that astrocytes and microglia prepared from aged animals may respond differently to stimulation by Aβ than glia prepared from young animals. Interestingly, astrocytes prepared from adult mice do not upregulate their production of MCP-1 in the same way as astrocytes prepared from young mice do in response to Aβ (Wyss-Coray *et al.*, 2003) and microglial cells from cognitively normal elderly donors exhibited several abnormalities within their cytoplasmic structure, including deramification, spheroid formation and gnarling and fragmentation of processes (Streit *et al.*, 2004). Indeed increased reactive gliosis in retinas of aged rats has been demonstrated in response to ischaemic damage, however, this upregulation did not serve to protect retinal cells from deterioration (Kim *et al.*, 2004). It is clear from these studies that a marked dysregulation of glial cell number and function occurs with age.

In an effort to address the question of the source of IL-1β and IFNγ in CNS, primary cultures of neurons and glia were grown separately, treated with Aβ and release of IL-1β and IFNγ assessed. The findings suggest that IL-1β is released from both cell types in response to Aβ stimulation, however IFNγ was not detectable in supernatant from these cells. It is generally accepted that IL-1β is released from glia *in vitro* (Yao *et al.*, 1992; Griffin *et al.*, 1994) however surprisingly neurons themselves have been consistently identified as a cell type that are capable of producing inflammatory mediators such as IL-1 (Orzylowska *et al.*, 1999), IL-6 (Murphy *et al.*, 1999) and TNFα (Breder *et al.*, 1993). It is therefore possible that neurons themselves contribute to inflammatory reactions in their vicinity and so contribute to their own degeneration in AD. There are no reports of neurons releasing or producing IFNγ however, IFNγ has been shown to be released from natural killer cells and T cells in the peripheral immune system.

Whether, IFNγ stimulates IL-1β production or the reverse is true, is not clear. Treatment of cultured cells with IL-1β alone is not sufficient to induce significant increases in IFNγ, however, inhibition of caspase-1 abrogates LPS-induced increases in IFNγ production (Fantuzzi *et al.*, 1998). NK cells are an early source of cytokines such as IFNγ, which can modulate monocytes to produce IL-1β. Additionally, IFNγ can cause upregulation of caspase-1 gene expression – the current study has identified that Aβ-stimulated cell signalling events are mediated, at least in part, by caspase-1 and therefore it may be possible that IFNγ primes neurons and glia to respond to Aβ by enhancing caspase-1 activity. How IFNγ may exert this effect is not clear however preliminary data from this laboratory indicate the presence of IFNγ receptor on neurons and glia in the brain. Considering the evidence, whether IFNγ or IL-1β is produced initially in response to Aβ is not known however, it seems reasonable to assume that they may act synergistically on cells in the CNS.

How Aβ affects neurons and glia to induce its effects is unclear. A number of receptors for Aβ have been postulated. Perhaps one of the most interesting is receptor for 'advanced glyacation end-products' (RAGE), which is present on neuronal and glial membranes. The current study indicates enhanced expression of RAGE in hippocampal tissue prepared from all Aβ-treated groups of rats in

comparison to vehicle-treated rats. RAGE, as its name implies, binds advanced glycation end-products, β-sheet fibrils characteristic of amyloid; pro-inflammatory cytokine-like mediators of the S100/calgranulin family and amphoterin. Binding of ligands to RAGE does not accelerate clearance or degradation but begins a sustained period of cellular activation mediated by receptor-dependent signalling (Schmidt *et al.*, 2001). Other distinctive features of this receptor include its ability to engage classes of molecules rather than individual ligands and its enhanced surface expression in environments rich in RAGE ligands. This last point is crucial since it explains how upregulation of the receptor contributes to the ascending spiral of RAGE-dependent cellular perturbation. RAGE expression is increased on neurons and glia in brains of AD patients (Lue *et al.*, 2001).

The consequences of Aβ ligation of RAGE appear different on neurons versus glia. Microglia become activated as a consequence of Aβ-RAGE interaction as reflected by increased motility and expression of cytokines (Yan *et al.*, 1996) while early RAGE-mediated neuronal activation is superseded by cytotoxicity at later stages (Sousa *et al.*, 2001). Moreover, RAGE mediates oxidative stress (Wautier *et al.*, 2004). Mice overexpressing neuronal RAGE and mutant amyloid precursor protein – the latter causing an Aβ-rich environment – show exaggerated cell stress at 3-5 months including activation of NFkB and IL-6 whereas cytotoxicity occurs at 8-10 months (Goova *et al.*, 2000). These findings are consistent with RAGE-mediated amplification of the effects of Aβ prior to extensive amyloid deposition. Additionally, ligation of RAGE by other inflammatory mediators amplifies the inflammatory response (Schmidt *et al.*, 2001).

In addition to Aβ upregulating RAGE, data from this study indicates an age-related upregulation of RAGE. Similar results of upregulation of RAGE expression have been reported in human heart tissue from elderly subjects and correlate well with reduced heart function (Simm *et al.*, 2004). This upregulation in RAGE expression may be a consequence of the increased accumulation of AGEs in tissues including the heart that occurs with age (Casselmann *et al.*, 2004). Binding of RAGE to its ligands can result in the production of pro-inflammatory mediators such as COX-2 and prostaglandin E_2 (PGE_2) that have been implicated in the pathogenesis of

several inflammatory diseases (Shanmugam *et al.*, 2003). Prolonged activation of RAGE has been shown to result in downregulation of activated ERK in osteoblast cells (Cortizo *et al.*, 2003). Surprisingly Aβ failed to induce an upregulation in RAGE expression in hippocampus of aged rats in this study. One possibility may be that further upregulation of RAGE expression in hippocampus of aged rats may require synthesis of RAGE – a process that may not be consistent with the time frame of Aβ treatment in this study. Additionally, some studies have indicated that exposure to AGEs may not modify expression of RAGE but alter the affinity of binding sites for AGEs under certain conditions (Cortizo *et al.*, 2003).

Other mediators of microglial activation include CD40 and its ligand CD154 (Grewal and Flavell, 1998). CD40 is expressed on many cell types and the interaction of CD40 expressed on antigen-presenting cells with CD154 is critical for a productive immune response. CD40 expression is upregulated by pro-inflammatory cytokines IFNγ and IL-1β and appears to be cell specific. Data from this study indicate an upregulation of CD40 in adult rats in response to 60 and 200μM Aβ administration, however, 20μM Aβ had little effect. Previous findings indicate that IFNγ is the most potent inducer of CD40 in macrophages and microglia (Nguyen *et al.*, 1998; Nguyen *et al.*, 2000; Nguyen and Benveniste, 2000). This finding postulates a mechanism by which Aβ induces an increase in CD40 expression in hippocampus of adult rats. Indeed transgenic mice overexpressing APP show improved spatial memory when treated with an anti-CD40 antibody (Todd Roach *et al.*, 2004). Since CD40 expression and IL-1β concentration are not altered by administration of 20μM Aβ it may be reasonable to propose that IL-1β and IFNγ must work synergistically to induce an upregulation in CD40 expression.

In the present study, CD40 expression was also increased with age however, no further increase occurred in hippocampus of aged rats treated with Aβ. Again this result may imply that IL-1β and IFNγ work synergistically to induce an increase in CD40 expression. These data support the idea that IL-1β and IFNγ may co-stimulate upregulation of CD40 expression since CD40 was only upregulated in situations where both IL-1β and IFNγ were increased e.g. in 60 and 200μM adult rats and in

aged control rats but not in 20μM Aβ-treated adult and aged rats in which either IL-1β or IFNγ was not increased.

Evidence from this laboratory has reported that impairments in LTP induced by LPS (Vereker *et al.*, 2000a) and stress (Vereker *et al.*, 2001) are accompanied by increases in IL-1β concentration in hippocampus of rats. Among the downstream effects of pro-inflammatory cytokines such as IL-1β is activation of JNK. IFNγ appears to operate in a co-stimulatory manner to induce activation of JNK (Rose *et al.*, 1997; Abreu Martin *et al.*, 1999; Chan and Riches, 2001). Aβ induced activation of JNK in hippocampus of adult rats – this result confirms findings from the initial study. This effect has been previously reported *in vitro* (Bozsycko-Coyne *et al.*, 2001; Morishima *et al.*, 2001; Troy *et al.*, 2001).

Data from this study reveal an age-related increase in JNK activation – reports from this laboratory support this finding (O'Donnell *et al.*, 2000; Vereker *et al.*, 2000; Lynch and Lynch, 2002) Interestingly JNK activation was not significantly increased in hippocampus of aged rats injected with Aβ in comparison to control-treated aged rats – this effect mirrors the effects of Aβ on RAGE and CD40 expression and consequently imply that JNK activation may occur downstream of RAGE and CD40 ligation. CD40 ligation of lymphocytes has been reported to result in activation of JNK (Brunner *et al.*, 2002; Ahmed-Choudhury *et al.*, 2003) and similarly ligation of RAGE has been shown to result in activation of the JNK signalling pathway in endothelial cells (Fiuza *et al.*, 2003; Murua Escobar *et al.*, 2003).

While the effects of age and Aβ on pro-inflammatory signalling seem to be somewhat similiar, the hippocampus of aged rats does not appear to undergo the same effects by Aβ as those induced in adult rats. This implies a dysregulation of some form of signalling that occurs with age. However, the effects of Aβ on IL-1β, IFNγ, RAGE and CD40 expression in hippocampus provide compelling evidence that not only do microglia become activated in response to Aβ stimulation but that neurons are capable of producing inflammatory cytokines in response to Aβ.

In an effort to determine whether prevention of activation of microglia may abrogate the Aβ-induced effects, primary cortical glia were treated with Aβ in the presence of minocycline hydrochloride which is a potent inhibitor of microglial

activation. The results from this study indicated that IL-1β release was increased from glia treated with Aβ but prevented by minocycline hydrochloride. Similarly, minocycline has been shown to modulate the function of reactive glia (Zhang *et al.*, 2003). Indeed the neuroprotective effect of minocycline has been reported to occur as a result of inhibition of iNOS and caspase-1 expression in a mouse model of Parkinson's disease (Du *et al.*, 2001). These data suggest that Aβ induces an inflammatory response mediated by IL-1β, released from either neurons or glia, that mediates the subsequent impairment in LTP.

A number of recent studies have identified that n-3 polyunsaturated fatty acids, (PUFAs) possess neuroprotective properties (Babcock *et al.*, 2000; Moon and Pestka, 2003). In addition to their neuroprotecitve effects against insult, PUFAs appear to be required for neuronal function. In animal models n-3 fatty acid deficiency causes memory deficits (Gamoh *et al.*, 1999), learning disabilities (Yoshida *et al.*, 1997) and visual acuity loss (Neuringe, 2000). n-3 fatty acid deficiency is associated with various neurological disease states in humans (Martinez 1992; Hoffman and Birch, 1998) lending further support to the essential role of n-3 fatty acids in neuronal function. Preterm infants with underdeveloped brains show improved visual attention on inclusion of n-3 fatty acids in their formula (Birch *et al.*, 1998). Additionally n-3 fatty acids are required for the survival of rat retinal photoreceptors (Rotstein *et al.*, 1997) and exert a protective effect on apoptosis of retinal photoreceptors during development (Rotstein *et al.*, 1996).

Consumption of PUFAs in fish oil-containing diets has been associated with modulation of a diverse array of immunological events that include suppression of lymphocyte proliferation, antigen presentation, MHC II expression, cytotoxic T lymphocyte activity, NK cell activity, macrophage-mediated cytotoxicity and neutrophil/monocyte chemotaxis (Wu and Meydani, 1998; Kehn and Fernandes, 2001). These effects have been the rationale for experimental and clinical attenuation of acute inflammatory, delayed-type hypersensitivity, autoimmune and transplant rejection responses by n-3 PUFAs (Otto *et al.*, 1990; Taki *et al.*, 1992; Harbige and Fisher, 2001; Ergas *et al.*, 2002). The mechanisms for these effects may be related to the capacity of n-3 PUFAs to modulate inflammatory mediator profiles and

concentrations as well as immune cell populations by altering apoptotic signalling, cell-to-cell communication and signal transduction (Meydani *et al.*, 1991; von Schacky, 1996; Jolly *et al.*, 2001). Given the wealth of data on the neuroprotective actions of n-3 fatty acids, it seemed reasonable to assess the ability of one such fatty acid, eicosapentaenoic acid (EPA), to protect against Aβ-induced cellular effects in hippocampus.

The attenuation of both early and later responses of perforant path-granule cell synapses to tetanic stimulation in adult rats treated with 60 and 200μM Aβ provides further confirmation of findings previously reported both in this study and in the literature (Cullen *et al.*, 1997; Freir *et al.*, 2001). Significantly however, it was found that prior treatment with EPA prevented the inhibitory effect of Aβ administration so that LTP was sustained in a manner similar to the vehicle-treated cohorts. Similarly EPA prevented the age-related deficit in LTP and the Aβ-induced inhibition of LTP in hippocampus of aged rats. Given the fundamental importance of increased IL-1β concentration in inhibiting LTP, it was not surprising to find that EPA prevented the Aβ-induced increase in IL-1β in hippocampus of adult and aged rats and returned IL-1β concentration in hippocampus of vehicle-treated aged rats to values similar to those reported in hippocampus of vehicle-treated adult rats. Similarly, EPA prevented Aβ-induced and age-related increases in IFNγ concentration. Significantly, this is the first report for a protective effect of EPA in neuronal tissue *in vivo* following Aβ administration.

EPA abrogates LPS-induced (Lonergan *et al.*, 2004) and γ-irradiation-induced (Lonergan *et al.*, 2002) inhibition of LTP. Several reports have indicated that the reported increase in glutamate release may be a significant contributory factor in expression of LTP in dentate gyrus (Bliss and Collingridge, 1993; Lynch, 1998; McGahon *et al.*, 1999a, 1999b, 1999c). Interestingly it has been reported that IL-1β inhibits glutamate release *in vitro* (Vereker *et al.*, 2001). These findings are consistent with the idea that enhanced glutamate release may be one element leading to the successful expression of LTP, at least in dentate gyrus.

It has been reported that age-related deficits in LTP and accompanying changes in IL-1β concentration are reversed by prior treatment with n-3 PUFAs

(Martin *et al.*, 2000; O'Donnell *et al.*, 2000). Similarly, EPA has been shown to reverse the age-related increase in IL-1β concentration and restore LTP in hippocampus of aged rats (Martin *et al.*, 2002). Dietary n-3 PUFAs decrease synthesis of TNFα and IL-1β in mononuclear cells (Endres *et al.*, 1989; Meydani *et al.*, 1991; Caughey *et al.*, 1996). Previous studies have indicated that EPA can inhibit IL-1β production in human monocytes *in vitro* (Baldie *et al.*, 1993), in rodent macrophages (Yaqoob and Calder, 1995, Wallace *et al.*, 2000) and also in mice following intraperitoneal administration of LPS (Sadeghi *et al.*, 1999). Circulating levels of IFNγ have been reported to be decreased in patients receiving EPA following bone marrow transplantation (Takatsuka *et al.*, 2001) and the reported modulatory function of EPA on NK cell activity may underpin the ability of EPA to suppress IFNγ production in hippocampus in response to Aβ administration. Indeed dietary supplementation with other n-3 fatty acids has been shown to reduce IL-1β, IFNγ and IL-6mRNA in a contact hypersensitivity model in mice *in vivo* (Tomobe *et al.*, 2000) and in human lymphocytes *in vitro* (Purasiri *et al.*, 1997).

Ageing is a complex process involving many factors including downregulation of enzyme activity and increased membrane rigidity and consequently it is probable that the ability of EPA to restore LTP in hippocampus of aged rats does not reside with any one of these factors. Significantly higher levels of cholesterol and decreased concentration of total PUFAs in neuronal membranes contribute to deficits in neuronal function with age (Yehuda *et al.*, 2002). Interestingly, cholesterol concentration of neuronal membranes in hippocampus of aged rats has been reported to be increased and is attenuated by treatment with PUFAs (S. Little and A. Nicolai, personal communication). Rigidity of neuronal membranes increases with increasing membrane concentration of cholesterol (Angelie *et al.*, 2001) – hippocampus, cerebellum and cortex being most affected (Terracina *et al.*, 1992; Ohyama *et al.*, 1995).

Normal physiological functioning of neuronal membranes is highly dependent on their structure. Complex learning in aged rats results in decreased concentration of membrane cholesterol and decreased membrane rigidity in hippocampus and frontal cortex (Kessler *et al.*, 1985). Most studies report decreases in

PUFA composition and turnover especially in hippocampus, cortex, striatum and hypothalamus with age (Pu *et al.*, 1999; Ulmann *et al.*, 2001). Age-related loss of membrane PUFAs is believed to occur as a result of the low rate of penetration of PUFA from blood to brain and impaired biochemical machinery that incorporates and elongates fatty acids (Yehuda *et al.*, 2002). Structural changes in blood-brain barrier in ageing and AD have been demonstrated (de la Torre and Mussivand, 1993; Ginsberg *et al.*, 1998) and some studies have indicated that biochemical machinery e.g. desaturase activity is different in adult and aged rats (Terracina *et al.*, 1992). These same studies have shown that incorporation of fatty acids into the membrane is inhibited and turnover rate is slow. Additionally the activity of desaturases and other elongating enzymes is reduced with age (Kumar *et al.*, 1999) therefore supplementation with EPA may bypass the age-related decline in desaturase activity and provide direct supplementation for membrane composition. It is clear from several studies that members of the n-3 family of PUFAs can enter the brain from the blood (Pawlosky *et al.*, 1996, 1997; Greiner *et al.*, 1997; Ward *et al.*, 1998, 1999; Innis, 2000).

Babcock and colleagues (2000) have proposed that EPA treatment may reverse age-related increases in IL-1β concentration through substitution of EPA for arachidonic acid (AA) in phospholipids reducing the production of cytokines such as IL-1β. Production of pro-inflammatory cytokines can occur through the phospholipase A_2-dependent liberation of AA from phospholipids; this may result in the cyclooxygenase-dependent conversion of the resultant free AA to PGE_2. PGE_2 has the ability to stimulate cytokine production and therefore it was argued that substitution of EPA for AA might reduce PGE_2 activity through reduction of free AA (Babcock *et al.*, 2000). Thus, the effects of EPA on membrane composition and IL-1β production may explain the ability of EPA to prevent Aβ-induced and age-related impairments in LTP.

Since the MAP kinase family has been identified as a significant player in cellular signalling, and data from this study implicated JNK as having a pivotal role in Aβ-induced deterioration of neuronal function, it was appropriate to assess the effect of EPA on JNK activity. Nuclear expression of phosphorylated JNK was

unaffected by Aβ administration however upon translocation of JNK to the nucleus it is unclear whether it is degraded or returns to the cytoplasm (Kallunki *et al.*, 1996). Therefore it is possible in the time frame of this experiment there was not sufficient time for translocation of JNK or alternatively that JNK on interacting with substrates in the nucleus was degraded or returned to the cytoplasm. Significantly cytosolic expression of phosphorylated JNK was increased following Aβ administration. This result supports the previous findings in this study, which indicated that JNK phosphorylation was upregulated in response to Aβ. Of particular interest was that EPA abrogated the Aβ-induced activation of JNK and that EPA restored LTP following Aβ administration. Similar results have been reported from this laboratory in which, LPS-induced activation of JNK is prevented by EPA (Lonergan *et al.*, 2004). Moreover, EPA has been shown to reverse the age-related increase in JNK activity (Martin *et al.*, 2002). These data taken with those of the previous study in which inhibition of JNK with DJNKI1 attenuated apoptotic changes induced by Aβ *in vitro* identify a key role for JNK in Aβ-induced impairment of LTP and associated cellular deterioration.

The anti-inflammatory properties of IL-4 have been described both *in vivo* and *in vitro.* IL-4 attenuates activation of a number of immune cells including neutrophils, monocytes and macrophages, by limiting the production of pro-inflammatory cytokines (Standiford *et al.*, 1990; te Velde *et al.*, 1990; Wertheim *et al.*, 1993). Interestingly, IL-4 antagonises many of the actions of the pro-inflammatory cytokine, IFNγ, through inhibition of Th1-cell differentiation (Callard *et al.*, 1991; Chatelain *et al.*, 1992; Maggi *et al.*, 1992; Mosmann and Sad, 1996). Evidence from this laboratory has indicated that LPS-induced inhibition of LTP is accompanied by a decrease in IL-4 concentration in hippocampus (Kavanagh *et al.*, 2004) an effect that is attenuated by EPA. Similarly, IL-4 concentration has been shown to be decreased in hippocampus of aged rats that failed to sustain LTP (Maher *et al.*, 2004). In the present study *ex vivo* analysis of IL-4 concentration in hippocampal tissue from adult rats 3 hours after central injection of Aβ revealed no effect of Aβ on IL-4 concentration. While IL-4 has proven to be protective against Aβ insult (Chao *et al.*, 1994) no reports have been made on modulation of the activation of this cytokine by Aβ. Downregulation of IL-4

concentration in transgenic mice overexpressing APP has been reported however, this effect may result from reduced levels of the neuroprotective sAPPα rather than directly from Aβ-induced neurotoxicity (Abbas *et al.*, 2002).

Results from the present study show a small but not statistically significant age-related decrease in IL-4 concentration – an effect attenuated by treatment with EPA. These data suggest that in addition to a parallel increase in IL-1β-induced signalling in aged rats, downregulation of IL-4 may contribute to the age-associated impairment of LTP. Downregulation of IL-4 concentration in hippocampus of aged rats has been reported (Maher *et al.*, 2004). IL-4 has been shown to inhibit IL-1β-mediated increase in caspase-3 activity in cultured hippocampal neurons (F.O. Maher, personal communication). IL-4 attenuates the LPS- and IL-1β-induced inhibition of LTP and subsequent signalling events (Y.A. Nolan and M.A. Lynch, personal communication). Additionally, IL-4 may mediate its protective effects through inhibition of IFNγ-associated signalling since IFNγ has been shown to upregulate genes associated with inflammation (Paludan, 1998). Taken together these results indicate that EPA may only affect IL-4 concentration if it is downregulated such as occurs with age and LPS administration.

PUFAs are considered to be the primary endogenous ligands leading to activation of peroxisome proliferator-activated receptors (PPARs) and because of their anti-inflammatory effects it was considered that EPA may exert its protective effects through these receptors. PPARs regulate many cellular and metabolic processes and are transcription factors belonging to the ligand-inducible nuclear receptors (Wahli, 2002). Three isotypes have been identified (PPARα, PPARβ and PPARγ) in lower vertebrates and mammals and these display differential tissue distribution. While little is known about the main function of PPARβ, it seems that PPARα and PPARγ control energy homeostasis and inflammatory responses. Both of these have been implicated in chronic conditions such as diabetes, obesity and atherosclerosis. While PPARα is expressed primarily in liver, heart, muscle and kidney PPARγ is enriched in adipocytes and macrophages and can be activated by the thiazolidinedione class of anti-diabetic drugs (Forman *et al.*, 1995; Kliewer *et al.*, 1995; Lehmann *et al.*, 1995).

In this study, PPARγ expression was found to be downregulated by Aβ in hippocampus of adult rats. While no such effects of Aβ on PPARγ have been reported PPARγ agonists have been shown to attenuate Aβ-induced secretion of pro-inflammatory products from microglia (Combs *et al.*, 2000). Although, Aβ has been shown to increase PPARγ activity of human glioma cells, expression of PPARγ was unaltered by Aβ treatment *in vitro.* In the present study, PPARγ expression was decreased in hippocampus of aged rats – reports of reduced abundance of PPARγ in preadipocytes with age have also been made (Kirkland *et al.*, 2002). Similarly, decreased activity of PPARγ has been reported in myoblasts from aged rats compared to myoblasts isolated from adult rats (Taylor-Jones *et al.*, 2002). Of interest is the finding that IL-1β and LPS inhibited differentiation of brown adipose tissue and that this effect was accompanied by a downregulation in PPARγ expression (Mracek *et al.*, 2004). This data indicates that PPARγ expression may be downregulated in situations where IL-1β concentration is increased and that stressors such as Aβ may prevent attenuation of the inflammatory response by endogenous proteins by stimulating their downregulation. In support of this argument is the finding that PPARγ agonists protect against IL-1β-stimulated production of PGE_2 (Cheng *et al.*, 2004). Interestingly, JNK has been shown to decrease the transcriptional activity of PPARγ by phosphorylation at a serine residue (Camp *et al.*, 1999). This finding suggests that pro-inflammatory cytokines such as IL-1β may downregulate PPARγ activity through phosphorylation by JNK.

Findings from this study indicate that EPA is able to attenuate the Aβ-induced and age-related decreases in PPARγ expression. This finding is of significance since it proposes that EPA's suppressive effects on pro-inflammatory signalling may result from increased expression of PPARγ and subsequent activation. EPA upregulates PPARγmRNA in isolated adipocytes and strong correlations between plasma EPA concentrations and PPARγmRNA expression exist in adipose tissue (Chambrier *et al.*, 2002). Recently it has been proposed that the anti-inflammatory actions of PPARγ may be mediated, in part, by the ability of this receptor to induce the expression of Inhibitor (I)-kB (Delerive *et al.*, 2000). The presence of high levels of IkB would serve to block translocation of nuclear factor

(NF)-kB to the nucleus and subsequently act to inhibit pro-inflammatory gene expression. PPARγ acts to prevent AP-1 binding to promoter regions of the COX-2 gene though two independent mechanisms. Firstly, PPARγ ligands block the expression of the c-Jun gene preventing the formation of a functional AP-1 complex (Subbaramaiah *et al.*, 2001). Secondly, PPARγ associates with co-activators of this complex and precludes interaction of the co-activators with the AP-1 complex (Subbaramaiah *et al.*, 2001). These findings comprise evidence for the involvement of PPARγ in the mechanism of the neuroprotective action of EPA.

Agonists of PPARγ promote lipid accumulation and also suppress cytokine-induced macrophage activation (Jiang *et al.*, 1998; Nagy *et al.*, 1998; Tontonoz *et al.*, 1998; Ricote *et al.*, 1999). Indeed prostaglandin (PG)-J_2 and anti-diabetic thiazolidinediones (TZDs) can inhibit LPS- or IFNγ-induced macrophage activation (Jiang *et al.*, 1998; Ricote *et al.*, 1999) – this inhibiting action results in suppression of iNOS and a failure to release inflammatory cytokines such as TNFα, IL-1β and IL-6. It has been suggested that PPARγ activation interacts with signalling pathways involving STAT1, Ap-1 and NFkB to block their downstream target genes. Since these reports PPARγ agonists have been shown to inhibit inflammatory responses in other cell types including endothelial cells, smooth muscle cells and T lymphocytes (Yang *et al.*, 2000; Plutzky, 2001). However, the anti-inflammatory properties of PPARγ agonists TZDs and PGJ_2 at high concentrations may not require PPARγ since PGJ_2 and TZDs managed to block LPS-stimulated production of cytokines from macrophages (Chawla *et al.*, 2001; Moore *et al.*, 2001).

Data from the present study show a suppressive effect of the PPARγ agonist troglitazone on Aβ-induced IL-1β secretion from glia *in vitro*. This finding has been reported elsewhere with troglitazone attenuating the Aβ-stimulated release of IL-6 and TNFα *in vitro* (Combs *et al.*, 2000). Very few studies have investigated the actions of PPARγ agonists on neurons. Treatment of primary cerebellar granule cell cultures with LPS and IFNγ resulted in induction of iNOS expression and subsequent apoptotic death of neurons arising from the action of NO and its immediate metabolites (Heneka *et al.*, 2001). PPARγ agonists prevented iNOS expression and enhanced neuronal survival was observed (Heneka *et al.*, 2001). These studies have

been extended to animal models where injection of LPS and IFNγ resulted in induction of neuronal iNOS and death of neurons at the sites of injection. Simulataneous injection of PPARγ agonists such as troglitazone attenuated this effect (Heneka *et al.*, 2000).

Findings from the current study suggest that increases in pro-inflammatory cyokines in hippocampus are induced by Aβ, occur with age and are accompanied by impairment of LTP. Significantly, EPA has the ability to abrogate the Aβ-induced and age-related increases in IL-1β concentration and to restore LTP in both of these conditions. Compelling evidence *in vitro* has implicated IL-1β in activation of the stress-activated protein kinase, JNK. Consequently it is reasonable to suggest that activation of JNK is a crucial step underlying the impairments in LTP observed in this study. Recent findings in this laboratory indicate that inhibition of JNK attenuates LPS-induced inhibition of LTP (C.E. Barry and M.A. Lynch) and data from the current study indicate that inhibition of JNK abrogates the downstream signalling events stimulated by Aβ *in vitro.*

JNK activation leads to cell death in a number of cell types (Mielke and Herdegen, 2000). It is likely that the subsequent cell loss is evidenced by an impairment of synaptic function, specifically the impairment identified here in LTP. EPA prevented the Aβ-induced and age-related deficit in LTP and its primary effect in restoring LTP under these conditions may result from its capacity to suppress pro-inflammatory cytokines IL-1β and IFNγ.

The initial hypothesis that an increase in IL-1β concentration and IL-1β-induced signalling mediate Aβ-induced neurotoxicity and enhance susceptibility of hippocampus of aged rats to Aβ seems reasonable since Aβ had a significantly greater effect on both LTP and IL-1β concentration in these circumstances. EPA supplementation successfully attenuated Aβ-induced and age-related changes associated with IL-1β and its subsequent signalling, highlighting the importance of n-3 fatty acids in neuronal function.

There is compelling evidence that IL-1β underpins the deleterious effects of both Aβ and age. While this study furthers our understanding of the role of pro-

inflammatory cytokines in hippocampus several important questions arise from the resulting data:

1. How Aβ increases IL-1β in hippocampus still remains to be established. Currently, it is believed that Aβ stimulates microglia to secrete pro-inflammatory cytokines but the underlying mechanisms of Aβ-induced microglial activation are unclear.

2. How age-related increases in pro-inflammatory signalling occur remains to be resolved. Alterations in membrane structure and function occur with age but the primary mechanism resulting in enhanced IL-1β concentration in hippocampus is yet to be identified.

3. Aβ (20μM) induces inhibition of LTP in hippocampus of aged rats while having a minimal effect on LTP in hippocampus of adult rats. However, apart from IL-1β, all other parameters investigated show no increased activation in hippocampus of aged rats subsequent to Aβ administration. Other signalling pathways must be involved in the Aβ-induced impairment of LTP in hippocampus of aged rats observed here.

4. The underlying mechanisms of the neuroprotective action of EPA remain unknown. While EPA appears to suppress pro-inflammatory cytokine production and in some cases promote an anti-inflammatory effect how it achieves these effects is not clear.

Figure 4.1 Suggested scheme of events leading to Aβ and age-associated impairment of LTP

Intracerebroventricular administration of Aβ induces increases in IL-1β and IFNγ, which upon binding to their receptors, leads to activation of JNK. Age-related increases in IL-1β and IFNγ concentration may be responsible for the upregulated activity of JNK observed with age. Activation of JNK by Aβ and age leads to apoptotic cell death as characterised by cytochrome c release, caspase-3 activation, PARP cleavage, c-Jun phosphorylation and impairment in LTP in dentate gyrus. It is postulated that the ability of EPA to block the increased IL-1β and IFNγ concentrations is responsible for the effect of EPA observed downstream.

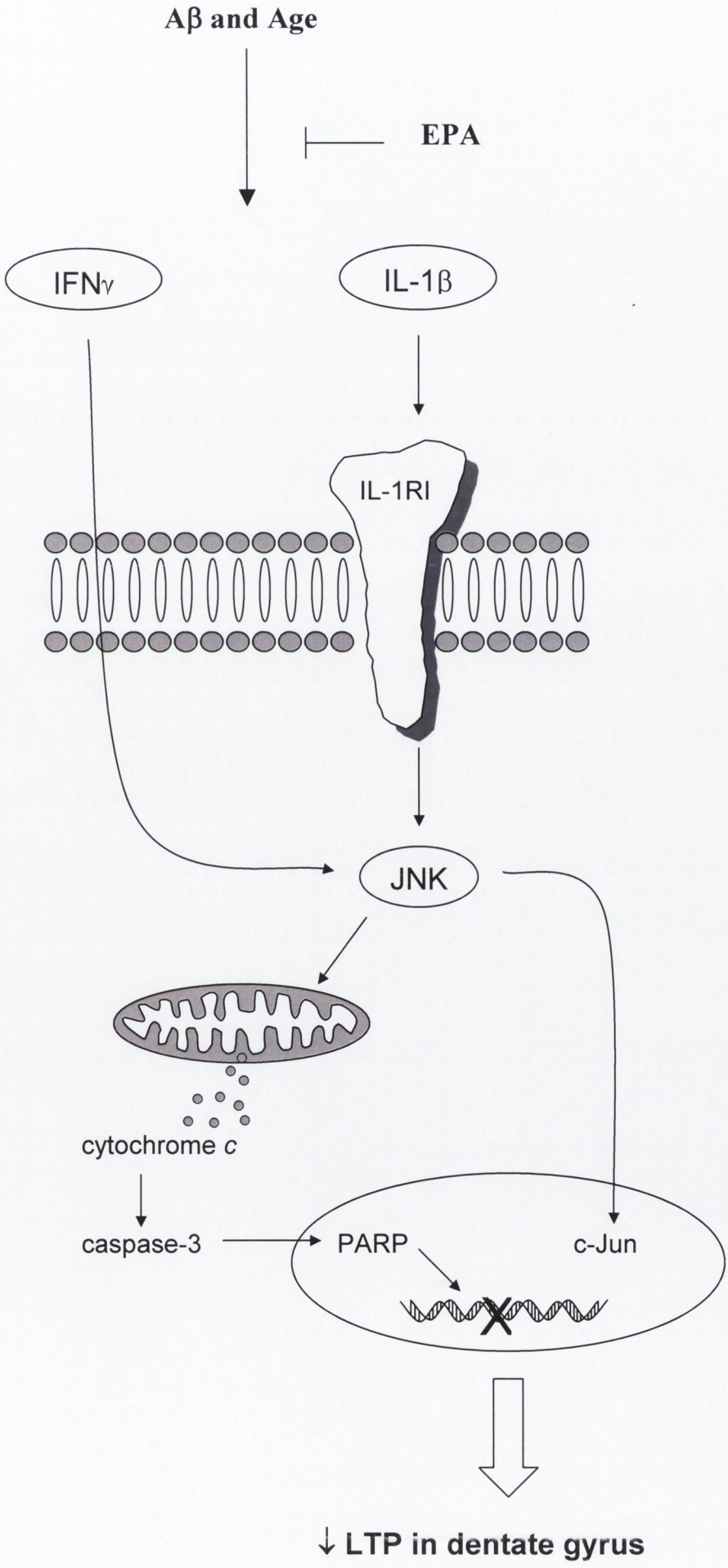

Aβ and Age
EPA
IFNγ
IL-1β
IL-1RI
JNK
cytochrome c
caspase-3
PARP
c-Jun
↓ LTP in dentate gyrus

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Table 1A. The effect of Aβ administration in hippocampus (values are expressed as means ± standard error of the means).

	Vehicle-treated	**Aβ-treated**	**Units**
IL-1β	132.80 ± 11.28	215.70 ± 17.65	pg/mg
pJNK	8.32 ± 1.58	11.84 ± 3.34	Arbitrary Units
JNK1	2.51 ± 0.46	2.73 ± 0.34	
p-c-Jun	2.98 ± 0.11	5.22 ± 0.79	
Bax	5.51 ± 0.12	9.43 ± 1.14	
cytochrome *c*	3.62 ± 0.02	5.08 ± 0.53	
PARP	5.37 ± 0.30	3.94 ± 0.56	
FasL	3.32 ± 0.04	5.36 ± 0.43	

Table 1B. The effect of Aβ and caspase-1 inhibition *in vitro* in cultured cortical neurons (values are expressed as means ± standard error of the means).

	Control	**Aβ**	**Caspase-1 inhibitor**	**Aβ + caspase-1 inhibitor**	**Units**

IL-1β	3.99 ± 0.72	7.13 ± 0.54	4.5 ± 1.4	2.74 ± 0.83	pg/ml

Table 1C. The effect of IL-1β and DJNKI1 *in vitro* on cultured cortical neurons (values are expressed as means ± standard error of the means).

	Control	**IL-1β**	**IL-1β + DJNKI1**	**DJNKI1**	**Units**
c-Jun	3.19 ± 0.22	4.88 ± 0.46	3.71 ± 0.3	3.32 ± 0.02	Arbitrary Units
FasL	3.43 ± 0.21	5.12 ± 0.26	3.87 ± 0.51	3.63 ± 0.40	

Table 2A. The effect of Aβ administration and age on LTP in the first 2 min (0-2 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means ± standard error of the means of 5 or 6 observations.

3 months				14 months		24 months		
Vehicle	**Aβ 20μM**	**Aβ 60μM**	**Aβ 200μM**	**Vehicle**	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Units**
140.00 ± 5.62	143.90 ± 10.57	117.89 ± 3.15	118.91 ± 4.70	153.54 ± 15.44	123.90 ± 3.54	133.735 ± 4.80	120.89 ± 6.74	EPSP slope (%)

Table 2B. The effect of Aβ administration and age on LTP in the last 5 min (0-2 min) of recording post tetanic stimulation (compared with the 5 min period immediately preceding tetanus). Values are expressed as means ± standard error of the means of 5 or 6 observations.

3 months				14 months		24 months		
Vehicle	**Aβ 20μM**	**Aβ 60μM**	**Aβ 200μM**	**Vehicle**	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Units**

132.96 ± 9.36	116.79 ± 6.96	95.18 ± 3.70	95.19 ± 3.81	113.39 ± 2.20	94.68 ± 4.03	116.33 ± 7.57	103.21 ± 3.26	EPSP slope (%)

Table 2C. The effect of Aβ administration and age in hippocampus

	3 months				14 months		24 months		
	Vehicle	**Aβ 20μM**	**Aβ 60μM**	**Aβ 200μM**	**Vehicle**	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Units**

IL-1β	11.33 ± 3.81	12.60 ± 2.50	22.00 ± 7.26	26.25 ± 3.06			27.50 ± 2.40	55.67 ± 12.12	pg/mg
IFNγ	144.80 ± 71.82	416.72 ± 44.89	462.06 ± 68.52	518.34 ± 47.03	443.89 ± 61.12	439.88 ± 21.14	354.67 ± 39.25	373.77 ± 25.90	
RAGE	9.34 ± 0.16	14.68 ± 1.65	16.72 ± 1.52	13.66 ± 1.10	14.70 ± 0.37	13.78 ± 2.04	13.30 ± 0.82	16.12 ± 1.81	Arbitrary Units
CD40	14.00 ± 0.72	17.59 ± 1.46	21.33 ± 1.72	23.27 ± 2.41	22.95 ± 2.41	21.98 ± 2.21	20.53 ± 2.00	17.62 ± 1.02	
pJNK	5.59 ± 0.25	6.94 ± 0.43	7.00 ± 0.22	7.41 ± 0.23	7.81 ± 0.35	7.58 ± 0.45	6.67 ± 0.28	7.04 ± 0.90	

Table 2D. The effect of Aβ on cultured cortical neurons and glia

	Neurons		**Glia**		
	Control	**Aβ**	**Control**	**Aβ**	**Units**
IL-1β	4.47 ± 0.70	7.13 ± 0.55	35.92 ± 7.17	65.56 ± 10.39	pg/ml

Table 2E. The effect of Aβ and minocycline hydrochloride *in vitro* in cultured cortical glia (values are expressed as means ± standard error of the means).

	Control	Aβ	Aβ + minocycline hydrochloride	Minocycline hydrochloride	Units
Il-1β	105.05 ± 26.51	308.66 ± 42.47	216.14 ± 32.19	240.33 ± 97.77	

Table 3A. The effect of Aβ administration and dietary supplementation with eicosapentaenoic acid (EPA) on LTP 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 ± standard error of the means of 5 or 6 observations.

Control						EPA						Units
Vehicle		Aβ 60μM		Aβ 200μM		Vehicle		Aβ 60μM		Aβ 200μM		
0-2 min	*40-45 min*	*0-2 min*	*40-45 min*	*0-2 min*	*40-45 min*	*0-2 min*	*40-45 min*	*0-2 min*	*40-45 min*	*0-2 min*	*40-45 min*	
129.04 ± 3.12	137.01 ± 13.19	132.29 ± 12.65	109.19 ± 11.02	115.09 ± 10.59	107.21 ± 5.57	158.20 ± 3.83	138.72 ± 9.57	132.14 ± 7.77	131.28 ± 4.83	140.01 ± 2.03	109.39 ± 9.58	EPSP slope (%)

Table 3B. The effect of Aβ administration and dietary supplementation with eicosapentaenoic acid (EPA) on LTP 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) in middle-aged and aged rats. Values are expressed as means ± standard error of the means of 5 or 6 observations.

14 months								24 months								
Control				EPA				Control				EPA				
Vehicle		Aβ		Vehicle		Aβ		Vehicle		Aβ		Vehicle		Aβ		Units
0-2min	*40-45min*	*0-2 min*	*40-45min*	*0-2min*	*40-45min*	*0-2 min*	*40-45min*	*0-2min*	*40-45min*	*0-2 min*	*40-45min*	*0-2min*	*40-45min*	*0-2 min*	*40-45min*	EPSP slope (%)
139.70 ± 5.06	122.83 ± 10.52	142.33 ± 6.93	113.17 ± 8.39	138.61 ± 7.51	112.54 ± 5.34	143.71 ± 6.17	125.72 ± 5.61	129.42 ± 6.04	113.66 ± 8.52	125.24 ± 7.12	99.19 ± 5.03	141.89 ± 4.34	122.96 ± 3.78	138.84 ± 6.07	128.16 ± 5.76	

Table 3C. The effect of Aβ administration and dietary supplementation with eicosapentaenoic acid (EPA) in hippocampus of adult rats. Values are expressed as means ± standard error of the means of 5 or 6 observations.

	Control			EPA			
	Vehicle	**Aβ 60μM**	**Aβ 200μM**	**Vehicle**	**Aβ 60μM**	**Aβ 200μM**	**Units**
IL-1β	264.53 ± 4.76	485.23 ± 73.88	445.68 ± 39.84	283.58 ± 24.03	308.33 ± 33.31	339.73 ± 31.59	pg/mg
IFNγ	352.00 ± 51.72	963.75 ± 262.25	770.25 ± 213.63	436.79 ± 28.67	496.93 ± 109.09	525.96 ± 140.08	
IL-4	26.74 ± 4.29	26.19 ± 3.86	23.29 ± 4.65	23.88 ± 2.58	24.13 ± 3.65	24.93 ± 4.07	
Cytosolic JNK	14.84 ± 2.33	26.68 ± 2.09	26.70 ± 3.90	20.05 ± 0.54	18.12 ± 2.04	6.83 ± 0.34	Arbitrary Units

Nuclear JNK	1.40 ± 0.02	1.58 ± 0.09	1.85 ± 0.16	1.64 ± 0.27	1.73 ± 0.29	1.82 ± 0.38	
PPARγ	2.60 ± 0.12	1.89 ± 0.30	1.51 ± 0.34	2.54 ± 0.53	2.41 ± 0.48	2.65 ± 0.43	

Table 3D. The effect of Aβ administration and dietary supplementation with eicosapentaenoic acid (EPA) on LTP 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) in middle-aged and aged rats. Values are expressed as means ± standard error of the means of 5 or 6 observations.

	14 months				**24 months**				
	Control		**EPA**		**Control**		**EPA**		
	Vehicle	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Vehicle**	**Aβ 20μM**	**Units**
IL-1β	374.73 ± 26.20	355.37 ± 14.44	333.32 ± 21.04	354.59 ± 32.62	393.49 ± 29.27	575.43 ± 38.74	331.86 ± 12.83	415.51 ± 57.00	
IFNγ	438.91 ± 33.82	495.60 ± 71.19	512.94 ± 55.02	549.94 ± 75.88	600.14 ± 56.28	621.58 ± 127.83	436.62 ± 41.15	519.74 ± 22.01	pg/mg
IL-4	-	-	-	-	19.69 ± 2.49	20.97 ± 3.84	29.00 ± 4.66	21.38 ± 2.36	
PPARγ	-	-	-	-	1.85 ± 0.62	1.87 ± 0.70	3.22 ± 0.86	3.36 ± 0.93	Arbitrary Units

Table 3.E The effect of Aβ and troglitazone *in vitro* in cultured cortical glia (values are expressed as means ± standard error of the means).

	Control	**Aβ**	**Aβ + troglitazone**	**Troglitazone**	**Units**
IL-1β	126.82 ± 20.58	283.57 ± 27.38	128.93 ± 18.88	204.17 ± 32.52	

Appendix II. Addresses

Alexis	Alexis Corporation (UK) Ltd., P.O. Box 6757, Bingham, Nottingham NG13 8LS, United Kingdom.
Amersham	Amersham Biosciences UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, United Kingdom.
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD, United Kingdom.
Calbiochem	Calbiochem-Novabiochem Corp., 10394 Pacific Center Court, San Diego, CA 92121, USA.
Chemicon	Chemicon International Inc., 28820 Single Oak Drive, Temecula, CA 92590,

	USA.
Laxdale	Laxdale Ltd., Kings Park House, Laurelhill Business Park, Polmaise Road, Stirling FK7 9JQ, United Kingdom.
Lennox	Lennox Laboratory Supplies, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.
Molecular Probes	Molecular Probes Europe B.V., PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.
Pierce	Pierce Biotechnology Inc., 3747 N. Meridian Road, P.O. Box 117, Rockford, IL 61105, USA.
R&D Systems	R&D Systems, 614 McKinley Place NE, Minneapolis,

	MN 55413, USA.
Red Mills	Red Mills Food Ltd., Goresbridge, Co. Kilkenny, Ireland.
Santa Cruz	Santa Cruz Biotechnology Inc., 2161 Delaware Avenue, Santa Cruz, CA 95060, USA.
Sigma	Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset BH12 4QH, United Kingdom.
Vector	Vector Laboratories Inc., 30 Ingold Road, Burlingame, CA 94010 USA
Whatman	Whatman plc, Whatman House, St. Leonard's Road, 20/20 Maidstone,

Kent ME16 0LS,

United Kingdom.

Appendix III. Solutions

The following solutions were used:

Electrode running buffer

Tris base, 25mM

Glycine, 192mM

SDS, 0.1% (w/v)

Krebs solution containing $CaCl_2$

NaCl, 136mM

KCl, 2.54mM

KH_2PO_4, 1.18mM

$MgSO_4$, 1.18mM

$NaHCO_3$, 16mM

Glucose, 10mM

Containing $CaCl_2$, 2mM

Lysis buffer, pH 7.4

HEPES, 20mM

KCl, 10mM

EGTA, 1mM

$MgCl_2$, 1.5mM

EDTA, 1mM

DTT, 1mM

PMSF, 0.1mM

Leupeptin, 2μg/ml

Aprotinin, 2μg/ml

Sucrose, 200mM

Mitochondrial Buffer

Tris-Base 50mM

NaCl 150mM

EGTA 2mM

EDTA 2mM

PMSF 0.1mM

Triton 0.2%,

Igepal P-40 0.3%,

leupeptin 10μg/ml

aprotinin 2μg/ml

Phosphate buffered saline (PBS), pH 7.4

Na_2HPO_4, 80mM

NaH_2PO_4, 20mM

NaCl, 100mM

Phosphate buffered saline, pH 7.3 for ELISA

NaCl, 137mM

KCl, 2.7mM

Na_2HPO_4, 8.1mM

KH_2PO_4, 1.5mM

Permeabilisation Buffer

sucrose 250mM

KCl 70 mM

NaCl 137mM

Na_2HPO_4 4.5mM

KH_2PO_4 1.4mM

PMSF 0.1mM

leupeptin 10μg/ml,

aprotinin 2μg/ml,

digitonin 200μg/ml

Sample buffer

Tris-HCl, 0.05M, pH 6.8

Glycerol 20% (v/v)

SDS 2% (w/v)

β-Mercaptoethanol 5% (v/v)

bromophenol blue 0.05% (w/v)

Stacking gel (4%), pH 6.8

Acrylamide/bis-acrylamide (30% stock), 13% (v/v)

dH_2O, 60% (v/v)

Tris-HCl, 0.05M, pH 6.8, 25% (v/v)

SDS (10% w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.5% (v/v)

Separating gel (12%), pH 8.8

Acrylamide/bis-acrylamide (30% stock), 40% (v/v)

dH_2O, 33% (v/v)

Tris-HCl, 0.05M, pH 6.8, 25% (v/v)

SDS (10% w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Separating gel (10%), pH 8.8

Acrylamide/bis-acrylamide (30% stock), 33% (v/v)

dH_2O, 40% (v/v)

Tris-HCl, 0.05M, pH 6.8, 25% (v/v)

SDS (10% w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Transfer buffer, pH 8.3

Tris base, 25mM

Glycine, 192mM

MeOH, 20% (v/v)

SDS, 0.05% (w/v)

Tris buffered saline (TBS), pH 7.4

Tris-HCl, 20mM

NaCl, 150mM

Appendix IV. Publications

Minogue, A.M., Schmid, A.W., Fogarty, M.P., Moore, A.C., Campbell, V.C., Herron, C.E. and Lynch M.A. (2003) Activation of the c-Jun N terminal Kinase signaling cascade mediates the effect of Amyloid-β on Long Term Potentiation *in vivo*: a role for Interleukin-1β? *Journal of Biological Chemistry*, **278**, 27971-27980.

Minogue, A.M. and Lynch, M.A. (2004) Endogenous interleukin-1beta synergises with beta-amyloid to block LTP in rat hippocampus. *FENS Abstracts*

Minogue, A.M., Herron, C.E. and Lynch, M.A. (2003) Age-related changes in endogenous Interleukin-1β may enhance susceptibility of rat hippocampus to beta-amyloid. *Society for Neuroscience Abstracts.* 584.14

Minogue, A.M., Kelly, A., Penney, L. and Lynch M.A. (2002) The effect of Interleukin-1β in hippocampus is enhanced when hippocampal Nerve Growth Factor concentration is decreased. *FENS Abstracts*.