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Analysis of the effect of different protocols of *amyloid-beta* treatment in the rat hippocampus

I. Declaration

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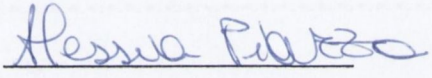

Alessia Piazza

II. Summary

Among the main characteristic features of AD is upregulation of inflammatory activities, increased production of pro-inflammatory cytokines and activation of microglia. Associated with dementia is the damage and loss of neurons in the hippocampus, a brain area involved in learning and memory processes. The evidence suggests that in conditions characterised by an underlying inflammation, such as in aging, the response to an inflammatory stimulus is exacerbated. The present study was undertaken to investigate the effect of different protocols of Aβ treatment in the rat hippocampus and to assess the possibility that age exacerbates the response to Aβ.

The results obtained from *in vitro* studies carried out on cultured glia demonstrate that treatment of glia with neurons reversed the LPS-induced microglial activation and suggest that LPS exerts its inflammatory activity, in part, by decreasing the expression of CD200R. Cotreatment of glia with LPS and Aβ induced a significant increase in IL-1β protein concentration which was not observed when either agent was used alone, suggesting an additive effect of LPS and Aβ.

The data obtained from *in vivo* work presented here indicate that acute icv injection of Aβ attenuated LTP and induced caspase-3 activity. Treatment with Aβ also induced up-regulation of some, but not all, of the markers of microglial activation such as iNOS and IL-1β. The effects of Aβ injection on LTP, caspase-3 activity and microglial activation were lost when Aβ was chronically infused for 28 days.

In a further experiment, rats were infused with Aβ and LPS, alone and in combination. The results demonstrate that chronic treatment with LPS significantly impaired LTP and induced MHCII and iNOS mRNA expression independent of Aβ. These findings do not support the hypothesis that LPS exacerbates the response to Aβ and rather suggest that LPS-primed microglia may be involved in Aβ clearance.

The evidence suggests that age is associated with impaired LTP and increased expression of some cell surface markers of microglial activation. The fact that some, but not all, of the markers of activated microglia were increased by

age and Aβ treatment suggests that microglial activation involves different states and that variation in the protocol of Aβ administration impacts on the toxicity of the peptide.

III. Acknowledgements

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Finally, thanks to my fiancé Tomaso whom this thesis is dedicated, for his support and for his patience.

Table of contents

V. List of figures

VI. List of abbreviations

aCSF	artificial cerebrospinal fluis
AD	Alzheimer's disease
APC	antigen presenting cell
ApoE	apolipoprotein E
APP	amyloid precursor protein
APP	amyloid-precursor protein
Aβ	amyloid beta
BACE	β-site APP-cleaving enzyme
BBB	blood-brain barrier
bp	base pair
CaMKII	Ca^{2+}/calmodulin-dependent protein
kinase II	
CD200R	CD200 receptor
CD40L	CD40 ligand
cDNA	copy DNA
CNS	central nervous system
COX	cycloxygenase
CSF	cerebral spinal fluid
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
ECL	enhanced chemiluminescence
EPSP	excitatory postsynaptic potential
FAD	familial AD
GFAP	glial fibrillar acidic protein
HFS	high-frequency stimulation
HPLC	high performance liquid chromatography
ICAM-1	intracellular adhesion molecule-1
ICE	IL-1β converting enzyme
Icv	intracerebroventricular
IEGs	immediately early genes

IFNγ	interferon-gamma
Ig	immunoglobulin
IL-10	interleukin-10
IL-12	interleukin-12
IL-18	interleukin-18
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
ip	intrperitoneal
LPS	lipopolysaccharide
LRP-1	low-density lipoprotein receptor-related protein
LTP	long term potentiation
MAPK	mitogen activated protein kinases
MHCII	major histocompatibility complex class II
mOD	millioptical density
mRNA	messenger ribonucleic acid
NBM	neurobasal medium
NF-κB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NSAIDs	non steroidal anti-inflammatory drugs
OD	optical density
PBS	phosphate buffered saline
PBS-T	PBS-Tween
PCR	polymerase chain reaction
PGE_2	prostaglandin E2

PI3K	phosphatidylinositol-3 kinase
PK	protein kinase
PPARγ receptor γ	peroxisone proliferator activated
PS	presenilin
RAGE	advanced glycation endproducts
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SNAP-25	synaptosome-associated protein 25 kDa
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SRs	scavenger receptors
STAT	signal transducers and activators of transcription
TBS	Tris buffered saline
TBS-T	TBS-Tween
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	Toll like receptor
TNF	tumor necrosis factor
v	volume
w	weight

Chapter 1

Introduction

1.1 The central nervous system

The central nervous system (CNS) includes the main information-processing organs of the nervous system, consisting of the brain and spinal cord. It is organized into different compartments consisting of the parenchyma proper, the ventricles and the meninges. The entire CNS is bathed both externally and internally by cerebrospinal fluid (CSF) which circulates through the ventricular spaces. The production and composition of the CSF are mainly regulated by the choroid plexus, a villous structure of blood vessels that protrudes into the ventricles. The outer surface of the CNS is surrounded by the triple-membrane system of the meninges.

The CNS has traditionally been regarded as an immunologically privileged organ because of a relatively impermeable blood brain barrier (BBB) formed by endothelial tight junctions, and an immunosuppressive microenvironment which, together, limit immune cell entry and function (Galea et al., 2007). This assumption implies that CNS has no intrinsic immune system of its own and is separated from the general immune system of the organism as long as the BBB is intact. However, immune-effector cells are present throughout the CNS and, in respond to immunological stimuli and neuronal damage, they release a number of physiological mediators known as chemokines which attract leucocytes, mainly $CD8^+$ and $CD4^+$ T cells, through the BBB to the site of injury (Babcock et al., 2003; Eugenin and Berman, 2003).

1.2 Hippocampus

The hippocampus, first labelled as such by Giulio Cesare Aranzi (1564) because of its similarity in shape to a seahorse (Greek: hippos= horse, kampi= curve), is a structure of the brain located in the medial temporal lobe which is crucial for storage and retrieval of certain types of information involved in spatial and recognition memory (Broadbent et al., 2004). The hippocampal formation consists of two main parts namely the hippocampus proper (CA1-CA3 pyramidal cell layers) also known as Ammon's horn and the dentate gyrus. Input to the hippocampus from the cortex via the entorhinal cortex represents the major cortical input to the hippocampus and allows the formation of memories as well as learning process. The entorhinal cortex ultimately receives information from all

sensory organs as well as from many areas of the cortex. Axons from this area project through the perforant path to the granule cells of the dentate gyrus (for review see see Miller and O'Callaghan, 2005).

There are several internal connections within the hippocampus that are essential for the transmission of information between different sub-regions, especially the circuit of dentate gyrus in conjunction with the CA1 and CA3 sub-regions. Neurons of the entorhinal cortex innervate the granule cells of the dentate gyrus which send mossy-fibers to the pyramidal cells of the CA3 region, which in turn synapse via the Schaffer collateral pathway with the CA1 neurons (see figure 1.1). Each of these sub-regions is specialised for different tasks involved in memory and spatial navigation (Gilbert et al., 2001; for review see Miller and O'Callaghan, 2005).

Until the late nineteenth century it was believed that there were direct physical connections between nerves and that impulses were transmitted from one nerve to another through direct physical connection. However, studies by Golgi and Ramón y Cajal proved evidence that nervous cells are close but not continuous and communicate with each other via specialized junctions, known as synapses (for review see Grant, 2007; De Carlos and Borrell 2007). It is now known thanks to the experiments of Otto Loewi, that chemical transmission is the main means by which nerves communicate with one another in the nervous system (for review see Davenport, 1991). The exocytosis of neurotransmitter-filled vesicles at the synapses represents the signaling mechanism that underlies the transmission of electrical activity from one neuron to another.

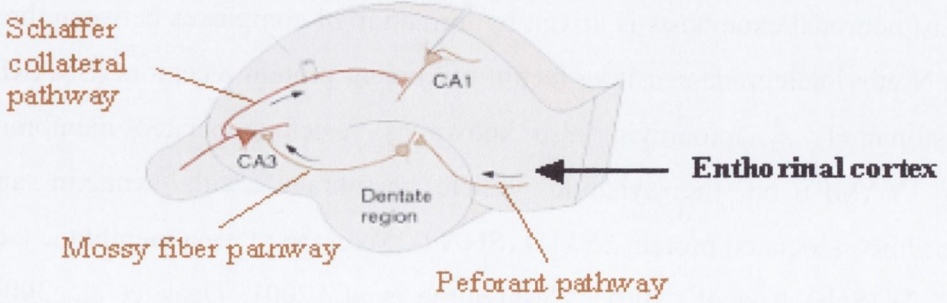


Figure 1.1 Afferent pathways of the hippocampus
(adapted from www.pitt.edu/~super1/lecture/lec14361/img023.GIF)

1.3 Intracellular trafficking of synaptic vesicles

For the purpose of analysis of the underlying mechanisms synaptic transmission can be considered in terms of pre- and postsynaptic components. The presynaptic neurons emit a signal via the regulated release of neurotransmitters and the postsynaptic cells perceive that signal by receptors that are localized precisely adjacent to the site of neurotransmitter release. Synapses are characterized by accumulation of synaptic vesicles and pre- and postsynaptic plasma membrane thickening at the point of synaptic contact, known as the active zone. Fast exocytosis of the vesicular neurotransmitter requires activity of and interactions between a complex set of proteins that regulate Ca^{2+}-dependent fusion of the vesicular membrane with the plasma membrane and rapid recycling of small number of these vesicles (Pyle et al., 2000; Sara et al., 2005; Aravanis et al., 2003; for review see Schweizer and Ryan, 2006).

In order to prepare for neurotransmitter release, synaptic vesicles undergo a membrane-trafficking cycle that starts with vesicular uptake of neurotransmitter through an electrochemical gradient. Filled vesicles move to the plasma membrane where they dock to a specific active zone. At this stage partial pre-fusion between the vesicle and the nerve membranes takes place to enable rapid response to Ca^{2+} signal. Arrival of an action potential eventually induces Ca^{2+} influx into the nerve terminal and complete fusion between vesicle and cell membranes leading to neurotransmitter release. Empty synaptic vesicles are then endocytosed by clathrin-coated pits and dynamin and transfer back to the nerve terminal (Hill et al., 2001; Hu et al., 2002; Granseth et al., 2007; Newton et al., 2006; Ferguson et al., 2007; for review see Harata et al., 2006).

Fast neuronal exocytosis is driven by formation of complexes between three soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins, namely synaptobrevin, also known as vesicle-associated membrane protein (VAMP) on the synaptic vesicle membrane, with syntaxin and synaptosome-associated protein 25 kDa (SNAP-25) on the plasma membrane (see figure 1.2) (Schoch et al., 2001; Washbourne et al., 2001; Deak et al., 2004; Siddiqui et al., 2007). Synaptobrevin is associated with synaptophysin, a multispanning membrane protein of synaptic vesicles thought to be involved in the processes of docking and fusion of synaptic vesicles (Washbourne et al.,

1995). SNARE proteins are characterized by domains of 60-70 amino acids, known as SNARE motifs, which readily assemble into bundles of α–helices leading to membranes connection (Antonin et al., 2002). After neurotransmitter release SNARE complexes have to be disassembled for the next fusion reaction through a process that involves palmitoylation of SNAP-25 cysteine residues (Washbourne et al., 2001).

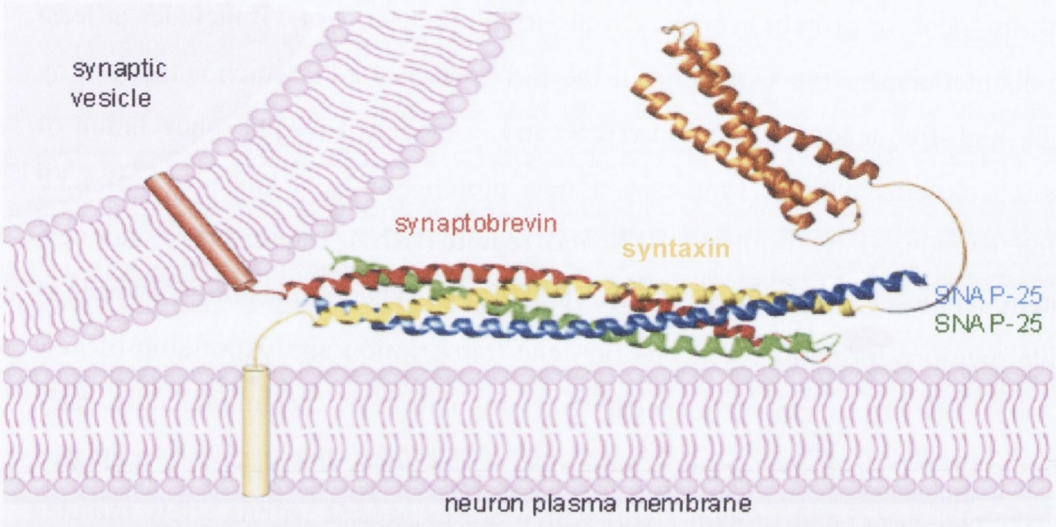


Figure 1.2 SNARE proteins complex.
(adapted from Nature Reviews, Neuroscience)

1.4 Long term potentiation

The processes of learning and memory formation, acquisition and retrieval of information respectively, involve adaptive changes in the synapses in response to a stimulus. Memory, in particular, is defined as explicit when it deals with conscious recall of facts requiring the hippocampus and the enthorinal cortex, whereas it is categorized as implicit when involving non conscious recall of skills involving the cerebellum and basal ganglia (for review see Robertson, 2002; Lynch, 2004).

The ability of the synapse to change in strength in response to a stimulus is known as synaptic plasticity and depends on neuronal mechanisms which can occur pre-synaptically to alter neurotransmitter release or post-synaptically to modify responsiveness to transmitter release or both. When these changes induce increased efficacy, represented by an increase in amplitude of the excitatory post-synaptic potential (EPSP), they are said to induce potentiation of synaptic plasticity, whereas if the output is a reduction in efficacy it is called depression.

Long term potentiation (LTP) is an increase in synaptic transmission induced by a brief, large magnitude rise in post-synaptic Ca^{2+} and represents a useful cellular correlate of learning and memory. LTP is particularly robust in all afferent inputs to the hippocampus (for review see Lynch, 2004).

Memory storage involves two main phases: a protein synthesis independent short-term phase which lasts 2-3 hours and a long-term phase lasting weeks and requiring *de novo* protein synthesis. Similarly, hippocampal LTP includes at least two distinct phases: the short-term of the early phase of LTP which is induced by single high-frequency stimulation (HFS) and is mediated by phosphorylation of existing proteins without synthesis of new proteins and the late phase of LTP which is induced by multiple HFS and requires RNA transcription and new protein synthesis. Thus, long lasting expression of synaptic plasticity, as well as stable memory formation, depends on gene transcription and expression of new proteins. The first change to appear is up regulation of the immediately early genes (IEGs) including c-fos and the gene encoding activity-regulated cytoskeletal-associated protein (Arc) which are expressed within a few minutes after induction of LTP (Guzowski et al., 2005; Kee et al., 2007). Modulation of different phases of LTP is carried out by Ca^{2+} signaling. LTP induction is initiated by Ca^{2+} influx into the post-synaptic dendritic spine through activation of N-methyl-D-aspartate (NMDA) receptor channels in response to glutamate release. In the case of early-phase LTP, subsequent activation of Ca^{2+}-dependent protein kinases such as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and protein kinase (PK) C, may result in the phosphorylation of existing proteins. On the other hand, late-phase LTP requires activation of PKA and new protein synthesis (for review see Robertson, 2002; Lynch, 2004). It has been suggested that synthesis of new proteins may be regulated by activation through phosphorylation of the transcription factor cAMP response element-binding protein (CREB) (Casey et al., 2002). Moreover, production of stable LTP involves expression of proteins involved in neurotransmitter release resulting in the generation of new synaptic contents (Mullany and Lynch, 1997; Kelly et al., 2000; Chen et al., 2007).

1.5 Neurons

Neuronal cells represent the basic cellular units of nervous tissue. It is estimated that there are 100 billion neurons in the CNS with their afferent and efferent extensions, dendrites and axons, all closely enveloped by glial cells. Their purpose is to receive, conduct and transmit impulses in the nervous system. It is known that the neuronal population usually is established shortly after birth and that neurons do not divide. Thus, in most regions of the mammalian brain, the production of neurons is mainly confined to the prenatal phase however, in the dentate gyrus of several species, including rat and mouse, granule cells are generated postnatally and are recruited into circuits involved in spatial memory (Kuhn et al., 1996; Bischofberger, 2006; Kee et al., 2007). Recently neurogenesis has been decribed which continues albeit at a low rate throught life (van Praag et al., 2002). Creation and maintenance of neuronal shapes are effected by the cytoskeleton consisting of three distinct complexes known as microtubules, neurofilaments and microfilaments. Microtubules are thought to be the substrate for the majority of mitochondrial transport in both axons and dendrites (Heidemann, 1996). Their core structure consists of a polymer of α- and β-tubulin units that are arranged in a cylindrical tube. Important microtubule-associated proteins include tau proteins that constitute several isoforms and play an important role in the assembly of tubulin monomers into microtubules and in maintaining the cytoskeleton and axonal transport.

Evidence suggests that neurons are involved in cytokine physiology: a number of receptors for cytokines with immune functions such as interferon-gamma (IFNγ) and interleukin-1 (IL-1) have been reported on neurons *in vitro* (Neumann et al., 1997b; Nolan et al., 2004). IFNγ has been shown to be produced by dorsal root ganglion (DRG) neurons and to exert an autocrine/paracrine regulatory activity on the cells (Neumann et al., 1997a). Physiologically active neurons can modulate activation of brain immune cells via local interaction through several mechanisms including soluble mediators and cell adhesion molecules (Neumann et al., 1998; for review see Neumann, 2001). Neurons rely on glial cells for the maintenance of an extracellular environment, for production of growth factors and removal of toxic substances.

1.6 Glia

Glial cells were initially described as a non-cellular connective tissue holding neurons together. Today it is known that glia represent the most numerous group of cells in the brain, there are ten times more glial cells than neurons, and their roles in providing structural, metabolic and trophic support to neurons are well established. Two broad groups of glial cells are recognised: macroglia including astrocytes and oligodendrocytes of ectodermal origin, and microglia of mesodermal origin. Glial cells differ from neurons in that they possess non synaptic contacts and retain the ability to divide through life, especially in response to injury. Growing evidence demonstrates the ability of glia to respond to, and send signals to neurons and synapses in the CNS and the peripheral nervous system suggesting that glial cells, and not only neurons, are actively involved in processing information and synaptic integration (Bezzi and Volterra, 2001; Todd et al., 2006). Although oligodendrocytes are necessary for efficient transmission of electrical current between neurons by forming the insulating myelin sheath of axons, astrocytes and microglia represent the highly reactive parenchymal cell population which is involved in the immune response against infectious and inflammatory stimuli (for review see Aloisi, 2001).

1.6.1 Immune cells of the CNS

1.6.1.1 Astrocytes

Amongst the glial cells astrocytes are the most numerous and, in the normal brain, they play essential roles in providing glia-neuron contact, ionic homeostasis, buffering excess neurotransmitters, secreting neurotrophic factors and serving as a critical component of the BBB (Ullian et al., 2001; Siddharthan et al., 2007; for review see Allen and Barres, 2005). It has been shown that astrocytes are involved in the regulation of synaptic activity through a mechanism potentiating the excitatory transmission in dentate granule cells (Jourdain et al., 2007). One well known function of astrocytes is concerned with repair. Following trauma or in response to brain inflammation, astrocytes invariably proliferate and undergo fibrosis by the accumulation of filaments, expressed as an increase in glial fibrillar acidic protein (GFAP) (Rabchevsky et al., 1998; Faulkner et al., 2004). The reactive gliosis that occurs after an injury to the CNS has been

considered as an impediment to axonal regeneration. However, recent studies have reported that, in certain conditions, reactive astrocytosis plays a key role in determining axonal elongation via release of various cytokines and neurotrophic factors (Ridet et al., 1997). It has been suggested that astrocytes contribute to their own activation in an autocrine fashion involving transforming growth factor α (TGFα) and activation of the epidermal growth factor receptor (Rabchevsky et al., 1998). Astrocytes have been implicated in development and maintenance of pain in response to tissue injury by producing pro-inflammatory cytokines which, in turn, act on neurons to facilitate central sensitization (Guo et al., 2007). Contrasting data indicate that astrocytes are involved in regulation of microglial activity either by enhancing or reducing their immune competent activity through mechanisms which include release of soluble factors rather than direct cell to cell interaction (Shaffer et al., 1995; Sola et al., 2002; von Bernhardi and Eugenìn, 2004; Aloisi et al., 1997).

1.6.1.2 Microglia

1.6.1.2.a Introduction

Microglial cells were first described by del Rio Hortega (1932) as a unique cell type typified by different morphology than other glia and neurons. They represent about 5-10% of all glia and derive from mesodermal precursor cells of hematopoietic lineage that enter the brain during the embryonic and early post-natal phases of development (for review see Barron, 1995). During these phases of brain remodelling and maturation, microglia are involved in the clearance of neuronal cells through programmed cell death. In the mature brain and under physiological conditions resting microglia serve the role of immune surveillance and host defence and are characterized by ramified morphology, lack of phagocytic activity, down-regulated expression of membrane ligands and receptors (for review see Aloisi, 2001). Ramified microglia are very sensitive to changes in their microenvironment and readily respond to CNS injuries or immunological stimuli.

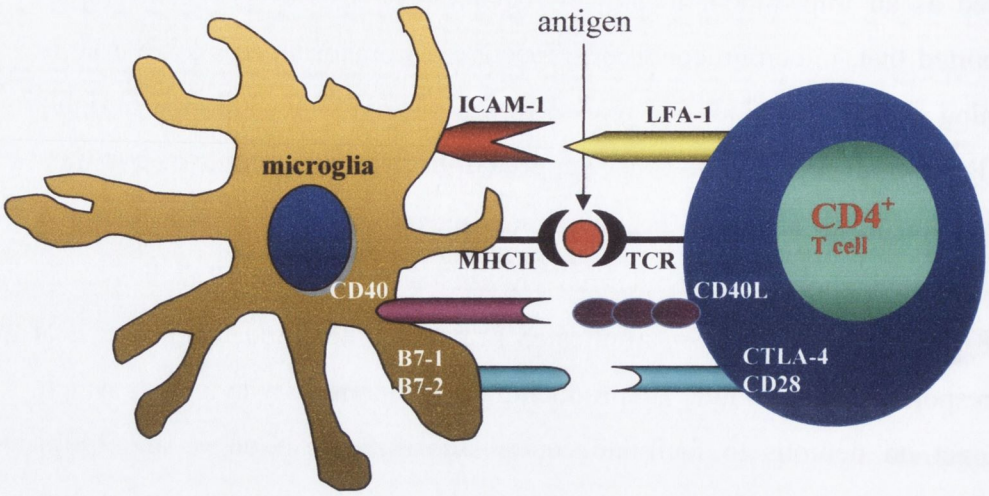


Figure 1.3 Presentation of processed antigen to infiltrated $CD4^+$ T cells by activated microglia. Antigen presentation is mediated by MHCII and other costimulatory molecules such as CD86, CD80, CD40 and ICAM-1.

1.6.1.2.b Microglial activation

Upon stimulation by inflammatory modulators microglia undergo a process of gradual maturation called activation that is characterised by proliferation and formation of multinucleated giant cells (Beyer et al., 2000). Surveillance microglia migrate to the site of injury where they undergo functional changes such as expression on the surface of several receptors that trigger or amplify immune response. These include, amongst others, pattern recognition receptors and cytokine receptors (Lee et al., 2002; for review see Aloisi, 2001). The pattern recognition receptors are involved in identification and targeting of microbial molecules and include the integrin CD11b/CD18, also known as complement receptor type 3 (CR3). In the normal CNS low levels of CD11b are expressed on resting microglia but CD11b expression is upregulated as a consequence of microglial activation (Aloisi et al., 2000a; Fischer and Reichmann, 2001). CD11b has been implicated in modulation of cell-mediated immunity (Marth and Kelsall, 1997).

Microglial activation also involves drastic morphological changes as microglia change from resting ramified to active amoeboid (Zhang et al., 1997). Under specific stimulation they become phagocytic and serve as antigen presenting cells (APC) (Carson et al., 1998; for review see Streit, 2002). Presentation of processed antigens to infiltrated T cells requires interaction

between the T cell receptor (TCR)/CD3 complex and the major histocompatibility complex (MHC) class II antigens (see figure 1.3). In the normal brain, expression of these molecules is minimal or absent whereas upon inflammatory stimulation microglia up-regulate expression of MHCII (de Groot et al., 2001; Lyons et al., 2007a; Lynch et al., 2007; for review see Aloisi et al., 2000b). A second set of costimulatory signals required for functional activation of T cells includes the interaction of T cells surface molecules CD28/CTLA-4 with their specific B7 counterpart. B7 is a family of membrane glycoproteins which comprises mainly two members: B7-1 (CD80) and B7-2 (CD86) (Wille et al., 2002). B7-2 has been implicated in the regulation of Th1/Th2 cells differentiation and activation (Kuchroo et al., 1995) and its up-regulation has been demonstrated in response to pro-inflammatory stimuli (Bohatschek et al., 2004; Ponomarev et al., 2005; Clarke et al., 2007; for review see Aloisi et al., 2000b). Human microglia have been shown to constitutively express considerable levels of B7-2 but not B7-1 which is minimally detectable in resting microglia but is increased after exposure to IFNγ, a key modulator of the immune response (Satoh et al., 1995). Other key molecules for APC activation are CD40 and its ligand CD40L. CD40 is a membrane protein member of the tumor necrosis factor (TNF) receptor family which is normally expressed on the surface of B-cells and other antigen presenting cells as the receptor for CD40L, a 39 kDa glycoprotein transiently expressed on the surface of activated T cells (for review see Chen et al., 2006a). The binding of CD40L to its receptor CD40 induces B cell activation and differentiation. CD40 is expressed by multiple cell types within the CNS including microglia, astrocytes, endothelial cells and neurons. CD40/CD40L interaction has been shown to regulate microglial activation by shifting their activation from the phagocytic to the APC phenotype leading to secretion of pro-inflammatory cytokines and neurotoxins which, in turn, may act on a feed-back loop to further inhibit microglial phagocytic activity (Ke et al., 2005; for review see Aloisi et al., 1998; Townsend et al. 2005).

Another common feature associated with microglial activation is expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) which plays an important role in immune-mediated cell adhesion and intracellular signal transduction (for review see Dietrich, 2002). It is expressed in leukocytes, endothelial cells and many other tissues at low levels but its expression is

upregulated in the presence of pro-inflammatory mediators (Wang et al., 1995; Shen et al., 1997). ICAM-1 is involved in the control of leukocyte traffic into the CNS by mediating leukocyte-endothelial cell adhesive reactions.

1.6.1.2.c Microglial activation controversy

An interesting point about microglia is their ability to perform both neuroprotective and neurotoxic functions. The primary purpose of microglia is to support neuronal function and aid their development and survival by secreting a number of trophic factors such as brain derived neurotophic factor (BDNF) and transforming growth factor beta (TGF-β) (Morgan et al., 2004; Polazzi et al., 2001). As a result of their phagocytic activity microglia eliminate apoptotic neurons. Phosphatidylserine (PS) is a phospholipid normally expressed on the inner side of the plasma membrane and expressed on the cell surface during apoptosis. Interaction of PS-expressing apoptotic cell with PS-receptor (PS-R) expressed on microglial cells, induces safe removal of dying neurons and release of TGF-β1 (Minghetti et al., 2005). However, if the activated microglia are not able to suppress the cause of neuronal injury, dying or damaged neurons repeatedly activate microglia to produce neurotoxic factors. This process of persistent activation of microglial cells leads eventually to a self-propelling cycle of neuronal damage (for review see Liu and Hong, 2003). Thus, given that neuronal death is a common characteristic of neurodegenerative diseases, it is evident that microgliosis, persistent microglial activation, plays a key role in the progression of many neurodegenerative diseases. Moreover, there are numerous studies *in vitro* and *in vivo* demonstrating that activated microglia release toxic substances such as nitric oxide (NO) and pro-inflammatory cytokines (Merrill et al., 1993; Viviani et al., 1998; Dasgupta et al., 2002; Nolan et al., 2003; Gibbons and Dragunow, 2006; for review see Vilhardt, 2005)

The protective or toxic effects of microglia on neurons may therefore depend on the nature of the stimulus, the molecules released and the cell to cell interactions between microglia and neurons.

1.6.1.2.d Regulation of microglial activation: CD200

The presence of inhibitory factors, intrinsic to the CNS, provides a beneficial mechanism in restricting disease progression and controlling immune activity within the brain. One mechanism of regulation of microglial activation includes interaction between CD200 and its receptor CD200R (see figure 1.4). CD200 is a membrane glycoprotein of 41-47 kDa broadly expressed in diverse cell types including lymphoid cells, vascular endothelial cells and neurons (for review see Wright et al., 2003). CD200R is very similar in structure to its ligand in that they both contain two immunoglobulin like domains but it has a longer cytoplasmic domain and contains three tyrosine residues that can be phosphorylated suggesting a role in signal transduction (Wright et al., 2000; Gorczynski et al., 2004). Moreover, CD200R is preferentially expressed on the surface of cells of the myeloid/monocyte lineage suggesting a role for CD200/CD200R interaction in the regulation of immune cell activity (Jenmalm et al., 2006; Chitnis et al., 2007; for review see Barclay et al., 2002). This observation is supported by studies conducted on mice lacking CD200 which display more numerous activated microglia and increased susceptibility to autoimmune diseases (Broderick et al., 2002; Hoek et al., 2000; Copland et al., 2007).

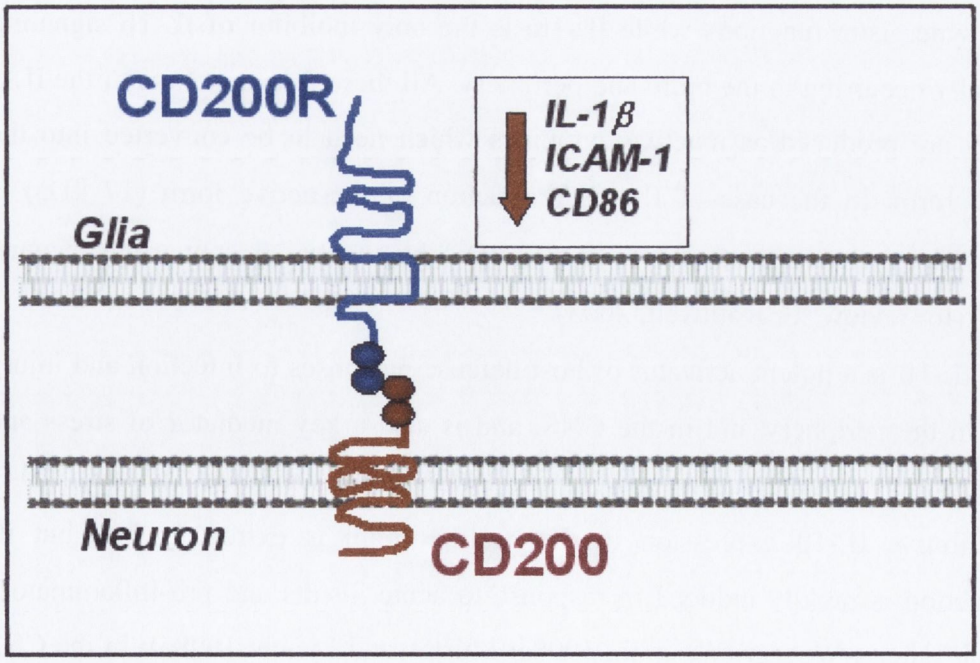


Figure1.4 Schematic representation of CD200 and CD200R expression. Interaction between CD200 and its receptor has been implicated in the

maintenance of glia in a quiescent state. (adapted from Downer et al., unpublished)

1.7 Cytokines

Cytokines are physiological mediators that exert diverse actions in the brain and play an important role in the modulation and mediation of both systemic host response to diseases and local changes induced by inflammation, infection and injury of the CNS (for review see Rothwell and Hopkins, 1995). The balance between pro-and anti-inflammatory cytokines is one determinant for inducing and regulating glial cell activation and their immune regulatory functions.

1.7.1 Pro-inflammatory signaling: interleukin-1β

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is released from antigen-presenting cells during infection and inflammation and, although its effects were originally considered to be confined to the immune system, it is now know to exert profound effects in the CNS. Some of these effects include modulation of thermoregulation, sleep and appetite (for review see Lynch, 2002). The IL-1 family consists of the ten proteins including the four most studied IL-1α, IL-1β, IL-18 and IL-1ra, the endogenous receptor antagonist. The first two have synergistic functions while IL-1ra is the only inhibitor of IL-1β signaling naturally occurring in the brain and periphery. All these four members of the IL-1 family are produced as inactive cytokines which need to be converted into the active form. In the case of IL-1β, production of the active form (17 kDa) is achieved by cleavage of pro-IL-1β (31 kDa) by the IL-1β converting enzyme (ICE) (for review see Rothwell, 2003).

IL-1β is a potent activator of host defense responses to infection and injury both in the periphery and in the CNS, and is also a key mediator of stress and sickness behaviour (Murray and Lynch, 1998a; for review see Dantzer, 2004). Constitutive IL-1β expression in the healthy brain is extremely low but its production is rapidly induced in response to acute insults and pro-inflammatory stimuli (Hauss-Wegrzyniak et al., 1998a; Murray and Lynch, 1998a). In the CNS the primary source of IL-1β are microglia but the cytokine is also produced to a lesser extent by astrocytes. This cytokine acts on astrocytes and microglia to

induce production of additional cytokines and chemokines which is indispensable in recruiting leukocytes to sites of infection or injury while it exerts toxic effects on neuronal cells. It has been suggested that IL-1β-induced signaling responses may be cell type-specific promoting inflammatory responses in glia and influencing function of neurons (Srinivasan et al., 2004). Consistent with this, there are numerous studies demonstrating that IL-1β influences synaptic function in hippocampal neurons and it exerts adverse effects on LTP (Murray et al., 1997; Vereker et al., 2000; Kelly et al., 2001; Vereker et al., 2001; Kelly et al., 2003).

In order to exert its inflammatory action IL-1β binds to the cell surface IL-1 receptor (IL-1R) which, within the brain, is mainly found in the hypothalamus and hippocampus and is expressed on both glia and neurons (for review see Lynch, 2002). Two main receptor subtypes for IL-1β have been identified: the 80 kDa type I (IL-1RI), found predominantly in T cells and fibroblasts and the 68 kDa type II (IL-1RII), also found in monocytes, neutrophils and bone marrow cells (reviewed in Heguy et al., 1993). However only type I receptor has a long cytoplasmic tail of about 200 amino acids which allows for signal transduction whereas IL-1RII has a short intracellular tail of 29 amino acids and it is thought to play a role in the inhibition/modulation of IL-1β activity by binding it and reducing its interaction with IL-1RI (for review see Fitzgerald and O'Neill, 2000; O'Neill and Dinarello, 2000; Lynch, 2002). Upon ligand binding IL-1RI recruits from the cytosol some serine/threonine protein kinases termed the interleukin-receptor associated kinases (IRAKs) which, in turn bind to a high affinity binding complex containing the IL-1RI, the IL-1R associated protein (IL-1RAcP) and the adaptor protein MyD88, which are indispensable for initiation of IL-1β-induced signal transduction. Once phosphorylated IRAKs interact with TRAF-6, a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family, inducing activation of multiple signaling pathways involved in regulation of transcription factors like nuclear factor kappa B (NF-κB) responsible for production of other inflammatory cytokines (see figure 1.5) (Kanakaraj et al., 1998).

1.7.2 Anti-inflammatory signaling

In contrast to IL-1β, interleukins 10 (IL-10) and 4 (IL-4) have been shown to possess anti-inflammatory properties. IL-10 was first described as cytokine synthesis inhibitor factor because of its ability to inhibit production of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα by activated macrophages. In humans it is expressed by activated $CD8^+$ peripheral blood T cells and activated $CD4^+$ Th2 cells (for review see Haddad et al., 2003). IL-10 acts as a potent modulator of initiation of pro-inflammatory response by Th1 cells (Allavena et al., 1998). Expression of this cytokine is elevated during inflammation and promotes survival of neurons and glia by limiting the production and activity of pro-inflammatory mediators (Kelly et al., 2001; Lynch et al., 2004). IL-10 exerts its anti-inflammatory action by binding to its cell surface receptor IL-10R inducing recruitment to the cell membrane of transcription factors, known as signal transducers and activators of transcription (see figure 1.5) (STAT1-STAT6). These molecules, in turn, form homo- and heterodimers and migrate to the nucleus where they induce transcription of cytokine inducible genes most likely through activation of the transcription factor NF-κB.

Similar to IL-10, IL-4 is a pleiotropic cytokine produced mainly by Th2 like cells. It plays a critical role in the inhibition of Th1 cells generation, promoting instead differentiation of T naïve cells into Th2 like cells (Kuhn et al., 1993). Upon binding to its cell surface receptor IL-4 triggers its dimerisation which in turn induces recruitment from the cytosol of STAT6 and subsequent activation of signal transduction (Kammer et al., 1996). Both IL-4 and IL-10 have been shown to modulate microglial activation in response to pro-inflammatory stimuli and subsequent IL-1β production *in vivo* (Ledeboer et al., 2002; Lyons et al., 2007a) and *in vitro* (Szczepanik et al., 2001; Iribarren et al., 2003; Butovsky et al., 2005). Moreover, it has been suggested that decreased levels of IL-4 and IL-10 signaling may contribute to inflammation in the brain (Maher et al., 2005; Moore et al., 2005; Nolan et al., 2005).

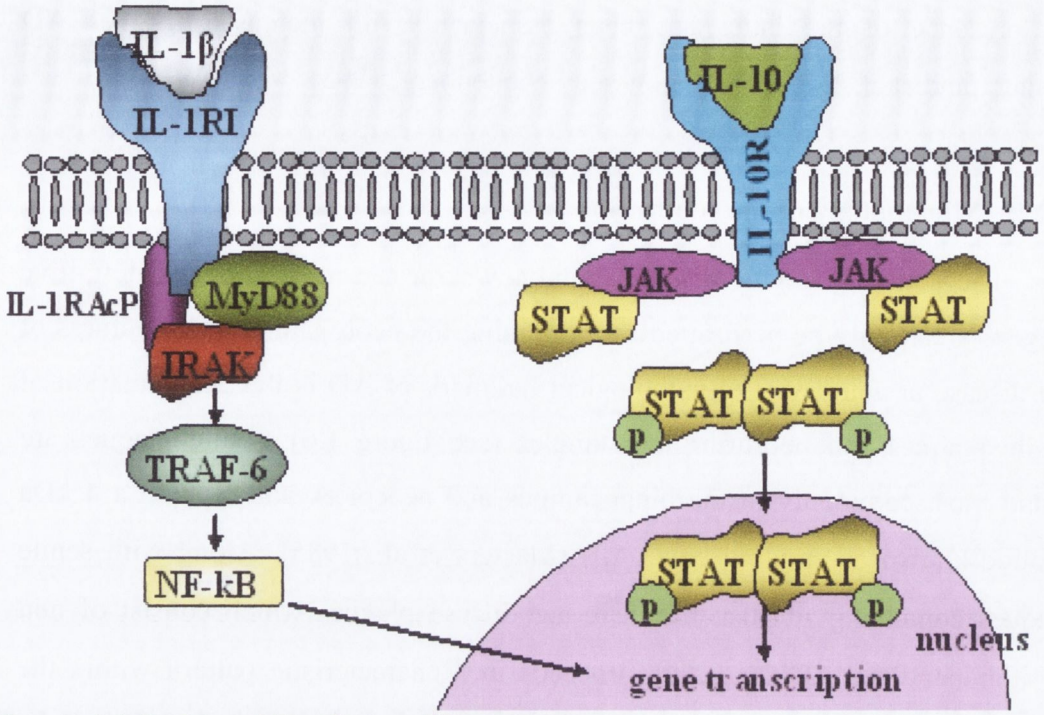


Figure 1.5 IL-1β and IL-10 signalling pathways.

1.8 Alzheimer's Disease

1.8.1 Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease clinically characterised by memory loss and cognitive decline. It is named after Alois Alzheimer who first described the neurofibrillary tangles and amyloid plaques that have come to be considered the hallmark of the disease. AD is now know as the leading cause of dementia in the elderly: it is estimated that about 10% of individuals over age 65 and nearly 50% over age 85 are affected with AD (Mount and Downton, 2006). While the majority of AD cases are sporadic late-onset cases, developing after 65, familial AD (FAD) is a rare form of AD which affects about 5% of patients and develops in individuals in their 30s and 40s, and certainly before the age of 60. Along with advancing age, sporadic AD has been linked to polymorphism of the apolipoprotein E (ApoE) gene whereas dominant mutations in three different genes, namely amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2), have been identified to account for FAD. ApoE is a lipid transport protein of 34 kDa, encoded on chromosome 19 and normally expressed on glial cells. Among the three allelic variants ε2, ε3 and

ε4, presence of the latter has been associated with increased risk of AD (Sunderland et al., 2004; for review see Schellenberg, 1995).

1.8.2 Morphology of the AD brain

The aetiology of the disease is unknown for the most part and definitive diagnosis can only be performed by examining the neuropathological features of the disease at autopsy. The pathological hallmark of AD is the accumulation of senile plaques and neurofibrillary tangles (see figure 1.6): senile plaques are found most commonly in the hippocampus and neocortex and contain a 4 kDa peptide known as amyloid beta (Aβ) (Masters et al., 1985). Along with senile plaques containing fibrillar Aβ there are diffuse plaques which consist of non fibrillar peptides. Aβ deposition proceeds in a characteristic pattern within the brain with the appearance of plaques first in the basal neocortex, followed by deposition in the frontal cortex and hippocampal formation until all areas of the cortex contain Aβ deposits, which may account for the progressive clinical symptoms of the disease (for review see Golde et al., 2000). Neurofibrillary tangles are intra-neuronal bundles of hyperphosphorylated tau: hyperphosphorylation reduces the ability of tau to bind microtubules leading to cytoskeleton degeneration and neuronal death. Another feature of the AD brain is Aβ accumulation in vessel walls as cerebral amyloid angiopathy. Evidence suggests that Aβ deposition in the periarterial pathways, along which interstitial fluid drains from the cortex, significantly contributes to cerebral amyloid angiopathy in AD (Weller et al., 1998).

The typical macroscopic picture of AD brain is massive cortical atrophy caused principally by synaptic loss which is most pronounced in the frontal and temporal cortices; this is accompanied by reactive gliosis. Neuronal loss, particularly evident in the hippocampus, is accompanied by significant decline in neocortical synapses which may account for the decline in memory and cognitive function which accompanies AD (for review see Mattson, 2004).

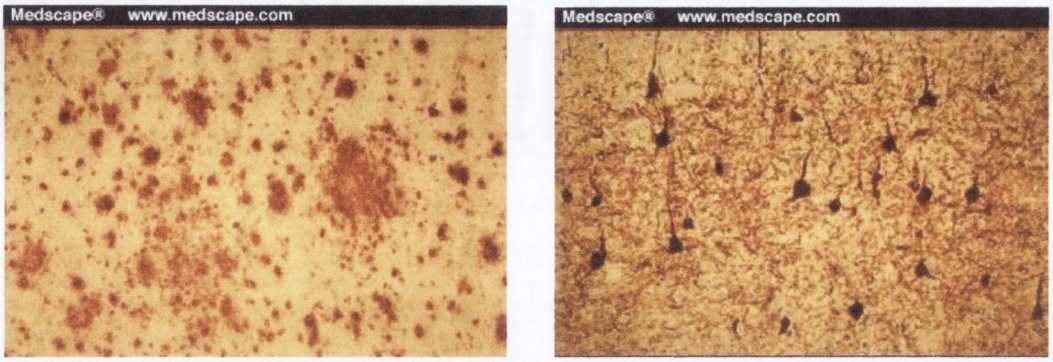


Figure 1.6 Senile plaques and neurofibrillary tangles.
High power view of a section from the cerebral cortex of an AD patient showing the high density of plaques (left panel) and tangles (right panel).

1.8.3 Aβ hypothesis

The Aβ hypothesis states that the progressive increase in Aβ cerebral levels, due to either increased production or decreased clearance, is the central event in the pathogenesis of AD. Although the density of neurofibrillary tangles correlates more with the cognitive symptoms than the distribution and density of Aβ plaques, it is now believed that tangles are a secondary feature of the disease process (Oddo et al., 2003; Ingelsson et al., 2004; Oddo et al., 2007; for review see Braak and Braak, 1995; Gotz et al., 2004). In support of this view there is evidence that while senile plaques are a specific feature of AD, neurofibrillary tangles are seen in a variety of neurodegenerative conditions (Hof and Perl, 2002; Taniguchi et al., 2004). Apart from analysis of changes in post mortem AD brains, where Aβ is the main constituent of senile plaques, the Aβ hypothesis is further supported by genetic data. FAD associated mutations of PS1, PS2 and APP genes have been linked to abnormal Aβ production or aggregation (Borchelt et al., 1997; Xia et al., 1998; for review see Schellenberg, 1995). AD is also characterised by a decrease in CSF levels of Aβ with respect to control subjects which adds to the hypothesis that Aβ accumulation in the brain plays an important role in the development of the disease (Sunderland et al., 2004). However, there is mounting evidence indicating the involvement of inflammation in the development of AD suggesting that the deposition of Aβ by itself is not sufficient to produce AD symptoms (for review see Block and Hong, 2005; Mrak and Griffin, 2005).

1.8.4 AD and neuroinflammation

The relevance of inflammation in AD neurodegeneration has been established by multiple studies (Nathan et al., 2005; Ojala et al., 2007, for review see Sastre et al., 2006). The evidence indicates that up-regulation of inflammatory mechanisms co-localizes with those areas of the brain which are mainly affected by the disease and are low or minimal in brain regions less affected by Aβ deposition (Verbeek et al., 1994; Smith et al., 1997; Apelt et al., 2001). Post-mortem analysis of AD brains revealed that DNA damage in the hippocampus exhibited a high degree of colocalization with neuronal nitric oxide synthase (nNOS) in reactive astrocytes (Simic et al., 2000). Moreover, pro-inflammatory cytokines such as IL-1β, TNFα and IL-6 and the chemokine MIP-1α are upregulated in AD brain (for review see Akiyama et al., 2000). Evidence that inhibition of cycloxygenase 2 (COX-2) ameliorated the Aβ-induced impairment in hippocampal functioning adds to the hypothesis that inflammation plays a role in the development of AD symptoms (Cakala et al., 2007).

Evidence that treatment of individuals with non steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD adds to the hypothesis that inflammation plays a critical role in AD (for review see McGeer and McGeer, 2001). Several mechanisms have been proposed to explain the beneficial effects of NSAIDs in AD: protection against Aβ aggregation, negative effect on APP processing and inhibition of pro-inflammatory cytokines production. Interestingly, treatment with NSAIDs has also been shown to significantly reduce plaque burden and microglial activation in APP-expression transgenic mouse model (Yan et al., 2003).

The principal targets of NSAID action are thought to be the cyclooxygenases, the enzymes responsible for the conversion of arachidonic acid into inflammatory mediators, including prostaglandin E2 (PGE_2) (for review see Pasinetti, 1998). However, it has been argued that the efficacy of NSAIDs in the treatment of AD is likely to be attributable to their action on the peroxisone proliferator activated receptor γ (PPARγ) rather than on the cyclooxygenases (for review see Sastre et al., 2006). PPARγ is a ligand-activated transcription factor and, upon ligand activation, forms heterodimers with the retinoid X receptors (RXR). The PAR/RXR heterodimers in turn recruit coactivators and bind to

specific consensus sequences present in the promoter domain of target genes (Tugwood et al., 1992). PPARγ activation can also inhibit specific gene expression by antagonizing the activity of other transcription factors such as NF-κB (Su et al., 1999, Landreth et al., 2001). Although PPARγ is expressed in adipose tissue and is important in adipocyte differentiation, its presence has also been detected in the brain, particularly, at neuronal level, in the hippocampus and cortex. A role in the regulation of the inflammatory response was suggested by the finding that its activation resulted in the inhibition of a number of inflammatory events including production of IL-1β, TNFα, IL-6 and inducible nitic oxide synthase (iNOS) in monocytes and the production of IL-2 and IFNγ from mitogen-activated splenocytes and T cells (Cunard et al., 2002, Clark et al., 2000). PPARγ agonists have been shown to modulate the Aβ-stimulated production of pro-inflammatory cytokines (Combs et al., 2000). It has been suggested that PPARγ may be involved in the regulation of Aβ turnover by reducing the levels of secreted and intracellular peptide without affecting expression or activity of any of the secretases involved in the generation of the peptide (Camacho et al., 2004). A possible mechanism by which PPARγ agonists prevent the neuronal damage and impairment in response to Aβ involves activation of the Wnt pathway with subsequent stabilisation of microtubule network that triggers an increase in neurite length and axonal calibre (Inestrosa et al., 2005).

1.8.5 Amyloid precursor protein

In the last years of the 20th century it was elucidated that Aβ, the major component of the AD plaques, was a cleavage product of a much larger membrane spanning protein, the amyloid-precursor protein (APP). APP is a type I transmembrane glycoprotein of up to 770 amino acids with a single transmembrane region, a large extracellular domain and a short cytoplasmic tail. Its gene is localized on chromosome 21 and is expressed abundantly in a variety of tissues: immunocytochemical studies provided evidence of APP expression on the plasma membrane of neurons and subpopulations of non neuronal brain cells (for review see Mattson, 1997). Because of its membrane localization it was originally thought to function as a receptor: however no ligand for APP has been

identified and there is no evidence that APP is linked to intracellular signal transduction. The functions of APP in normal brain are thought to be important in regulating neurite outgrowth, neural cell migration, synaptic plasticity and cell adhesion (Priller et al., 2006; for review see Mattson, 1997; Walsh et al., 2007). Evidence from the literature also suggests a role for APP in protecting the CNS against acute as well as chronic injury from endogenous or environmental toxins (Masliah et al., 1997; Masliah et al., 1998).

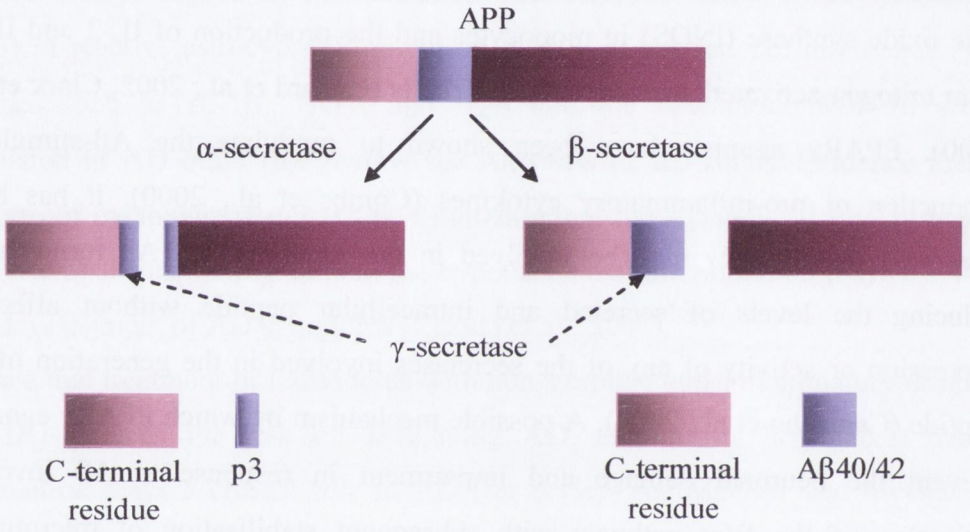


Figure 1.7 Schematic representation of the proteolytic processing of APP by the α-, β- and γ-secretases. The large N-terminal ectodomain is shown in purple, the C-terminal intracellular domain in pink.

1.8.5.1 APP metabolism

Metabolism of APP occurs via a complex process involving the activity of three proteases termed secretases. Cleavage of secretases can occur via two main pathways, namely the amyloidogenic and non-amyloidogenic pathways. APP processing via the non-amyloidogenic pathway takes place in the plasma membrane where cleavage by α-secretase releases a soluble fragment termed sAPPα, while leaving a 83 residue C-terminal fragment (CT83) with a truncated Aβ sequence (see figure 1.7). The C-terminal fragment, in turn, is cleaved by γ-secretase resulting in a truncated Aβ fragment known as p3. The candidates for α-secretase activity are three members of the ADAM (a disintegrin and metalloprotease) family: ADAM-9, ADAM-10 and ADAM-17. The production of

amyloidogenic peptide is due to the action of β-secretase, also known as the β-site APP-cleaving enzyme (BACE) and occurs mainly within endosomes and lysosomes.

BACE cleaves APP at its N-terminal end leading to formation of a 100 kDa soluble segment termed sAPPβ and a 12 kDa membrane anchored C-terminal fragment termed CT99. Subsequent cleavage of the C99 fragment by γ-secretase leads to formation of the 40-42 amino acid fragments. BACE1, located on human chromosome 11, encodes an aspartyl protease with a single transmembrane domain. Several studies have shown that BACE1 protein and activity levels are increased in AD brain, particularly in those regions affected by amyloid deposition such as the neocortex and the hippocampus suggesting a critical role for BACE1 in Aβ biosynthesis (Fukumoto et al., 2002; Chiocco et al., 2004). Moreover, in support of the hypothesis that inflammation contributes to the progression and severity of neurodegeneration in AD, it has been shown that glial activation reported in APP mice is accompanied by increased BACE1 expression and activity (Heneka et al., 2005). Increased BACE1 activity, in turn, has been reported to alter APP processing pathway in favor of Aβ deposition pattern (Chiocco et al., 2004). Consistent with these data there is evidence that lower BACE1 transcription is associated with relatively lower Aβ production in normal conditions (Li et al., 2006). A possible regulatory role of APP on BACE transcripts has also been suggested by Zohar and co-workers (2005). Moreover, it has been shown that NF-κB acts as a transcription activator for BACE in activated astrocytes and Aβ-exposed neurons (Bourne et al., 2007). These data suggest the existence of a positive feed-back loop between chronic inflammation, astrocyte activation, increased NF-κB activation of BACE transcription and further inflammation.

γ-secretase is a heterogeneous population of large membrane protein complexes containing the presenilins PS1 and PS2, nicastrin, anterior pharynx defective-1 (Aph1) and presenilin enhancer-2 (PEN2) which cleave their substrate along their transmembrane regions at the aspartyl residue (Wolfe et al., 1999; for review see Zhou et al., 2007). PS1 and PS2 are 467 and 448 amino acid polypeptides with about 60 % homology. Current research indicates an important role for PS in normal neurogenesis and neuronal survival as well as APP

proteolytic processing (for review see De Strooper, 2007). In a majority of familial cases determined by APP and PS mutations, a significant overproduction of Aβ and an increase in the $A\beta_{1-42}/A\beta_{1-40}$ ratio are observed (Borchelt et al., 1996).

1.8.6 Amyloid beta

Amyloid beta (Aβ), in the form of extracellular aggregates, is the main component of AD senile plaques. Aβ-related peptides consist of 39-43 amino acids derived from APP and can be detected as soluble products in numerous milieus such as the cerebral spinal fluid (CSF), plasma and brain in the range of low ng/g of gray matter (Weller et al., 1998). Although the majority of Aβ peptides produced exist as soluble low molecular weight monomers/dimers, senile plaques are mainly composed of insoluble fibers of Aβ, characterized by β–sheet structure. Indeed, both $A\beta_{1-40}$ and $A\beta_{1-42}$ (consisting of 40-42 amino acids, respectively) have been shown to naturally aggregate into high molecular weight structures known as protofibrils which are thought to be important intermediates in the fibrillogenesis process, the conversion of low molecular weight Aβ into fibrils (Walsh et al., 1997; Walsh et al., 1999). Among the main species of Aβ peptides, $A\beta_{1-42}$ is shown to be the most abundant form deposited in the senile plaques. Consistent with the hypothesis that $A\beta_{1-42}$ is more amyloidogenic than $A\beta_{1-40}$, it has been shown that $A\beta_{1-42}$ is more hydrophobic and forms precipitated fibrils faster than $A\beta_{1-40}$ and that degradation rate of $A\beta_{1-40}$ is greater than that of $A\beta_{1-42}$ (Snyder et al. 1994; Shaffer et al., 1995; Walsh et al., 1997). However, it has been reported that following injection of Aβ peptides in the rat brain, the abundance of the $A\beta_{1-40}$ aggregates was greater than the $A\beta_{1-42}$ aggregates suggesting that assembly of $A\beta_{1-42}$ into fibrils may be regulated *in vivo* and *in vitro* by different mechanisms (Shin et al., 1997). According to the authors, $A\beta_{1-40}$ and $A\beta_{1-42}$ differ in their ability to form amyloid fibrils *in vivo*: while $A\beta_{1-40}$ is mainly found in core plaques characterised by abundant fibrils, $A\beta_{1-42}$ is prominent in diffuse plaques with few Aβ fibrils (Shin et al., 1997). The initial phase of oligomerization of Aβ monomers involves formation of pentamer/hexamer units, known as paranuclei which have not been observed for

$A\beta_{1-40}$ (Bitan et al., 2003). It has been reported that the process of Aβ oligomerization begins intraneuronally and that further aggregation into fibrils and fibrillar aggregates is regulated by many factors including concentration, temperature, pH and Aβ species (Walsh et al., 2000; Stine et al., 2003). The total Aβ concentration may be the critical determinant of insoluble fibril formation. In young and healthy brains, amyloid peptide is fully catabolised immediately after its secretion from the cells before it can be deposited. By contrast, studies show a consistent decrease in Aβ concentration in the CSF of AD patients compared with controls and this is thought to be due to increased levels of insoluble Aβ that aggregates into Aβ fibrils and accumulates in extracellular deposits (Kuo et al., 1996; Wang et al., 1999). Other mechanisms which have been shown to promote fibrillation and peptide toxicity involve the interaction of amyloidogenic peptides with membrane lipids and certain metal ions such as Cu^{2+} and Zn^{2+} which are highly concentrated in the neocortex of most AD patients and further concentrated within Aβ plaques (Yanagisawa et al., 1997; Cherny et al., 1999).

1.8.6.1 Aβ neurotoxicity

Evidence suggests that Aβ–induced neurotoxicity is exerted prior to the appearance of extracellular senile plaques (Hsia et al., 1999) and that low molecular weight species of aggregated Aβ are also toxic (Pike et al., 1993; Lorton et al., 1996; Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 1999). Exposure of microglia and astrocytes to diverse Aβ species initiates a complex signal transduction cascade leading the cells to acquire a reactive phenotype and synthesize toxic products (Combs et al., 2001; Walker et al., 2001; Butovsky et al., 2005). It has been demonstrated that the microglial response to Aβ involves perturbation of Ca^{2+} homeostasis and subsequent activation of a tyrosine kinase-dependent signaling response rendering neurons more vulnerable to neurotoxicity (Mattson et al., 1992; Good et al., 1996; Combs et al., 1999; Ferreiro et al., 2006). Activation of phosphatidylinositol 3-kinase (PI3K)/Akt has been found to be involved in the Aβ-induced inflammatory response by inducing production of chemokines and metalloproteinases essential for leukocyte extravasation and migration, and propagation of inflammatory mechanisms (Ito et al., 2006; Ito et al., 2007). Activation of caspase-3 has been implicated in the process of

programmed cell death induced by neuronal exposure to Aβ (St. John, 2007). There is growing evidence suggesting that Aβ neurotoxicity is mediated by reactive oxygen species (ROS) which may account for damaged neuritic trees and compromised expression of synaptic vesicle proteins (Klegeris et al., 1994; McDonald et al., 1997; Combs et al., 2001; Abramov et al., 2004; Paradisi et al., 2004; Ferreiro et al., 2006). In addition, evidence that up-regulation of iNOS co-localizes with Aβ plaques (Luth et al., 2001) and is stimulated in response to Aβ with subsequent production of NO adds to the hypothesis that NO also is an important mediator of peptide neurotoxicity (Tran et al., 2001; Floden et al., 2005; Jang and Surh, 2005). Another mechanism involved in neuronal death includes the production of ceramide that accompanies the Aβ-induced membrane lipid peroxidation (Culter et al., 2004). Moreover, exposure of neurons to an oxidation product of homocysteine has been shown to induce accumulation of Aβ inside the cells and this was correlated with their vulnerability (Hasegawa et al., 2005).

1.8.6.2 Aβ receptors

A number of cell surface Aβ-binding receptors have been identified including: scavenger receptors (SR), the receptor for advanced glycation endproducts (RAGE) and the integrin $\alpha_5\beta_1$ (for review see Verdier et al., 2004). There are three classes of SR expressed on microglia: class A including type I, II and III, class B (CD36) and macrosialin (CD68). SRA has been shown to participate to Aβ uptake whereas SRB is though to be important for activation of intracellular signaling (Christie et al., 1996; Paresce et al., 1996; for review see Bamberger and Landreth, 2001). It has been demonstrated that microglial interaction with extracellular Aβ is mediated by a complex of cell surface receptors including integrin $\alpha_6\beta_1$, SRB and the integrin associated protein (IAP), also called CD47 (Bamberger et al., 2003; Koenigsknecht and Landreth, 2004). CD47 is made of a single immunoglobulin extracellular domain and five transmembrane segments terminating in a short cytoplasmic tail. It is expressed in a variety of cells including neurons and endothelial cells as well as myeloid and lymphoid cells and it has been shown to augment Fas-dependent apoptosis (Manna et al., 2005). Two ligands have been described for CD47, namely signal

regulatory protein α (SIRPα), involved in modulation of macrophage phagocytosis and adhesion and migration of several cell types including dendritic cells (DC), and the thrombospondins, a family of glycoproteins that regulates cell migration and proliferation in response to inflammation (Hagnerud et al., 2006; Lamy et al., 2007). Because of its physical association with the β integrin subunits CD47 is thought to play a role in regulation of the integrin function. Fibrillar engagement of the $\alpha_6\beta_1$, SRB and CD47 receptors complex has been shown to result in tyrosine phosphorylation of the proto-oncogene Vav which, in turn, induces production of ROS and phagosome formation through reorganization of actin cytoskeleton (Wilkinson et al., 2006).

Another receptor involved in Aβ uptake is the low-density lipoprotein receptor-related protein-1 (LRP-1), a member of the low density lipoprotein (LDL) receptor family which is central for the transport and metabolism of cholesterol and ApoE-containing lipoproteins. LRP-1 acts as a clearance receptor for ApoE-Aβ complexes at the BBB mediating transport of the peptide from brain into the blood. It has been suggested that changes in the relative distribution of RAGE, normally found on neurons and microglia, and LRP-1 may account for Aβ accumulation in the AD brain (Yan et al., 1996; Donahue et al., 2006). Overexpression of LRP in a mouse model of AD has been shown to increase neuronal endocytosis of Aβ (Zerbinatti et al., 2006). The high affinity of the Nogo-66 receptor (NgR), involved in limiting injury–induced axonal growth, for Aβ and its presence in AD plaques imply that NgR may be implicated in Aβ burden (Park et al., 2006).

1.8.6.3 Aβ and LTP

One important issue about Aβ peptide is the determination of the assembly state that may be responsible for the decline of cognitive functions in AD. Although fibrillar Aβ is the main component of senile plaques there is poor correlation between Aβ fibrils and the severity of clinical dementia and growing evidence suggests a role for oligomeric Aβ, primarily dimers and trimers, as major contributor to impaired memory. Oligomeric forms of Aβ have been shown to exert inhibitory effect on hippocampal LTP *in vivo* and *in vitro* (Walsh et al., 2002; Klyubin et al. 2005; Yun et al., 2006; for review see Walsh and Selkoe,

2007). Evidence that immunization using antibodies against Aβ oligomers prevented LTP inhibition *in vivo* further adds to the hypothesis that low molecular weight species of Aβ contribute to the cognitive impairment associated with AD (Rowan et al., 2005). It has been suggested that Aβ-mediated inhibition of LTP involves activation of microglia which results in release of TNFα and subsequent activation of metabotropic glutamate receptors and p38 kinase as well as production of ROS (Wang et al., 2004a; Wang et al., 2004b; Wang et al, 2005). Also, PPARγ activation has been shown to attenuate the Aβ-induced deficit of LTP *in vitro*, suggesting an interaction between Aβ, PPARγ and the expression of hippocampal LTP *in vitro* (Costello et al., 2005).

Aβ-induced memory deficits in AD involve inhibition of both early- and late-phase LTP through a mechanism independent of neurotoxicity and peptide aggregation. Studies show that acute application of diverse Aβ species induced LTP impairment and that this effect was most likely due to transient intracellular Ca^{2+} rises and enhanced Ca^{2+}oscillations (Chen et al., 2000; Freir et al., 2001; Chen et al., 2002). Moreover, in light of the fact that AD is characterized by debilitating cognitive deficits perhaps associated with loss of synaptic density, it has been reported that Aβ soluble oligomers could catalyze a stage of synaptic dysfunction that precedes synapse loss and neurodegeneration (Hartley et al., 1999). Exposure of neurons to Aβ oligomers has been shown to induce activation of the protease calpain through disruption of extracellular Ca^{2+} influx mediated by NMDA receptor (NMDAR) (Kelly and Ferreira, 2006). Activation of calpain led to dynamin disruption and hence, to a stage of synaptic dysfunction in hippocampal neurons. AD brain has been characterized by inhibition of NMDARs and perturbed Ca^{2+} homeostasis (Wang et al., 2004a; for review see Mattson, 2004). Similarly, NMDARs have been implicated in the Aβ-induced impairment of LTP although evidence suggests that NMDAR may not always be affected by Aβ or even, that involvement of NMDAR in Aβ-induced electrophysiological changes depends on the aggregation state of the peptide (Ye et al., 2004; Nomura et al., 2005; Szegedi et al., 2005). Phosphorylation of Ca^{2+}/calmodulin-dependent PKII has been linked to the inhibitory effect of soluble $A\beta_{1-42}$ on LTP (Zhao et al., 2004).

1.8.6.4 Aβ plaques and microglia

Microglial cells exhibiting an activated, reactive phenotype are found clustered within and adjacent to the AD brain senile plaques (Tooyama et al., 1990, Verbeek et al., 1994). It has been suggested that accumulation of mononuclear phagocytes to the site of Aβ deposition takes place before formation of Aβ deposits. MCP-1 (also known as Ccl-2, CC-chemokine ligand 2) is the main ligand for the microglial CC-chemokine receptor 2 (Ccr2) and is produced by microglia and astrocytes in response to Aβ. Production of MCP-1 by activated microglia has been implicated in the recruitment of further microglial cells (El Khoury et al., 2007).

Activated microglia secrete a diverse range of acute-phase proteins including α-anti-chymotrypsin, complement components, ApoE and serum amyloid P (SAP). Accumulation of most of these Aβ associated proteins is dependent on the degree of fibril density of the Aβ deposits and precedes the appearance of clusters of activated microglia and neuronal tau-related changes. Activated microglia are observed only in those Aβ deposits that have accumulated C1q and SAP: when exposed to a mixture of $A\beta_{1-42}$, C1q and SAP, adult human microglia secrete higher levels of pro-inflammatory cytokines *in vitro* than cells treated with $A\beta_{1-42}$ alone (Veerhuis et al., 2003).

Cultured microglia exhibit chemotaxis to pre-aggregated $A\beta_{1-42}$ and peripheral monocytes migrate across BBB model when Aβ is present on the other side (Giri et al., 2000). Putative mediators of microglial chemotaxis to Aβ are formyl peptide receptor (FPR), macrophage scavenger receptor (MSR) and RAGE (Christie et al., 1996; Yan et al., 1996). Soluble mediators secreted by Aβ-activated microglia such as macrophage colony stimulating factor (M-CSF) can also contribute to further microglial chemotaxis. Exposure of microglia to Aβ leads to increased release of chemokines IL-8, macrophage inflammatory peptide-1alpha (MIP-1α), MIP-1β and MCP-1 (for review see Rogers and Lue, 2001). It has been reported that a large portion of plaque-associated microglia are of blood origin (Simard et al., 2006). These bone marrow-derived cells have been shown to migrate toward Aβ deposits when the plaques have reached a certain size and eliminate them by phagocytosis both *in vivo* and *in vitro*. Resident microglia are present at the onset of plaque formation and hence they may play a role in this

phase while blood-derived microglia appear at later stage of the disease and may therefore try to clear the senile plaques (Mackenzie et al., 1995; Sheng et al., 1997). The fact that newly recruited microglia are more efficient than resident ones may represent a beneficial mechanism in restricting disease progression. It is important also to consider that the attachment and survival of microglia to Aβ implies that the peptide is not directly toxic to the cells.

1.8.6.5 Aβ and microglial phagocytosis

A paradox about the role of microglia in AD is their inability to clear up the Aβ deposits in spite of their activated phenotype. Although there is compelling evidence suggesting that microglia are capable of phagocytosing Aβ (Shaffer et al., 1995; Paresce et al., 1997; Kopec and Carroll, 1998) there are contrasting data in regard to microglial ability to efficiently degrade the Aβ deposits. Microglia have been shown to efficiently phagocytose fibrillar and soluble Aβ *in vitro* within days of its administration and deliver it to late endosomes and lysosomes, however degradation of the peptide has been proved incomplete (Weldon et al., 1998). It has been suggested that inability of microglia to degrade Aβ may be due to low hydrolytic activity of late endosomes and lysosomes within the cells (Majumdar et al., 2007b). However it has been reported that activation of microglia by pro-inflammatory stimuli acidify microglial lysosomes thereby enabling degradation of fibrillar Aβ (Majumdar et al., 2007a).

Reactive astrocytes in the AD brain have been shown to interfere with Aβ clearance by releasing proteoglycans which colocalise with the peptide in the senile plaques, and conferring resistance to enzymatic digestion (Shaffer et al., 1995). It has been suggested that the modulatory effect of astrocytes on activated microglia may account for the inability of the cells to phagocytose Aβ deposits (von Bernhordi and Eugenin, 2004). By contrast, extracellular accumulation of heat shock proteins (HSP) close to the microglia in senile plaques have been reported to induce IL-6 and TNFα production and $A\beta_{1-42}$ phagocytosis (Kakimura et al., 2002).

The mechanism by which Aβ peptide activates microglia has not been fully understood, however, it has been suggested that the cells might respond to some component, not sequence specific, but related to the structure of β-sheets of the

peptide (Lorton et al., 1996; Yates et al., 2000). By contrast, it has been suggested that material specific to Aβ dense core plaques rather than fibrillar Aβ is responsible for microglial activation. Dense core plaques are associated with neuritic changes such as lysed cell bodies where lysosomal enzymes, cellular DNA and advanced glycation endproducts, which are known to be sufficient to induce microglial activation, are secreted by injured or dying neurons. ATP or ADP and complement components are also released and may play a role in microglial chemotaxis (for review see D'Andrea et al., 2004). Although microglia can detect and respond to fibrillar Aβ through activation of intracellular signaling leading to production of ROS and pro-inflammatory cytokines their interaction with the peptide does not activate receptor systems involved in phagocytic activity (El Khoury et al., 1996; for review see Liu and Hong, 2003). This suggests that microglia cannot phagocytose Aβ when it is in an highly fibrillar form and, as a result, cells are continuously being stimulated by the persistent Aβ to produce pro-inflammatory cytokines which, in turn, might further exacerbate their activated phenotype (Lee et al., 2002; Familian et al., 2007).

1.9 Aging

1.9.1 The aging brain

Aging is inevitable and is most conveniently defined as the advance of chronological years. It is known that brain aging is associated with a progressive decline of the cognitive and memory functions accompanied by an overall reduction in the brain volume and weight and enlargement of the brain ventricles (for review see Anderton, 2002). These changes are partly the result of decreased nerve cell density, which in the rodent brain appears to be a localised event affecting particularly the CA regions, and attenuated proliferation of the granule cell precursors of the dentate gyrus (Kuhn et al., 1996; Simic et al., 1997). Although hippocampal neuron loss is viewed as a hallmark of normal aging and neuronal degeneration is thought to contribute directly to age-related deficits in learning and memory supported by the hippocampus, it has been demonstrated that loss of hippocampal neurons is not an inevitable consequence of normal aging (Rapp and Gallagher, 1996). Mechanisms rather than neuronal death have been implicated in the age-related cognitive decline, such as shrinkage of

neuronal soma, loss or regression of dendrites and dendritic spines and changes in electrophysiological properties (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Dickstein et al., 2007). However, other changes may be indicative of a compensatory increase in hippocampal function and include increased firing frequencies of pyramidal cell, elevated postsynaptic efficacy of granule cell synapses, changes in mass distribution and branching of dendritic trees, and increased NMDAR function (Dickstein et al., 2007; for review see Miller and O'Callaghan, 2005). It has been suggested that the age-related increased vulnerability of neurons to neurodegenerative conditions may rely on altered Ca^{2+} homeostasis (Raza et al., 2007).

Another mechanism that contributes to neuronal impairment and damage associated with the aging brain includes the progressive intracellular accumulation of ROS. Although the cells rely on antioxidant enzymes such as superoxide dismutase (SOD) to deal with oxidative stress and associated oxidative damage, the aging brain has been shown to display compromised activity of these enzymes (O'Donnell et al., 2000; Navarro and Boveris, 2004; for review see Dröge and Schipper, 2007). Imbalance between antioxidant defences and intracellular ROS accounts for increased lipid peroxidation, protein oxidation and DNA damage characteristic of the aging brain (Forster et al., 1996; Murray and Lynch, 1998b; O'Donnell and Lynch, 1998; Murray et al., 1999; Hamilton et al., 2001; for review see Bertlett and Stadtman, 1997).

1.9.2 Aging brain and inflammation

Inflammation appears to play a central role among the various processes that have been connected to brain aging: normal aging in the rodent and human brain is accompanied by activated astrocytes and microglia, especially in the hippocampus (Deng et al., 2006; Lynch et al., 2006; Stichel and Luebbert, 2006; Hayakawa et al., 2007). Increased MHCII and CD86 mRNA expression have been reported in the hippocampus of aged rats pointing to a pro-inflammatory CNS microenvironment in older animals (Frank et al., 2006). Normal aging, in particular, is accompanied by increasing numbers of activated microglia overexpressing IL-1β (Moore et al., 2007). Because IL-1β has been shown to inhibit LTP, it is suggested that the age-related increase in IL-1β and subsequent

interaction with its signaling receptor IL-1RI, induces a downstream signaling cascade which induces the LTP deficits that have been reported in aged rats (Griffin et al., 2006). These changes, predictably, have been coupled with activation of the stress-activated kinases JNK and p38 which, in turn, are associated with activation of caspase-3, a marker of apoptotic cell death, leading to the notion that cell death in the aged brain occurs via apoptosis (Lynch and Lynch, 2001). Increased IL-1β concentration and up-regulation of IL-1β-induced cell signalling cascades has been shown to be attributable to down-regulation of survival signals such as ERK and PI3K as a consequence of age-related alterations of the anti-inflammatory cytokine IL-4 (Maher et al., 2004; Maher et al., 2005). Thus, the balance between pro-and anti-inflammatory processes in the non pathological CNS shifts from a predominantly anti-inflammatory to a pro-inflammatory microenvironment during the normal aging process via down-regulation of molecules involved in modulation of microglial activation such as IL-10 and CD200 (Frank et al., 2006).

Accumulation of ROS in the aging brain represents another important mechanism involved in the impairment of cognitive function (Murray and Lynch, 1998b; Vereker et al., 2001) and increased microglial responsiveness to inflammatory stimuli which contribute to render the aging brain more susceptible to neurodegeneration (Long et al., 1998; Deng et al., 2006; Lecanu et al., 2006; Liang et al., 2007). Moreover, aging has been shown to increase the LPS-induced release of pro-inflammatory cytokines (Xie et al., 2003). Accumulation of DNA damage, as a consequence of cumulative insults that occur over time, has been shown to drive microglial activation (Unger, 1998).

It has been suggested that the age-related increase of glucocorticoids may be in part responsible for the age-related deterioration of the hippocampus (Murray and Lynch, 1998a). Also, stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to produce immunodepression mediated by increased corticosterone concentration and dysregulation of the Th1/Th2 cytokine profile (Viveros-Paderas et al., 2006). Furthermore, brain aging is associated with structural and functional changes of the BBB and, as a consequence of these alterations, there is an increase in permeability with serum leakage and leukocyte infiltration into the parenchyma which may account for propagation of the inflammatory processes (Stichel and Luebbert, 2006; Chang-Ling et al., 2007).

1.9.3 Aging brain and microglia

It has been suggested that microglial cells are prone to undergo senescent changes and that this could contribute to the age-related brain dysfunction. Dystrophic microglia in the human aging brain differ from activated microglia in that they are characterised by marked loss of processes without the concomitant cytoplasmic hypertrophy that usually accompanies activation, as well as spheroid formations, tortuousity of the processes and cytoplasmic fragmentation (Streit et al., 2004). Moreover it has been suggested that microglial cells in the healthy aging brain are neither resting nor fully activated, but primed (Godbout et al., 2005). Thus, when an organism with primed glial cells is challenged with a stimulus that would normally activate glia, such as peripheral infection, the pro-inflammatory response in the brain is exaggerated compared to that obtained from non primed cells (Tateda et al., 1996; Combrinck et al., 2002; Barrientos et al., 2006). Primed microglia show characteristic alterations of morphology and surface markers expression typical of the activated state but do not produce pro-inflammatory mediators such as IL-1β or iNOS (Cunningham et al., 2005). These data add to the hypothesis that the progressive up-regulation of glial inflammatory cytokines with aging may raise the vulnerability of brain cells to develop neurodegenerative diseases like AD in response to other genetic and environmental insults (Yu et al., 2002). Consistent with this, it has been reported that microglia isolated from animals exposed to uncontrollable stress exhibited a potentiated IL-1β response to pro-inflammatory challenge (Frank et al., 2007).

1.10 Experimental model of inflammation: lipopolysaccharide

1.10.1 Introduction

Lipopolysaccharide (LPS) is a gram-negative bacterial cell surface proteoglycan, also known as bacterial endotoxin, and is known to be a potent immunestimulator. Recognition of the bacterial toxin by the immune system leads to transcription of genes that encode for pro-inflammatory regulators of the immune response (Lund et al., 2006). Because of its ability to induce neuroinflammation in the brain, LPS has been widely used experimentally to produce inflammation as a model to study neurodegenerative diseases. The innate immune response is initiated by microglia in the brain. LPS binds to circulating

LPS binding protein (LBP) which subsequently transfers the LPS to membrane bound cluster differentiation 14 (CD14) present on microglia. CD14 is a glycoprotein bound to the outer cell membrane via a glycosylphosphatidylinositol (GPI) anchor and, although it has been described as a LPS receptor, it lacks transmembrane and intracellular domains so interaction with additional components is required for signal transduction (reviewed in Triantafilou and Triantafilou, 2002). Inhibition of CD14 *in vitro* has been shown to inhibit activation of downstream signaling pathway induced by LPS emphasizing the importance of LPS binding to CD14 in mediating the LPS-triggered gene induction (Hambleton et al., 1996). Moreover, it has been reported that LPS downregulated CD14 mRNA, suggesting a feed-back signal preventing over-stimulation (Becher et al., 1996). CD14 has also been linked to AD because of its ability to recognise and bind fibrillar Aβ, although with a much lower affinity than that for LPS (Fassbender et al., 2004). The Toll like receptor 4 (TLR4) and the myeloid differentiation protein MD2 form a complex through which LPS/CD14 transduce a signal leading to activation of multiple cascades involving the mitogen activated protein kinases (MAPK) and the transcription factor NF-κB as key players (figure 1.8) (Cario et al., 2000; Hornef et al., 2003; for review see Guha and Mackman, 2001). Activation of JNK and its substrate c-jun has been shown to be downstream consequence and critical step in the LPS-triggered attenuation of LTP (Barry et al., 2005).

In vitro studies of microglia stimulated with LPS have demonstrated increased levels of a wide range of pro-inflammatory molecules (Forloni et al., 1997; de Groot e al., 2001; Pawate et al., 2004; Jin et al., 2007). It has been shown that, within the brain, microglia are the only non neuronal cell type that express TLR4 and that presence of these cells is required for LPS-induced neurotoxicity and oligodendrocyte injury (Lehnardt et al., 2002; Lehhardt et al., 2003). Similarly, LPS administration *in vivo* stimulates a brain inflammatory response whether injected in the periphery or in the CNS (Qin et al., 2007). Microglial activation mediates the LPS-induced loss of entorhinal cells (Hauss-Wegrzyniak et al., 2002). Degeneration of these cells and loss of their projections into the hippocampus may underlie the impaired LTP which is associated with chronic exposure to LPS. Similarly, it is possible to speculate that the presence of chronic inflammation within the temporal lobe of patients with AD may account

for development of dementia. By contrast, it has been reported that *in vivo* prolonged activation of microglia by LPS caused alteration of neuronal function, assessed by step-through passive avoidance test, without leading to neuronal cell death in the hippocampal CA1 region (Tanaka et al., 2006).

A possible mechanism involved in LPS-induced learning and memory deficits without induction of neuronal loss includes attenuation of glutamatergic transmission. These findings were supported by evidence from the literature demonstrating that the widespread inflammatory response seen after intrahippocampal LPS administration did not cause detectable neurodegeneration (Szcepanik et al., 1996; Herber et al., 2006). The results showed by Herber and colleagues also demonstrated that the magnitude and timing of activation in response to LPS varies for different markers stressing the importance of "operational" definitions of microglial activation. It has been demonstrated that LPS-induced impairment of neuronal function is mediated by induction of iNOS (Yamada et al., 1999). Moreover, it has been shown that supranigral LPS administration induced acute microglial activation leading to dopaminergic neuron degeneration (Iravani et al., 2005). These findings reinforce the concept that LPS-induced neuronal damage is mediated by microglial activation.

1.10.2 LPS and Aβ

Although compelling evidence from the literature suggests a role for LPS in producing extensive inflammatory reactions within the brain associated with neuronal impairment and loss, data are emerging that some degree of brain inflammation aids in clearing Aβ deposits. Intrahippocampal administration of LPS has been shown to induce a strong reduction in the Aβ load in APP+PS1 transgenic mice (DiCarlo et al., 2001). Consistent with these findings is the evidence for a trend to lower levels of Aβ burden after systemic LPS challenge to mouse model of AD (Quinn et al., 2003). These data add to the evidence in favour of a role for inflammatory activity in the process of Aβ clearance. In addition, it has been shown that removal of parenchymal Aβ in transgenic APP mice follows a time-dependent manner following LPS administration suggesting that early microglial activation may be effective in removing Aβ or slowing the rate of deposition whereas, later microglial activation, once Aβ deposits have matured

and reached significant densities, may be insufficient to clear deposits and lead to a condition of chronic inflammation and neurodegeneration (Herber et al., 2004). Evidence that TLR signaling pathway may be involved in clearance of cerebral Aβ deposits and that microglial activation may be beneficial to AD patients comes also from *in vitro* studies. Activation of any of the three TLRs namely TLR2, TLR4 and TLR9, with their specific ligands has been shown to markedly boost uptake of Aβ by microglial cell line BV-2 (Tahara et al., 2006). Together these data suggest that some forms of microglial activation are able to increase the rate at which Aβ is being cleared.

On the other hand, some investigators have reported increased levels of Aβ in response to LPS. It has been shown that LPS-induced inflammation stimulates APP expression/processing thereby increasing the generation of Aβ (Sheng et al., 2003). Studies suggest that the augmented LPS-induced response to Aβ may be due to increased release of IL-1β possibly via up-regulation of the ICE (Yao and Johnson, 1997). Thus, it is thought that inflammation may not only increase the expression but also alter the processing of APP to generate larger concentrations of Aβ. Results from a recent work by von Bernhardi and colleagues (2007) suggest that *in vitro* inflammation may impair the clearance of APP by microglial cells and promote cytotoxic activation of microglial cells in response to APP exposure. Thus, reactivity to APP is potentiated in the presence of pro-inflammatory response induced by LPS suggesting that formation of senile plaques in the AD brain depends on the impairment of microglial function by both the induction of an abnormal inflammatory response and the impairment of APP processing. The increased levels of APP and Aβ-containing APP fragments could act as stimulators by further perpetuating the inflammatory response. Moreover, it has been shown that systemic inflammation induced by LPS enhanced COX-2 protein level in response to administration of $A\beta_{1-42}$ in the hippocampus of mice and significantly potentiated the memory impairment evoked by Aβ peptides. Whereas pre-treatment with LPS provided some protection against COX-2 production and memory deficit induced by Aβ (Cakala et al., 2007). Thus, systemic inflammation leads to the exaggeration of Aβ toxicity or to brain protection depending on time, duration and severity of the inflammation. In addition, it has been shown that LPS activation of cultured astrocytes elicited a marked concentration-dependent increase in the secretion of the soluble APP

through activation of a PKC-mediated signaling pathway (Small et al., 2005). These data, taken together, suggest that systemic infection may induce exaggerated inflammation in response to Aβ. Furthermore, it has been reported that Aβ and LPS act synergistically to induce microglial activation and release of pro-inflammatory molecules such as IL-1β and TNFα (Lotz et al., 2005; Gasic-Milenkovic et al., 2003).

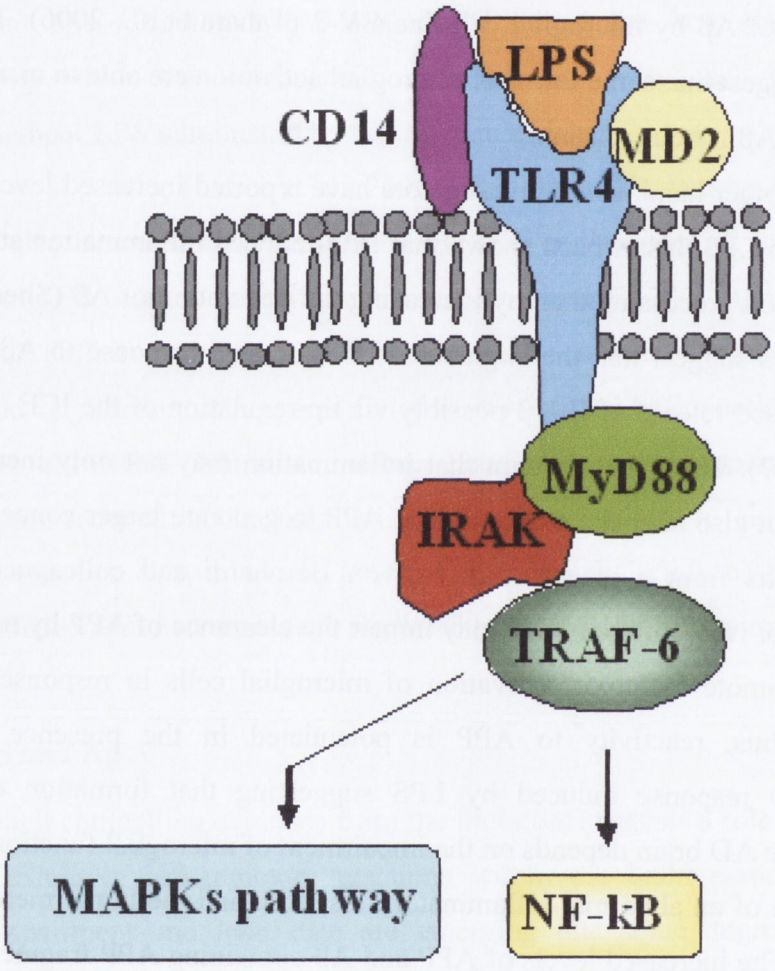


Figure 1.8 LPS signalling pathway.

1.11 Objectives

Evidence suggests that inflammation is central to the degenerative changes and cognitive impairments associated with AD. The hypothesis proposed is that Aβ induces microglial activation leading to the AD-related deficits of the brain.
The aims of this study were:

- to analyse the effect of different protocols of Aβ preparation and administration in the rat hippocampus;

- to investigate the role of microglial activation in response to Aβ;

- to analyse the effect of Aβ on neuronal function;

- to assess the possibility that age exacerbates the response to Aβ.

Chapter 2

Methods and materials

2.1 Culture of primary cells

2.1.1 Preparation of sterile coverslips

Glass coverslips (13mm diameter; Chance Propper, UK) were sterilized overnight in 70% ethanol, followed by an overnight exposure to UV light. Sterile coverslips were incubated for 1 hour in poly-L-lysine at 37 °C (40 μg/ml in sterile dH_2O) so as to provide cells with a surface to adhere to. Coated coverslips were air dried and placed in 24 well plates (Cruinn Diagnostic, Ireland) and stored at 4 °C until required.

2.1.2 Preparation of hippocampal neurons

Primary hippocampal neurons were established from post-natal one-day old Wistar rats, supplied by the BioResources Unit in Trinity College, Dublin. Rats were decapitated, the hippocampi were dissected free and the meninges were removed. The hippocampi were chopped using a sterile disposable scalpel and incubated in 0.3% trypsin in phosphate buffered saline (PBS; Sigma, UK) for 25 minutes at 37 °C. The tissue was triturated in PBS (Sigma, UK) containing 0.1% soybean trypsin inhibitor (Sigma, UK), DNase (0.2 mg/ml; Sigma, UK) and $MgSO_4$ (0.1M; Sigma, UK) and filtered through a sterile mesh filter (40 μm; BD Biosciences, USA). The suspension was centrifuged at 2000 xg for 3 minutes at room temperature (RT) and the pellets were resuspended in warm neurobasal medium (NBM; Gibco, UK) supplemented with penicillin (100 U/ml; Gibco, UK), heat-inactivated horse serum (10%; Gibco, UK), streptomycin (100 U/ml; Gibco, UK), glutamax (2 mM; Gibco, UK) and B27 (1%; Gibco, UK). Resuspended neurons were plated onto poly-L-lysine coated glass coverslips at a density of $0.25x10^6$ cells/ml and allowed to adhere for at least 2 hours in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. Warmed supplemented NBM (500μl) containing B27 was added to each well and the neurons were incubated for 3 days. After this, the medium was replaced with supplemented NBM containing 5 ng/ml cytosine-arabinofuranoside (ARA-C; Sigma-Aldrich, UK). After 24 hours the media was replaced with warmed supplemented NBM until the cells were ready to be used.

2.1.3 Preparation of cortical glia

Glial cell cultures were prepared from cortex of one-day old Wistar rats, supplied by the BioResources Unit in Trinity College, Dublin. Rats were decapitated with a sterile pair of scissors, the skull was exposed by cutting the skin in a straight line from the neck to the top of the head and removed to expose the brain. The cortex was dissected free, chopped using a sterile dispensable scalpel and incubated in warmed Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Dublin) supplemented with fetal bovine serum (Sigma-Aldrich, England), streptomycin (100 U/ml; Sigma-Aldrich, England) and glutamax (2 mM; Sigma-Aldrich, England) for 25 minutes at 37 °C. The solution was then triturated, filtered through a nylon mesh filter (40 μm; BD Biosciences, USA) and centrifuged at 2000 xg for 3 minutes at 20 °C. Following centrifugation the pellet was resuspended in warm DMEM. Resuspended glia were plated ($1x10^6$ cells/ml) onto each poly-L-lysine coated glass coverslips and allowed to adhere for at least 2 hours in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. Warmed supplemented DMEM was added to each well and cells were incubated for 13 days until ready for treatment, changing the medium every 3 days.

2.1.4 Treatment of cortical glia

All treatment agents were prepared by diluting the required concentration in pre-warmed supplemented DMEM. For the first study cortical glia were incubated in the presence of hippocampal neurons ($0.15x10^6$ cells/ml) for 4 hours after which neurons were removed and replaced with DMEM containing LPS (Sigma, UK). LPS was prepared as a stock solution in sterile PBS (composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5, pH 7.4-7.2; R&D System, USA) and diluted to a final concentration of 100 ng/ml. Cells were treated with LPS for 24 hours. For the LPS dose-response study cortical glia were treated with LPS at the concentration of 5, 20, 50 and 100 ng/ml for 24 hours. For the LPS+Aβ study cells were treated for 24 hours with LPS (10 ng/ml), $Aβ_{1-42}$ (2 μM; Biosource International Inc., USA) and a combination of LPS and $Aβ_{1-42}$. Aβ was dissolved in pure HPLC grade water to provide a 5 mM stock solution, diluted to 1 mM using sterile water and allowed to aggregate for 48 hours at 37 °C

according to the manufacturers instructions. Cells were treated with LPS and Aβ for 24 hours.

2.1.5 Harvesting of cells for mRNA extraction

After 24 hours of treatment, supernatants were removed and stored at -80 °C until further analysis was carried out (see section 2.4). Cells were lysed on 24 well culture plates by adding Tri-reagent (60 µl; Sigma-Aldrich, Dorset, UK) into each well and scraping the cells off coverslip using the rubber end of a 1ml syringe piston.

2.2 *In vivo* studies

2.2.1 Housing of animals

The in *vivo* studies were established on inbred strain male Wistar rats supplied by the Bioresources Unit in Trinity College, Dublin. The young rats were aged between 4 and 6 months and weighed approximately 250-350 g. The aged rats were aged between 18 and 22 months and weighed approximately 500-600 g. Young and aged animals were housed in groups of 4-6 and 2 respectively and were maintained under a 12-hour light-dark cycle in the BioResources Unit with the ambient temperature maintained between 22 °C and 23 °C. Food consisting of ordinary laboratory chow and water were available *ad libitum*. All animal experimentation was carried out 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 Aggregation and assessment of $A\beta_{1-40}$ and $A\beta_{1-42}$

$A\beta_{1-40}$ and $A\beta_{1-42}$ (Biosource International Inc., USA) were dissolved in HPLC grade water to provide 5 mM stock solution, diluted to 1 mM using sterile PBS and allowed to aggregate for 48 hour at 37 °C, according to the manufacturers instructions. The presence of fibrillar Aβ was demonstrated using a thioflavine T fluorescent assay. For the assay, aggregated samples of Aβ were added in triplicate to a black fluorescent plate and incubated with thioflavine T

(10 μM; 100 μM; Sigma, UK) in the presence of glycine (185 μl; 42.5 mM, pH 8.5; Sigma, UK). Fluorescence was read at 435 nm excitation and 485 nm emission (Spectramax Gemini, Molecular Devices, UK). The Aβ preparations were also assessed for oligomeric forms by gel electrophoresis and Coomassie blue staining. Samples (10 μl) were boiled for 2 minutes and loaded onto 15% SDS gel and the Aβ species separated by application of a 32 mA current. After electrophoresis the gel was rinsed in water and incubated overnight with the Coomassie solution (15 ml; GelCode Blue Stain reagent, Pierce, USA). The resultant bands were photographed (Gel-Doc-ItBioimaging System, Ultraviolet Products Inc., UK).

2.2.3 Preparation of Aβ and LPS

All treatment agents were diluted to the required concentration in artificial cerebrospinal fluid (aCSF; composition in mM: NaCl 150, KCl 3, $CaCl_2$ 1.4, $MgCl_2$ 0.8). LPS was prepared as a stock solution as described above (see section 2.1.4).

2.2.4 Anaesthesia

In the case of the acute study, animals were anaesthetised by intraperitoneal injection of urethane (1.5 g/kg; 33% w/v; Sigma, UK) and absence of pedal reflex was used to confirm deep anaesthesia. Animals were kept warm and, if needed, further top-up doses of urethane were administered to a maximum of 2.5 g/kg. In the case of the chronic and intra-hippocampal injection study, animals were anaesthetised with intraperitoneal injection of ketamine (2 μl/g; Pharmacia, Bela-Pharm GmbH and Co, Germany) and xylezene (2 μl/g; Bayer, UK) and the absence of pedal reflex was considered as an indicator of deep anaesthesia. Anaesthesia was maintained by administration of isoflurane (5% in 1.5 L/minute O_2; Abbott Laboratories Ltd., UK). In all cases anaesthetised animals were placed in a stereotaxic frame (ASI Instruments, UK).

2.2.5 Surgical procedures

2.2.5.1 Acute icv injection

A midline incision was made with a scalpel to reveal the skull. Bregma was identified and a dental drill was used to drill a small hole at 2.5 mm posterior and 0.5 mm lateral to bregma to facilitate placement of a syringe.

2.2.5.2 Chronic icv injection

After the skull was exposed using a sterile scalpel the outlet of the osmotic minipump was implanted into the third ventricle according to the following coordinates: 1.3 mm lateral and 0.9 mm posterior to bregma. The osmotic pump tanks were planted into a subcutaneous pocket in the midscapular area of the rats back. The cannula was fixed to the skull with dental cement and the cut was closed with metal sutures. At the end of the surgery animals were injected subcutaneously with Rymadil (1 μl/g; Grampian Pharmaceuticals Limited, Dundee, Scotland), as analgesic, and placed in heated cages and kept under observation for the following 24 hours. During the 28-days treatment period the rats were observed daily and were under veterinary supervision.

Chronic infusion of the drugs was conducted by means of osmotic minipumps (6 μl/day; Alzet, UK). Prior to implantation the pumps were primed for 48 hours in saline at 37 °C. Due to the presence of a high concentration of salt in a chamber surrounding the reservoir containing the test agents (isolated from it by an impermeable layer), water enters the pump through its outer surface (a semipermeable layer). Water entrance increases the volume in the osmotic layer thereby compressing the flexible reservoir and inducing delivery of the drugs into the brain ventricle through the flow moderator.

2.2.5.3 Intra-hippocampus injection

A midline incision was made with a scalpel to reveal the skull. Bregma was identified and a small hole was drilled at 3.9 mm posterior and 2.5 mm lateral to bregma to facilitate placement of a syringe. At the end of the surgery animals were injected subcutaneously with Rymadil (1 μl/g) and placed in heated cages and kept under observation for the following 24 hours.

2.2.6 Treatment groups

2.2.6.1 Acute icv injection

For the first study young rats were subdivided into two treatment groups: the first group received $A\beta_{40\text{-}1}$ (10 µl; 45.54 µM; Biosource International Inc., USA), the second group received a combination of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (10 µl; 18.94 µM and 26.6 µM, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively). For the second study animals were randomly assigned to two treatment groups (n=6 per treatment group): group I acted as control and received $A\beta_{40\text{-}1}$ (5 µl; 200 µM), group II received a combination of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (84 µM and 116 µM, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively). This study was carried out in conjunction with the PhD student Anne-Marie Miller. In the case of both studies, four hours after Aβ injection animals were assessed for their ability to sustain LTP (see section 2.2.5).

2.2.6.2 Chronic icv injection

For the first study groups of young and aged rats were subdivided into two treatment groups (n=6 per treatment group). The first group acted as controls and received $A\beta_{40\text{-}1}$ (63.8 µM), the other group received Aβ (26.9 µM and 36.9 µM $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively). This study was carried out in conjunction with doctor Antony Lyons. For the second study young rats were randomly assigned to four treatment groups: group I received aCSF or $A\beta_{40\text{-}1}$ (45.5 µM) as control treatment, group II received LPS (0.5 mg/ml), group III received Aβ (18.9 µM and 26.6 µM $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively), group IV was treated with a combination of LPS and Aβ. In the case of both experiments, animals were treated for 28 days.

2.2.6.3 Intra-hippocampus injection

Young and aged rats were randomly assigned to two treatments groups (n=6 per treatment group): one group acted as controls and received saline (5 µl) while the other group received $A\beta_{1\text{-}42}$ (5 µl; 200 µM in aCSF). Seven days after the injections animals were assessed for their ability to maintain LTP (see section 2.2.5).

2.2.7 Induction of LTP

2.2.7.1 Preparation of animals

Rats were anaesthetised as described above (see section 2.2.3.1). The head was positioned in a head holder in a stereotaxic frame. A middle line incision was made to reveal the skull and a dental drill was used to drill two halls to allow correct placement of the electrodes.

2.2.7.2 Electrodes implantation

A bipolar stimulating electrode and a unipolar recording electrode (Clark Electromedical, UK) were stereotaxically positioned on the surface of the brain according to the following coordinates: 4.4 mm lateral to Lambda and 2.5 mm lateral and 3.9 mm posterior to Bregma. The electrodes were lowered through the cortical and hippocampal layers until the stimulating electrode was positioned in the perforant path and the recording electrode in the dorsal cell body region of the dentate gyrus respectively. The positions of the electrodes were monitored by applying a 0.1 millisecond duration, 4 V pulse to the stimulating electrode at a frequency of 0.1 Hz. The evoked response was displayed on an Apple Mac computer through an analogue to digital converter (MacLab, Analog Digital Instruments) and the electrodes depth was adjusted so as to maximize EPSP. The final depth of the stimulating electrode was between 2.5 mm and 3 mm and of the recording electrode between 2.5 mm and 3.5 mm.

2.2.7.3 LTP induction and recording

After a period of stabilisation, test shocks (0.033 Hz) were delivered at 30 seconds intervals and recorded for 15 minutes before, and 45 minutes after, tetanic stimulation (three trains of 250 Hz shocks for 200 ms at 30 seconds intervals). The field EPSPs were displayed on-line and were analysed on completion of the experiments.

2.3 Preparation of tissue

2.3.1 Dissection

Animals were killed by decapitation and their brains rapidly removed. A longitudinal incision of the brain was made and the dissected quarter was coated in OCT compound (Sakura Tissue-Tek, Netherlands) and immersed in liquid nitrogen for later analysis. The hippocampus was quickly dissected free on ice and an aliquot was frozen in liquid nitrogen and stored at -80 °C for later analysis of messenger ribonucleic acid (mRNA; see sections 2.4 and 2.5).

2.3.2 Preparation of slices for freezing

Freshly dissected hippocampus was cross-chopped using a McIlwain tissue chopper (Mickle Laboratiory Engineering Co., UK). Slices were washed three times in ice-cold Krebs Ca^{2+} buffer (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) and stored at -80 °C in 1 ml Krebs solution containing 10% acid dymethylsulfoxide (DMSO; v/v; Sigma, UK) until required for analysis.

2.3.3 Protein quantification with BCA

Tissue was thawed rapidly and washed three times in Krebs solution. Tissue was homogenised in a 1 ml glass homogeniser (Jencons, UK). Protein quantification was performed using the BCATM Protein Assay Kit (Pierce, USA) based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein: A stock of 2000 μg/ml of bovine serum albumin (provided with the kit) was used to prepare the standards (0-2000 μg/ml) in Krebs solution. Samples and standards (25 μl) were added in triplicate to a 96-well plate (Sarsdet microtest plate, Ireland), working reagent (200 μl; 1:50 dilution of reagent B, containing sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide, and reagent A; containing 4% cupric sulfate) was added to each well and incubated at 37 °C for 30 minutes. The absorbance was read at 540 nm (Labsystems Multisken RC, UK) and a regression line was plotted (GraphPad, Prism, USA) in order to calculate the protein concentration in the samples and express it as mg protein/ml.

2.4 Analysis of mRNA with reverse transcription-PCR

2.4.1 Precautions

RNases, which degrade RNA, are ubiquitous in the environment and therefore all solutions were treated before use with diethyl pyrocarbonate (DEPC; 0.1% v/v; Sigma-Aldrich, UK) which inactivates RNases. All procedures were carried out on ice to inhibit RNA degradation by endogenous RNases.

2.4.2 RNA extraction

Extraction of total RNA was carried out on harvested cells in Tri-reagent and snap frozen hippocampal tissue which, on the day of extraction, was thawed rapidly and homogenized in Tri reagent using a 1 ml glass homogeniser. Samples were centrifuged at 12000 xg for 10 minutes to remove any insoluble material and the resulting supernatants were moved to newly autoclaved Eppindorf tubes and allowed to stand for 5 minutes at RT. Chloroform (0.2 ml/ml Tri-reagent; Sigma-Aldrich, Dorset, UK) was added to allow phase separation, samples were shaken vigorously and incubated for 15 minutes at RT. After incubation, samples were centrifuged at 12000 xg for 15 minutes allowing separation into a lower pink, phenol-chloroform phase, an interphase containing DNA and a colourless upper aqueous layer rich of RNA. The aqueous layer was moved to new Eppindorf tubes and RNA was precipitated by addition of isopropanol (0.5 ml/ml Tri- reagent; Sigma-Aldrich, Dorset, UK). Samples were mixed and incubated for 10 minutes at RT. After incubation, samples were centrifuged at 12000 xg for 10 minutes, the supernatants were removed and the resulting pellets were washed with 75% alcohol (ethanol diluted in autoclaved DEPC-treated water; Sigma-Aldrich, Dorset, UK). Samples were centrifuged at 7500 xg for 5 minutes and pellets stored at -80 °C until required for reverse transcription.

2.4.3 Analysis of isolated RNA by gel electrophoresis

To check that RNA was intact and did not undergo degradation, samples were run in 1% (w/v) agarose gel (Promega, Madison, USA). The gel was prepared by boiling 1 g of agarose in 100 ml of Tri borate EDTA (TBE) buffer (0.08 M Tri, 0.04 M boric acid, 1 mM EDTA). Ethidium bromide (5 μg/ml;

Sigma-Aldrich, Dorset, UK) was added and the gel cast into a horizontal stand and allowed to set. RNA samples (3 µl) were prepared for electrophoresis by mixing with H_2O (2 µl) and loading dye (1 µl; Promega, USA). Samples (5 µl) were then loaded into the wells and separated by application of a 90 V voltage for one hour. The gel was visualized under UV light and photographed using a UV transilluminator (Ultra Violet Products Ltd., UK).

2.4.4 Reverse transcription for cDNA synthesis

Samples (10 µl) were prepared by mixing with 1 µl of oligo dT primer (Invitrogen, Paisley, UK) and 1 µl of dNTP mix (containing 10 mM each of dATP, dTTP, dCTP and dGTP; Promega Corporation, Madison, USA). This mixture was heated at 65 °C for 5 minutes and then moved to ice (PTC-200 Peltier Thermocycler, Amersham, UK). 4 µl of 5x firt-strand buffer, 2 µl of dithiotreitol (DTT) and 1 µl of ribonuclease inhibitor (Invitrogen, UK) were added and samples mixed gently and incubated at 42 °C for two minutes. After incubation, superscript reverse transcriptase enzyme (1 µl; Invitrogen, UK) was added and the reaction was continued for 50 minutes at 42 °C for cDNA synthesis followed by 15 minutes at 70 °C to inactivate the reverse transcriptase. cDNA was stored at -20 °C until required.

2.4.5 Polymerase chain reaction

A mastermix PCR mixture was prepared with DNae-RNase-free water (16.5 µl; Sigma, Germany), 10x PCR buffer (2.5 µl; Invitrogen, UK), Mg^{2+} (1.5 µl; Promega Corporation, USA), nucleotide mix (1 µl; Promega Corporation, Madison, USA) forward and reverse primers (0.5 µl; Invitrogen, UK) and Taq Polymerase enzyme (0.5 µl; Promega Corporation, USA). Each sample (2 µl) was added to this master mix and PCR was run by using a thermocycler (Amersham, UK). Amplification started with an initial denaturing step at 95 °C for 1 minute followed by 25-35 cycles consisting of a denaturing step at 95 °C for 1 minute, an annealing step at 55-65 °C for 1 minute (see Table 2.1 for details) and an extension step at 72 °C for 2 minutes. A final extension step at 72°C for 10

minutes was applied to ensure complete extension of PCR products. PCR products and a 100 bp ladder were mixed with loading dye and run for 1 hour at 90 V voltage in a 2% (w/v) agarose gel containing ethidium bromide (5 μg/ml). Separated samples were visualised under UV light and photographed using a UV transilluminator.

2.4.6 PCR for IL-4

This PCR was carried out by adding to 17.5 μl of PCR mixture (IL-14 + 18s Dual-PCR Kit, Maxim Biotech Ltd., USA) 5 μl of molecular grade water and 0.5 μl of Jump Start Taq polymerase enzyme (Sigma, UK). cDNA (2 μl) was added to the mixture and PCR was run with a denaturing step at 95 °C for 3 minutes, followed by 44 cycles consisting of denaturing step at 95 °C for 1 minute, an annealing step at 60 °C for 1 minute and an extension step at 72 °C for 2 minutes. A final extension step at 72 °C for 10 minutes was applied to ensure complete extension of PCR products. PCR products and a 100 bp ladder were run for 2 hours as described above.

Target gene	Primer sequence	Annealing Temp. (°C)	Fragment Size (bp)
β-actin	for 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ rev 5′-CTTCTGCATCCTGTCAGCGATGC-3′	65	236
IL-1β	for 5′-GCACCTTCTTTTCCTTCATC-3′ rev 5′-CTGATGTACCAGTTGGGGAA-3′	59	447
MHCII	for 5′-CAGTCACAGAAGGCGTTTATG-3′ rev 5′-GATCGCAGGCCTTGAATGATG-3′	58	245
IL-10	for 5′-TGCCAAGCCTTGTCAGAAATGATCAAG-3′ rev 5′-GTATCCAGAGGGTCTTCAGCTTCTCTC -3′	65	127
IL-4	for 5'-TCCATGCACCGAGATGTTTGTACC-3'; rev 5'- CGTAGGATGCTCCCTTTATGAACG-3'	60	352

Table 2.1 Genes used for RT-PCR with respective annealing temperature and fragment size

2.4.7 Densitometry

Quantification of product bands was carried out by densitometric analysis using the ZERO-Dscan Image Analysis System (Scanalitics, USA). Values are expressed as a ratio of target gene to β-actin house-keeping gene mRNA expression and are presented as arbitrary units. In the case of IL-4, values are expressed as a ratio of target gene to 18s.

2.5 Analysis of mRNA with real time PCR

2.5.1 RNA extraction

Total RNA was purified from hippocampal tissue with a Nucleospin Kit (Macherey-Nagel, Germany). Samples were homogenized in RA1 solution containing β-mercaptoethanol by a polytron mixer. The mixtures were filtered through Nucleospin filter units by centrifugation for 1 minute at 1000 xg. The filter units were discarded and the homogenized lysates were washed with 70% ethanol (350 μl). The lysates were loaded on new filter units and spun down for 30 seconds at 8000 xg. Membrane desalting buffer (350 μl) was added to the filters and samples were centrifuged for 1 minute at 11000 xg. DNase reaction buffer containing 10% reconstituted DNase I (v/v) was added (95 μl) onto the centre of the silica membranes and allowed to incubate for 15 minutes at room temperature. Samples were centrifuged twice for 30 seconds at 8000 xg, the first time with addition of 200 μl of RA2 and the second time with 600 μl of RA3. The last wash was performed by addition to the filter units of 250 μl RA3 and centrifugation for 2 minutes at 11000 xg. The RNA of each filter unit was eluted in RNase-free water (60 μl) and centrifuged for 1 minute at 11000 xg. In order to obtain high concentrations of purified RNA, elutes were reapplied onto the filter units and spun down once more for 1 minute at 11000 xg. RNA samples were stored at -80 °C until required.

2.5.2 Analysis of isolated RNA

To check that RNA was intact and did not undergo degradation, samples were run on 1% (w/v) agarose gel as described above (see section 2.4.3). Concentration of RNA was measured due to its ability to adsorb light at 260 nm

using a spectrophotometer (DU730 Beckman Coulter, USA). Purity of samples was established by measuring their absorbance at 280 nm. A ratio of optical density (OD) 260/280 equal to 1.8-2.1 was considered indicative of pure RNA. Samples were diluted 1:200 in DEPC-treated water and added into a quartz cuvette.

2.5.3 Reverse transcription for cDNA synthesis

Total RNA was reverse transcribed into cDNA using high-capacity cDNA archieve kit (Applied Biosystems, Germany) according to the protocol provided by the manufacturer. Briefly, appropriate volumes of RNA were added to nuclease-free H_2O to obtain 1 µg into 50 µl. A 2x mastermix was prepared containing the appropriate volumes of 10x RT buffer, 25x dNTPs, 10x random primer, multicribe reverse transcriptase (50 U/µl) and nuclease-free H_2O. The mastermix (50 µl) was added to the RNA and the mixtures were heated at 25 °C for 10 minutes followed by 2 hours at 37 °C using a thermocycler (PTC-200 Peltier Thermocycler, Amersham, UK). Newly synthesized cDNA was stored long-term at -20 °C.

2.5.4 Real-time PCR

Real-time PCR primers were delivered as "Taqman Gene Expression Assays" for the rat genes listed in Table 2.2 (Applied Biosystems, USA). Real-time PCR was performed on Applied Biosystems 7300 Real-time PCR System with Applied Biosystems 7300 System SDS Software 1.3.1 in 96-well format and with 25 µl reaction volume per well. cDNA was mixed with PCR Mastermix Plus (Applied Biosystems, USA) and the respective gene primers. Rat β-actin (#4352340E; Applied Byosystems, USA) was used for normalization and each sample was measured in duplicate in a single real time PCR run. 40-45 cycles were run with the following parameters: 10 minutes at 95 °C and for each cycle 15 seconds at 95 °C for denaturation and 1 min at 60 °C for transcription. Analysis of gene expression values was performed using the efficiency-corrected comparative CT method, determining target gene expression relative to β-actin endogenous control.

Gene name	Taqman gene expression assay number	GenBank accession no.
MHCII	Rn01768597-m1	NM-198741.1
CD11b	Rn00709342_m1	NM-012711.1
iNOS	Rn00561646-m1	NM-012611.2
IL-1β	Rn00580432-m1	NM-031512.1
CD40	Rn00584362-m1	NM-053353.1
CD200	Rn00580478-m1	NM-031518.1

Table 2.2 Genes used for real-time PCR and respective assay number and GenBank accession no.

2.6 Analysis of cytokines

2.6.1 Preparation of samples

Analysis of cytokine concentration was carried out on hippocampal tissue which was dissected free from brain, cross chopped and frozen at -80 °C in Krebs solution containing 10% DMSO. Samples were washed three times in ice-cold Krebs solution and homogenized in Krebs solution using a 3 ml homogenizer. Protein concentration was assessed according to the assay described above (see section 2.3.3) and equalized using Krebs solution. Samples were stored at -80 °C until further required.

2.6.2 Analysis of IL-1β concentration

Analysis of IL-1β was carried out on supernatant of cortical glial cells and hippocampal homogenate using an Enzyme Linked Immunosorbent Assay (ELISA). 96-well plates (NUNC Immuno, Denmark) were incubated overnight at room temperature with capture antibody (100 µl; 0.8 µg/ml; goat anti-rat IL-1β in PBS). Plates were washed three times in PBS containing 0.05% Tween (PBS-T; Lennox, UK) and incubated at RT for 1 hour in blocking buffer (300 µl; PBS containing 1% BSA, 5% sucrose and 0.05% N_aN_3). A serial of dilutions (0-1000 pg/ml) of recombinant IL-1β (R&D System, USA) in PBS containing 1% BSA

was prepared to produce a standard curve. The samples were washed three times in PBS-T and standards and samples (100 µl) were added to each well in triplicate and incubated at RT for 2 hours. Plates were then washed in PBS-T and incubated for 2 hours with detecting antibody (100 µl; 350 ng/ml; biotinylated anti rat IL-1β IgG in PBS containing 1% BSA and 2% normal goat serum, NGS; R&D System, USA). Streptavidin horseradish-peroxidase (HRP) conjugated (R&D System, USA) was diluted in PBS containing 1% BSA to a concentration of 5 µl/ml. Plates were washed three times with PBS-T and 100 µl of the diluted streptavidin-HRP were added to each well and incubated for 20 minutes. After three washes with PBS-T plates were added with substrate solution (100 µl; 1:1 dilution of H_2O_2 and tetramethylbenzidine (TMB); R&D System, USA) and allowed to incubate in the dark for 20-30 minutes or until colour developed. The reaction was blocked by addition of H_2SO_4 (1 M) and plates were read immediately at 450 nm (Labsystem Multiskan RC, UK). A standard curve was made up by plotting the standards with their absorbance and interleukin concentration was expressed as pg IL-1β/ml or pg IL-1β/mg protein.

2.6.3 Analysis of IL-18 concentration

Analysis of IL-18 was carried out on supernatant of cortical glial cells using ELISA. 96-well plates were incubated overnight at 4 °C with capture antibody (100 µl; 2.5 µg/ml; anti-rat IL-18 in PBS). Plates were washed once in PBS-T and incubated at RT for 1 hour in blocking buffer (200 µl; PBS containing 0.5% BSA (w/v) and 0.1% Tween). A serial of dilutions (0-1000 pg/ml) of recombinant IL-18 (Biosource, USA) in PBS containing 0.5% BSA and 0.1% Tween, was prepared to produce a standard curve. The samples were washed once in PBS-T and standards and samples (100 µl) were added to each well in triplicate and immediately detection antibody was added into all wells (50 µl; 0.4 µg/ml; biotinylated anti rat IL-18 IgG in PBS containing 0.5% BSA and 0.1% Tween; Biosource, USA). After 2 hours incubation at RT, plates were washed five times in PBS-T and incubated for 30 minutes with streptavidin-HRP (100 µl; 1:3000 dilution in PBS containing 0.5% BSA and 0.1% Tween). After five washes with PBS-T plates were added with substrate solution (100 µl; 1:1 dilution of H_2O_2 and

TMB) and allowed to incubate in the dark for 20-30 minutes or until colour developed. The reaction was blocked by addition of H_2SO_4 (1 M) and plates were read immediately at 450 nm (Labsystem Multiskan RC, UK). A standard curve was made up by plotting the standards with their absorbance and interleukin concentration was expressed as pg IL-18/ml.

2.7 Immunohistochemistry

2.7.1 Preparation of brain slices

Sections were prepared from the portions of the brains which were coated in OCT, snap frozen in liquid nitrogen and stored at -80 °C. Sections (10 μm) were prepared using a cryostat (Leica, Meyer, UK), mounted on gelatine coated glass slides and stored at -20 °C for immunohistochemical analysis. Each slide contained three slices.

2.7.2 Immunostaining for MHCII

Frozen brain slices were fixed in ice-cold ethanol for 5 minutes and washed in Tris buffered saline (TBS; composition in mM: Tris HCl 20, NaCl 150). Samples were blocked in normal horse serum (NHS; 10:100 in 4% BSA-TBS; Vector, USA) for 30 minutes. The serum was removed and incubation continued overnight at 4 °C with primary antibody (mouse monoclonal anti-rat OX-6; 1:100 in 2% BSA-TBS; Serotec, UK). The following day the slices were washed in TBS and incubated for 2 hours with secondary antibody (biotinylated horse anti-mouse IgG; 1:200 in 2% BSA-TBS; Vector, USA) at RT. The samples were washed in TBS and the endogenous peroxidases were blocked by incubation for 15 minutes in 0.3% H_2O_2 (Sigma, UK) in TBS. Brain slices were washed three times for 5 minutes in TBS and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, USA). Colour was developed by incubation for 10 minutes with diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution). Slices were rinsed in double distilled water to terminate the reaction. Samples were counterstained with toluene blue, dehydrated in increasing concentrations of alcohol and mounted in DPX onto glass slides.

2.8 SDS-polyacrylamide gel electrophoresis

2.8.1 Preparation of samples

Analysis was carried out on hippocampal tissue which was dissected free from brain, cross chopped and frozen at -80 °C in Krebs solution containing 10% DMSO. Samples were thawed rapidly, washed three times in ice-cold Krebs solution and homogenized in Krebs solution using a 3 ml homogenizer. Protein concentration was assessed with protein quantification assay as described above (see section 2.3.3) and equalized accordingly using Krebs solution. Lithium dodecyl sulphate sample buffer (2.5 µl; NuPage, Invitrogen; UK) and reducing agent (1µl; NuPage, Invitrogen; UK) were added to each sample (6.5 µl) and the mixtures were heated at 70 °C for 10 minutes. Samples were stored at -80 °C.

2.8.2 Gel electrophoresis

Sample separation was carried out on 4-12% gradient SDS gels (NuPage Precast; Invitrogen, UK). These were mounted on an electrophoresis unit (Xcell II Surelock Mini Cell System, Invitrogen, UK) which was filled with NuPage running buffer (Invitrogen; UK). Samples (10 µg) and stained molecular weight marker (5 µl; Biorad, UK) were loaded into the wells and protein separation was carried out by application of 130 V for approximately 2 hours. The separation was terminated when the samples reached the bottom of the gels.

2.8.3 Western immunoblotting

After protein separation was achieved on SDS gels, samples were transferred onto nitrocellulose paper (0.45 µm pore size; Sigma, UK). The gel was placed on top of a nitrocellulose membrane and filter paper (Standard Grade, Whatman, UK) was placed on top and beneath the nitrocellulose and the gel forming a sandwich. Everything was soaked in transfer buffer (Invitrogen, UK) and placed in a blotting module which was filled with transfer buffer. The module was then placed into a transferring unit that was filled with distilled water. Sample transfer into nitrocellulose membrane was achieved through application of constant 30 V for 70 minutes. The nitrocellulose papers were removed from the sandwich and incubated for 2 hours at RT or overnight at 4 °C to eliminate non

specific bounding with a blocking solution consisting of PBS containing 5% dried milk (Marvel, UK) or 5% BSA.

2.8.4 Analysis of CD200

After blocking for two hours with 5% dried milk, the membranes were incubated with goat primary antibody (anti-rat IgG; 1:100 in TBS-T containing 1% dried milk; Santa Cruz, UK) overnight at 4 °C. Membranes were washed with TBS-T three times for 5 minutes and incubation continued for 1 hour with secondary antibody (HRP-conjugated anti-goat IgG; 1:1000 in TBS-T containing 1% dried milk; Sigma, UK). Prior to development of chemiluminescent signal, samples were washed for 6 times in TBS-T over a 1 hour period. Samples were incubated using the enhanced chemiluminescence (ECL; Amersham, UK) system for 1.5 minutes after which the membranes were exposed for 30 seconds to photographic film (Hyperfilm ECL, Amersham, UK) in the dark and the film developed using a Fuji X-ray processor.

2.8.5 Analysis of CD86

Nitrocellulose membranes were incubated with blocking buffer (5% BSA in TBS-T) for 2 hours at RT and left overnight at 4 °C with rabbit primary antibody (anti-rat IgG; 1:200 in 2% BSA TBS-T; Santa Cruz, UK). Membranes were washed with TBS-T and incubation continued for 1 hour with secondary antibody (anti-rabbit IgG; 1:1000 in 2% BSA TBS-T; Sigma, UK). Samples were washed and incubated with ECL for 1.5 minutes. The membranes were exposed for 1 minute to photographic film in the dark and the film developed.

2.8.6 Analysis of ICAM-1

Analysis of ICAM-1 protein density was carried out by blocking the membranes for non-specific binding with 5% BSA for 2 hours at RT. Incubation continued overnight at 4 °C with mouse primary antibody (anti-rat IgG; 1:200 in 2% BSA TBS-T; Santa Cruz; UK). Membranes were washed with TBS-T and incubated for 1 hour with secondary antibody (anti-mouse IgG; 1:1000 in 2% BSA in TBS-T; Sigma, UK). After 6 washes over a 1 hour period,

chemiluminescent signal was developed as described above and membranes were exposed to photographic film in the dark for 10 minutes after which the film was developed.

2.8.7 Analysis of actin

Following detection of target protein expression, samples were analysed for actin expression to assess loading of protein. The membranes were stripped by incubation for 5 minutes with stripping solution (1:10 in distilled water; Reblot Plus Antibody Stripping Solution, Chemicon, USA) and re-probed with mouse monoclonal primary antibody (anti-rat IgG; 1:5000 in 5% dried milk in TBS-T; Sigma, UK) for 1 hour at RT. Samples were washed 3 times in 15 minutes and incubation with secondary antibody (anti-rat IgG; 1:1000 in TBT-T containing 5% dried milk; Sigma, UK) was allowed for 1 hour. Membranes were washed with TBS-T and incubated for 1.5 minutes with ECL to allow chemiluminescent signal to develop. Membranes were exposed to photographic film in the dark for few seconds after which the film was developed.

2.9 Analysis of synaptic vesicle proteins

2.9.1 Preparation of synaptosomes

Analysis was carried out on hippocampal tissue which was dissected free from brain, cross chopped and stored at -80°C in Krebs solution containing 10% DMSO. Samples were washed three times in ice-cold Krebs Ca^{2+}solution and homogenized in lysis buffer (composition in mM: sucrose 320, HEPES 5; pH 7.4) containing aprotinin (2 μg/ml), leupeptin (10 μg/ml) and pepstatin (10 μg/ml) using a 1 ml homogenizer. Homogenised tissue was spun at 5000 xg for 15 minutes at 4 °C. Supernatant was centrifuged at 15,000 xg for 15 minutes at 4 °C. The pellets, crude synaptosomal fraction, were carefully resuspended in Krebs Ca^{2+} containing the cocktail of protease inhibitors (100 μl) and homogenised with 10 strokes. Protein concentration was assessed and equalized according to the assay described above (see section 2.3.3) using Krebs solution containing the cocktail of protease inhibitors. Samples were stored at -80 °C.

2.9.2 Western immunoblotting

The concentration of synaptic proteins synaptobrevin, synaptophysin, SNAP 25 and syntaxin were assessed from crude hippocampal synaptosomal preparation by gel electrophoresis and immunoblotting. Samples were prepared by addition of NuPAGE LDL sample buffer (Invitrogen, UK) containing NuPAGE reducing agent and heated at 70 °C for 10 minutes. Samples containing 3 or 5 μg of protein were loaded onto gradient 4-12% SDS gels (NuPAGE Novex Bis-Tris; Invitrogen, UK). Proteins were separated by application of a 130 V current constant for 90 minutes in the presence of NuPAGE MOPS SDS running buffer (Invitrogen, UK). The separated proteins were transferred onto nitrocellulose membrane (Whatman, Germany) at 30 V constant for 75 minutes in the presence of NuPAGE transfer buffer (Invitrogen, UK). The membranes were incubated for 2 hours in blocking buffer consisting of 5% dried milk powder in TBS-T in the case of syntaxin and actin, and 2% dried milk in the case of the other proteins.

2.9.2.1 Analysis of synaptophysin

Membranes were incubated in primary antibody mouse monoclonal anti-synaptophysin (1:500 in TBS-T containing 2% dried milk; Sigma, UK) overnight at 4 °C. Membranes were washed three times for 5 minutes in TBS-T and incubated in secondary antibody (anti-mouse IgG; 1:400 in TBS-T containing 2% dried milk; Sigma, UK) for 2 hours at RT. Membranes were washed three times for 10 minutes with TBS-T followed by 10 minutes wash with dH_2O. Protein complexes were visualised after 90 seconds incubation with ECL. Membranes were exposed for 5 seconds to photographic film in the dark and the film developed.

2.9.2.2 Analysis of syntaxin

Samples were incubated with monoclonal primary antibody (1:10000 in TBS-T with 5% milk; Sigma, UK) for 2 hours at RT. After three washes of 5 minutes incubation continued for 1hour at RT with secondary antibody (anti-mouse IgG; 1:1000 in TBS-T with 5% milk; Sigma, UK). Membranes were washed and the proteins visualised after 90 seconds incubation with ECL. The

photographic film was developed after exposure for 1 second to the membrane and developed with a photographic processor.

2.9.2.3 Analysis of SNAP 25

In the case of SNAP 25 membranes were incubated in mouse monoclonal primary antibody (1:1000 in TBS-T containing 2% dried milk; Abcam, UK) overnight at 4 °C. The membranes were washed with TBS-T and placed in secondary antibody (anti-mouse IgG; 1:1000 in TBS-T containing 2% milk; Sigma, UK) for 2 hours at RT. Samples were washed with TBS-T followed by 10 minutes wash with dH_2O before the protein complexes were visualised by 1.5 minutes incubation in ECL. Membranes were exposed for 20 seconds to photographic film and the film developed.

2.9.2.4 Densitometry

In all the cases the resultant protein bands were visualised under UV light and photographed using a UV transilluminator. Quantification of the bands was performed by densitometric analysis using the ZERO-Dscan Image Analysis System. Values are expressed as a ratio of target protein to actin and are presented as arbitrary units.

2.10 Analysis of caspase-3 activity

Caspase-3 activity was measured in hippocampal homogenate prepared as previously described (see section 2.6.1) using Caspase-3 Drug Discovery Kit (AK-700 a QuantiZyme Assay System, Biomol, UK) in 96 well format, according to the protocol provided by the manufacturer. Briefly, test samples (25 µl) were added in duplicate to each well followed by addition of caspase-3 (25 µl; 2 U/µl). The reaction was started by addition of Ac-DEVD-pNA substrate (50 µl; 200 µM) and the plate was read at 1 minute intervals for a total of 10 minutes (during which the reaction is linear) at 405 nm in a microplate reader. Enzyme activity was calculated with reference to a conversion factor, calculated from the optical density of the calibration standard, and a slope (mOD/min). Values are expressed as nmol/mg protein/minute.

2.11 Statistical analysis

Data are expressed as means ± standard error of the means (SEM). A one-way analysis of variance (ANOVA) or a two-way ANOVA was performed where appropriate to determine whether significant differences existed between conditions. If any significant change was detected, post hoc comparisons were performed using Newman-Keuls or Dunnett's test. Student's t-test for unpaired means was also performed when only two treatment groups where involved in the experiment. Data were deemed significant when $p<0.05$ (GraphPad Prism, USA).

Chapter 3

Analysis of the effect of neurons on LPS-activated microglia and of co-treatment with LPS and Aβ on cultured cortical glia

3.1 Introduction

One mechanism involved in maintaining microglia in a resting state is the interaction between CD200, a membrane glycoprotein mainly expressed on neurons, and its receptor CD200R found on the surface of cells of the myeloid lineage, including microglia (Copland et al., 2007, for review see Wright et al., 2003). Indeed, mice lacking CD200 display more numerous activated microglia and increased susceptibility to autoimmune diseases (Hoek et al., 2000; Broderick et al., 2002; Copland et al., 2007).

In normal physiological conditions microglia act as surveillance cells and are present in a quiescent state typified by low expression of cell surface antigens and reduced production of cytokines (for review see Aloisi, 2001). Exposure of microglia to pro-inflammatory stimuli, such as LPS, induces microglial activation and production of pro-inflammatory cytokines such as IL-1β and IL-18 (Conti et al., 1999). Activated microglia are also characterised by increased expression of MHCII antigens which, by interaction with TCR/CD3 complex, allow for antigen presentation to infiltrating T cells (for review see Aloisi, 2001). Expression of this molecule is minimal or absent in normal conditions but it is readily up-regulated upon inflammatory stimulation (Lyons et al., 2007a; Lynch et al., 2007; for review see Aloisi et al., 2000b).

Exposure of microglia to Aβ has been shown to initiate a cascade of events leading to the synthesis of toxic products such as IL-1β (Combs et al., 2001; Butovsky et al., 2005; Walker et al., 2001). The evidence has suggested that LPS is likely to exacerbate the response of microglia to Aβ by inducing increased expression of ICE and subsequent production of IL-1β (Yao and Johnson, 1997). In addition, it has been suggested that LPS and Aβ act in synergy to induce activation of microglial cells and production of pro-inflammatory cytokines (Lotz et al., 2005; Gasic-Milenkovic et al., 2003).

3.2 Methods and materials

The *in vitro* studies presented were carried out on cultured cortical glia established from new-born Wistar rats and grown in the presence of DMEM for 13 days (see section 2.1.3). Cultured neurons were also used and they were established from the hippocampi of new-born Wistar rats and grown in the presence of NBM for 5 days. For the first study mixed glia were incubated in the presence of neurons ($0.15x10^6$ cells/ml) for 4 hours after which neurons were removed and glia were treated with LPS (100 ng/ml) for a further 24 hours. In the case of the LPS dose-response study, glial cells were treated for 24 hours with a serial of LPS dilutions (5, 20, 50 and 100 ng/ml) wherease in the case of the LPS+Aβ study mixed glia were treated with a combination of LPS (10 ng/ml) and Aβ (2 μM) for 24 hours.

At the end of each treatment, supernatants were collected for analysis of cytokine concentration and cells harvested for RNA extraction (see section 2.1.5). Extraction of total RNA was carried out on harvested cells in Tri-reagent. Chloroform was added to allow separation of an aqueous layer rich in RNA which was precipitated by addition of isopropanol. Samples were centrifuged at 12000 xg for 10 minutes, the supernatants were removed and the resulting pellets were washed with 75% alcohol. Pellets were resuspended in DEPC-treated water and analysed for RNA integrity by electrophoresis. Synthesis of cDNA was performed by reverse transcription of RNA by addition of superscript reverse transcriptase enzyme. Newly synthesised cDNA was then amplified by RT-PCR (see section 2.4).

Analysis of IL-1β and IL-18 protein concentrations was performed on supernatants by ELISAs (see section 2.6).

3.3 Results

Pre-treatment of glia with neurons reverses the LPS-induced microglial activation

One proposed mechanism involved in maintaining microglia in a resting state includes the interaction between CD200, expressed on neurons, and its receptor CD200R found on microglial cells (Copland et al., 2007). Here the expression of CD200R mRNA was analysed by RT-PCR to investigate whether LPS-induced microglial activation may be related to modulation of the CD200/CD200R interaction. Figure 3.1 (A) shows that LPS treatment induced a significant increase of mRNA expression of MHCII, a marker of activated microglia ($p<0.05$, student's t-test for independent means). Figure 3.1 (B) shows that the LPS-induced microglial activation was associated with a significant decrease of CD200R mRNA expression ($p<0.05$, student's t-test for independent means).

In order to further investigate the role of CD200/CD200R interaction in the modulation of microglial activation, mixed glial cells were incubated in the presence of neurons followed by treatment with LPS. Analysis of markers of microglial activation was first achieved by analysis of MHCII and IL-1β mRNA. Results shown in figure 3.2 (A) demonstrate that treatment of cultured glia with LPS induced a significant increase of MHCII mRNA expression ($F_{(1,12)}=9.7$, $p<0.01$, ANOVA). Pre-treatment of glial cells with neurons induced a 1.7 fold decrease in mean values of MHCII mRNA expression, however, this effect did not reach statistical significance. Results presented in figure 3.2 (B) show that treatment with LPS induced a significant increase in IL-1β mRNA expression in glial cells ($F_{(1,12)}=9.9$, $p<0.01$, ANOVA). Neuman-Keuls analysis revealed that pre-treatment of glia with neurons significantly attenuated the LPS-induced increase in IL-1β mRNA.

Amongst the many functional markers of microglial activation are the pro-inflammatory cytokines IL-1β and IL-18 which have been shown to be released by cultured glia, mainly microglia, in response to pro-inflammatory stimuli (Conti et al., 1999; Moore et al., 2005). Here IL-1β and IL-18 protein concentration was

measured by ELISA in medium prepared from glia which were incubated in the presence of neurons for 4 hours followed by treatment with LPS for 24 hours. Results presented in figure 3.3 (A) show that treatment of glia with LPS significantly increased the levels of IL-1β concentration ($F_{(1,10)}$=46.39, P<0.0001, ANOVA). Pre-treatment with neurons significantly reversed the LPS-induced increase in IL-1β protein concentration ($F_{(1,10)}$=18.37, p<0.01, ANOVA). The ability of neurons to modulate glial activation in response to LPS was further investigated by analysis of IL-18 concentration. Figure 3.3 (B) shows that treatment of glia with LPS induced a significant increase in IL-18 protein concentration ($F_{(1,12)}$=21.61, p<0.05, ANOVA). Pre-treatment with neurons significantly attenuated the LPS-induced increase in IL-18 protein concentration ($F_{(1,12)}$=6.5, p<0.05, ANOVA).

Effects of LPS and Aβ, alone and in combination, on glial cells

The evidence suggests that LPS *in vitro* exacerbates the response of glial cells to Aβ possibly by stimulating the production of IL-1β (Yao and Johnson, 1997). Here it was of interest to understand whether LPS and Aβ may act in synergy to induce microglial activation assessed by analysis of MHCII mRNA expression and IL-1β release. In a first set of experiments mixed glial cells were treated with a series of LPS concentrations (5-100 ng/ml) in order to obtain the concentration of LPS required to initiate a pro-inflammatory response. Figure 3.4 shows that IL-1β concentration was significantly increased by treatment with LPS from a concentration of 20 ng/ml ($F_{(4,29)}$=7.462, p<0.001; ANOVA).

To study the possibility of a synergistic effect between LPS and Aβ, LPS was used at the concentration of 10 ng/ml in combination with Aβ at 2 μM, a threshold concentration which in this laboratory has been shown to induce a pro-inflammatory response in glia (Lyons et al., 2007a). Results presented in figure 3.5 (A) show that incubation of mixed glia with LPS resulted in a significant increase in MHCII mRNA expression compared to control ($F_{(1,20)}$=2.68, p<0.0001; ANOVA). However, a significant interactive effect between LPS and Aβ was not detected. By contrast, data reported in figure 3.5 (B) show that IL-1β protein concentration was significantly increased in the presence of Aβ ($F_{(1,18)}$=8.26, p<0.05, ANOVA), independent of LPS. Neuman-Keuls analysis

revealed that IL-1β concentration was increased only in the group treated with a combination of LPS and Aβ.

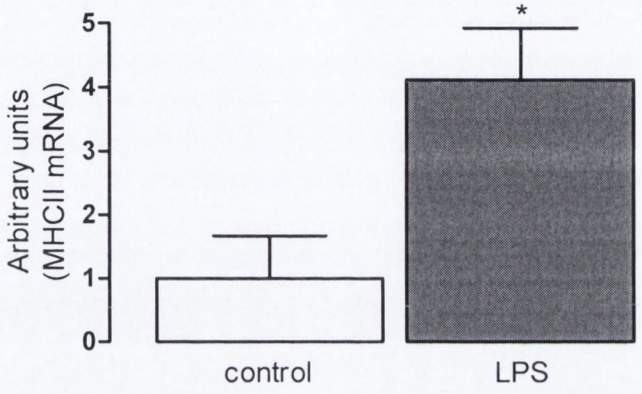
Figures

Figure 3.1 LPS treatment increases MHCII mRNA and decreases CD200R mRNA expression

A) Treatment of cultured mixed glia with LPS (100 ng/ml) induced a significant increase of MHCII mRNA expression (Student t-test for independent means, *$p<0.05$, n=3-4). Data are expressed as a ratio of MHCII to β-actin and are means ± SEM.

B) Treatment of cultured mixed glia with LPS (100 ng/ml) induced a significant decrease of CD200R mRNA expression (Student t-test for independent means, *$p<0.05$, n=3-4). Data are expressed as a ratio of CD200R to β-actin and are means ± SEM.

A)


Arbitrary units
(MHCII mRNA)
5
4
3
2
1
0
*
control
LPS

B)

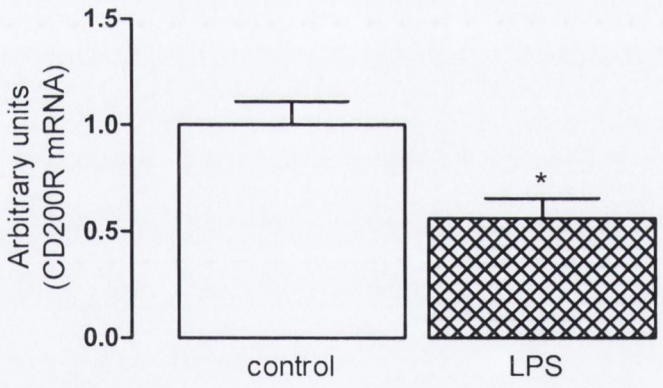
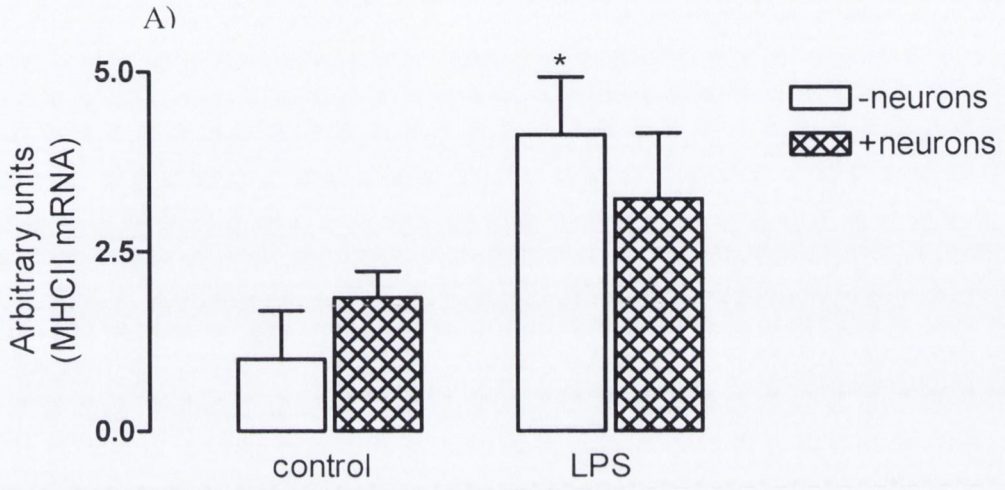

Arbitrary units
(CD200R mRNA)
1.5
1.0
0.5
0.0
*
control
LPS

Figure 3.2 Treatment of glia with neurons reverses the LPS-induced increase in IL-1β but not MHCII mRNA expression

A) A two-way ANOVA showed that treatment of glia with LPS (100 ng/ml) significantly increased MHCII mRNA expression ($F_{(1,12)}$=9.7, $p<0.01$, n=4). This change was not reversed by treatment of glia with neurons. Data are expressed as a ratio of MHCII to β-actin and are means ± SEM. *$p<0.05$ vs control (Newman-Keuls test).

B) A two-way ANOVA showed that treatment of glia with LPS (100 ng/ml) significantly increased IL-1β mRNA expression ($F_{(1,12)}$=9.9, $p<0.01$, n=4). Newman-Keuls analysis revealed that treatment of glia with neurons significantly attenuated the LPS-induced increase in IL-1β mRNA expression. Data are expressed as a ratio of IL-1β to β-actin and are means ± SEM. **$p<0.001$ vs control, +$p<0.05$ vs LPS, (Newman-Keuls test).


A)
5.0
2.5
0.0
Arbitrary units
(MHCII mRNA)
*
-neurons
+neurons
control
LPS

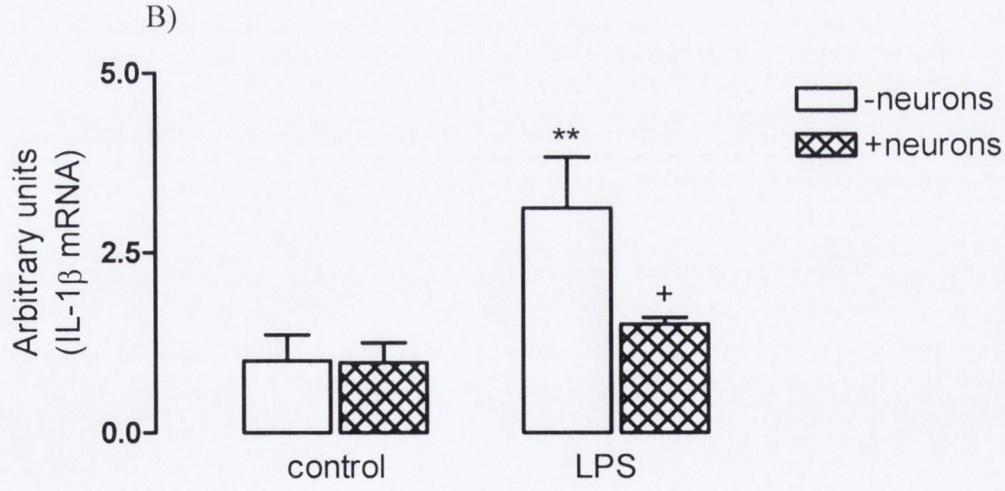
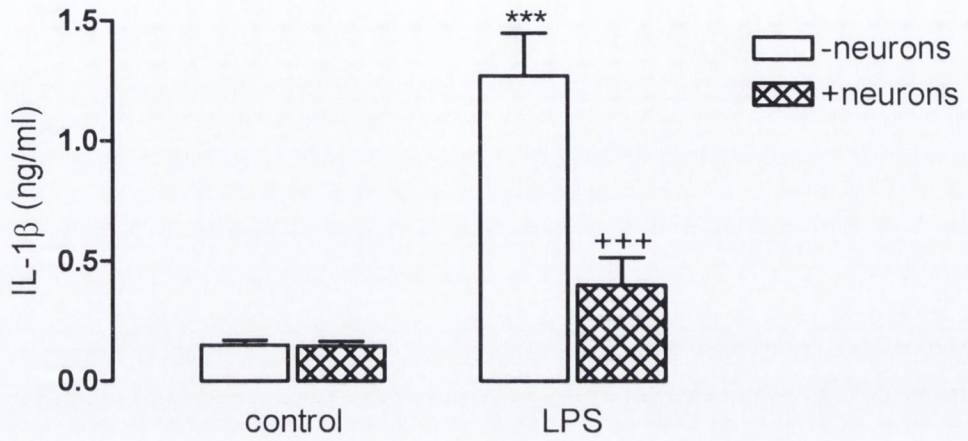

B)
5.0
2.5
0.0
Arbitrary units
(IL-1β mRNA)
**
+
-neurons
+neurons
control
LPS

Figure 3.3 Treatment of glia with neurons reverses the LPS-induced increase in IL-1β and IL-18

A) A two-way ANOVA showed that treatment of glia with LPS (100 ng/ml) significantly increased IL-1β concentration ($F_{(1,10)}$=46.39, P<0.0001, n=3-4). Treatment of glia with neurons significantly reversed the LPS-induced increase in IL-1β protein concentration ($F_{(1,10)}$=18.37, p<0.01). Data are expressed as means ± SEM ***p<0.001 vs control, +++p<0.001 vs LPS (Newman-Keuls test).

B) A two-way ANOVA showed that treatment of glia with LPS (100 ng/ml) significantly increased IL-18 concentration ($F_{(1,12)}$=21.61, p<0.05, ANOVA, n=4). Treatment of glia with neurons significantly reversed the LPS-induced increase in IL-18 protein concentration ($F_{(1,12)}$=6.5, p<0.05). Data are expressed as means ± SEM ***p<0.001 vs control, ++p<0.01 vs LPS (Newman-Keuls test).

A)


1.5
1.0
0.5
0.0
IL-1β (ng/ml)
***
+++
-neurons
+neurons
control
LPS

B)

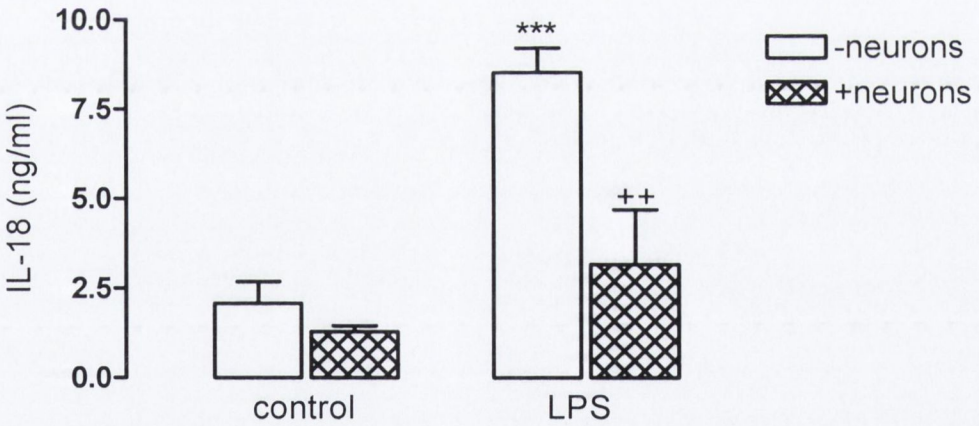

10.0
7.5
5.0
2.5
0.0
IL-18 (ng/ml)
***
++
-neurons
+neurons
control
LPS

Figure 3.4 LPS at low concentrations induces IL-1β release from glial cells

A one-way ANOVA showed that treatment of glia with LPS (20-100 ng/ml) significantly increased IL-1β concentration compared to untreated controls ($F_{(4,29)}$=7.46, $p<0.001$, n=6). Data are expressed as mean ± SEM. **$p<0.01$ vs control (Dunnett's test).

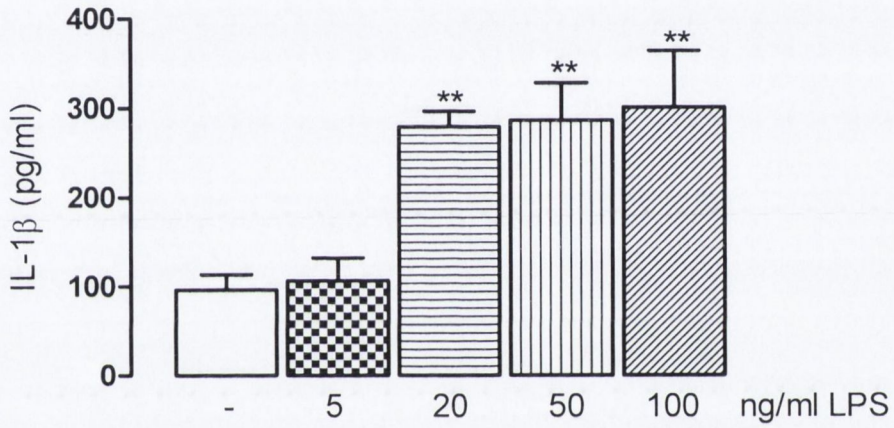
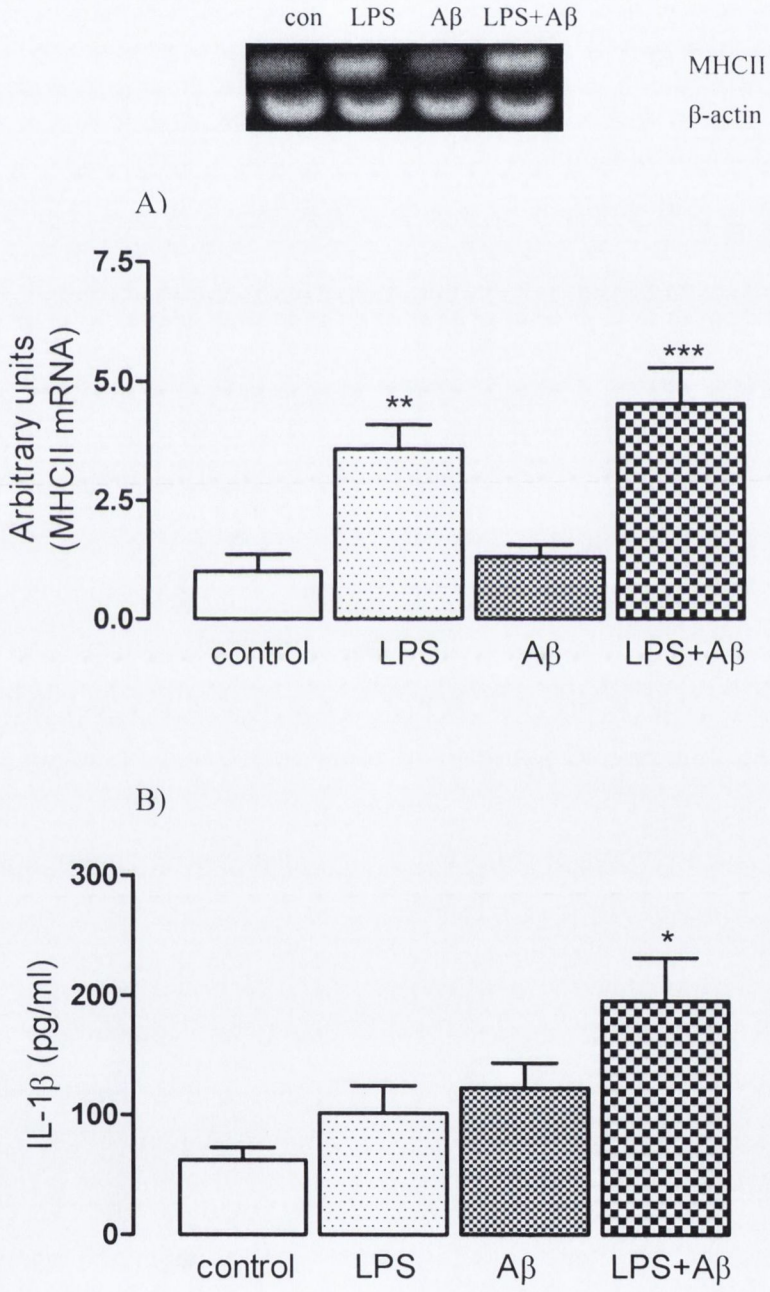

400
300
200
100
0
IL-1β (pg/ml)
**
**
**
-
5
20
50
100
ng/ml LPS

Figure 3.5 LPS and Aβ induce MHCII mRNA expression and IL-β production

A) A two-way ANOVA showed that treatment of glia with LPS (10 ng/ml) induced a significant increase of MHCII mRNA expression ($F_{(1,20)}$=32.68, $p<0.0001$, n=6) independent of Aβ. Data are expressed as a ratio of MHCII to β-actin and are means ± SEM. ***$p<0.001$ vs control, **$p<0.01$ vs control (Newman-Keuls test).

B) A two-way ANOVA showed that treatment of glia with Aβ (2 μM) induced a significant increase of IL-1β concentration ($F_{(1,18)}$=8.26, $p<0.05$, n=4-6) independent of LPS. However, post hoc Neuman Keuls test analysis showed that only LPS and Aβ in combination induced a significant increase of IL-1β levels compared to control. Data are expressed as means ± SEM. *$p<0.05$ vs control (Newman-Keuls test).


con
LPS
Aβ
LPS+Aβ
MHCII
β-actin
A)
Arbitrary units
(MHCII mRNA)
7.5
5.0
2.5
0.0
**
***
control
LPS
Aβ
LPS+Aβ
B)
IL-1β (pg/ml)
300
200
100
0
*
control
LPS
Aβ
LPS+Aβ

3.4 Discussion

The aim of this study was to analyse the effect of neuronal pre-treatment on LPS-induced activation of microglia. The results obtained show that treatment of glia with neurons reversed the LPS-induced microglial activation. Microglial activation, associated with increased expression of MHCII mRNA, was coupled with a significant decrease in CD200R mRNA expression. The data demonstrate that the pro-inflammatory effect of LPS is exerted at a transcriptional level and suggest that LPS may act to induce immune response by compromising CD200/CD200R interaction.

It is known that within the CNS there are intrinsic inhibitory factors providing a beneficial mechanism in restricting disease progression and controlling immune activity. Amongst these mechanisms is the interaction between CD200, which is expressed on neurons, and its cognate receptor CD200R found on cells of the myeloid/monocyte lineage (Copland et al., 2007). TLRs regulate innate immune responses and are widely expressed on microglia as well as astrocytes. The evidence provided here, demonstrating that treatment of glia with neurons reversed the LPS-induced microglial activation, further consolidates the notion that CD200/CD200R interaction is involved in the regulation of microglial activation and supports the data from a recent study by Lyons and colleagues (2007b). A specific role for neuronal–glial interaction in maintaining glial cells in a quiescent state was demonstrated in that study. The data are also consistent with previous reports describing exaggerated microglial activation in response to pro-inflammatory stimuli and increased susceptibility to autoimmune diseases in mice lacking CD200 (Hoek et al., 2000; Broderick et al., 2002; Copland et al., 2007). Addition of antibodies raised against CD200 to the neuronal-glial preparation would validate the results obtained by confirming the role of CD200 in the attenuation of the LPS-induced changes in glia. Moreover, the LPS-induced increase of IL-1β mRNA and protein confirms previous findings which demonstrate the importance of IL-1β in the LPS-induced pro-inflammatory response (Gayle et al., 2002; Lynch et al., 2004). However, it is important to point out that the glial cultures used for this experiment included microglia as well as astrocytes. It is thereby possible that the LPS-induced increase of IL-1β signal may come from astrocytes and not only from microglia. Indeed astrocytes express

TLR and are able to produce IL-1β (Corsini et al., 1996). Attention must be paid when assessing microglial activation by analysis of molecules that are also produced by other cell types. In addition, although the evidence suggests a clear role for neuronal CD200 in the regulation of glial activation, neither the neuronal phenotype nor the absence of other cell types was assessed.

It has been suggested that LPS acts to induce IL-1R1 mediated signalling pathway by stimulating IL-1β production. This, in turn, leads to phosphorylation of MAPKs JNK and p38 followed by activation of caspase-3 and subsequent deterioration of cell function (Kelly et al., 2003; Nolan et al., 2003; Barry et al., 2005). Among the mechanisms involved in LPS signalling is activation of NF-κB, a transcription factor that regulates several cell functions including cell growth, differentiation and apoptosis. LPS binds to its receptor TLR4 and induces activation of downstream molecules which eventually lead to removal of the inhibitory IκB subunit and translocation of NF-κB to the nucleus where it modulates gene expression (Griffin and Moynagh, 2006; Miggin et al., 2007). As a consequence, pro-inflammatory cytokines are produced in response to LPS (Kim et al., 2007a; Li et al., 2007).

Analysis of MHCII mRNA expression revealed that there was a significant LPS-induced increase which, perhaps surprisingly, was not reversed by pre-treatment with neurons. This is in contrast with results published in a recent work by Lyons and colleagues (2007b) which demonstrated that the addition of neurons to Aβ-treated glia decreased MHCII mRNA expression. In that study cultured glia were cotreated with neurons and Aβ whereas in the present study mixed glial cells were pre-treated with neurons and only after the supernatant was removed LPS was added to the cells. Although it would be natural to speculate that variations in the duration of neurons/glia treatment may account for this difference it is important to consider that neurons most likely remained attached to the glia culture after their addition, however this was not assessed. Thus, no real difference exists between the two experiments, apart from an extra pre-incubation of 4 hours in the study presented here. It should be noted that there was marked variations between observations (as indicated by the high SEM values) and this probably impacted on the stats. It has been mentioned that IL-1β is produced in response to LPS by cells other than microglia, such as astrocytes, however microglia are considered the main source of this cytokine and increased

concentration of IL-1β has often been associated with increased microglial activation. By contrast, it is arguable whether MHCII mRNA expression is the most reliable marker of microglial activation. The process of microglial activation includes several stages each of them characterized by expression of a large array of molecules and changes in microglial activation state may not necessarily involve the antigen presentation pathway which requires interaction between the T cell receptor and the processed antigen bound to MHCII (Beyer et al., 2000; for review see Aloisi, 2001). MHCII is a surface marker important in immune regulation which is constitutively expressed at low levels on ramified quiescent microglia (for review see Gehrmann et al., 1995). However, in response to a variety of stimuli, MHCII expression is rapidly upregulated and enables presentation of processed antigens to infiltrating T cells (Carson et al., 1998).

Treatment of glia with LPS induced a significant increase of the levels of IL-18 concentration consistent with other reports showing an LPS-induced expression of the pro-inflammatory cytokine (Conti, 1999). IL-18 belongs to the IL-1 family and, similar to IL-1β, it has been shown to exert an inhibitory effect on LTP in the rat hippocampus in addition to contributing to the upregulated microglial activation observed in the brain of aged animals (Curran and O'Connor, 2001; Griffin et al., 2006). Since it has been shown that IL-18 is mainly produced by activated microglia (Mori et al., 2001), levels of cytokine concentration were measured to further analyse whether pre-treatment with neurons could reverse the LPS-induced microglial activation. Consistent with the IL-1β results, incubation of glia with neurons reversed the increase of IL-18 expression caused by LPS, suggesting that neuronal-glial interaction is necessary for maintaining microglia in a resting state.

Minogue and colleagues (2007) reported that the effect of Aβ was more pronounced in aged compared to young rats suggesting that in conditions with underling inflammation, such us the aged brain, the response to Aβ is more robust. I considered that Aβ might also exert an additive effect with LPS and therefore, in a second set of experiments, the effect of LPS and Aβ, alone and in combination, were analysed on cultured cortical glia. The data demonstrate that exposure of mixed glia to LPS induced a significant increase of MHCII mRNA expression that was independent of Aβ. However, analysis of IL-1β protein concentration revealed that only the combination of LPS and Aβ induced a significant increase

of the cytokine expression compared to control. Although it has been argued that Aβ is not a potent immunestimulator *per se* (Veerhuis et al., 2003), the same concentration of Aβ used here has previously proven effective to induce glial activation, as assessed by increased MHCII mRNA expression and IL-1β and TNFα protein levels (Lyons et al., 2007a; Lyons et al., 2007b). It is possible that differences between batches of $A\beta_{1-42}$ may account for the different result. Moreover, it is possible that differences exist between the effect induced by $A\beta_{1-40}$ and the effect induced by $A\beta_{1-42}$, as suggested by Minogue and colleagues (2007). The authors showed that 5 μM of fibrillar $A\beta_{1-40}$ was able to induce microglial activation, as assessed by analysis of IL-1β mRNA and protein, whereas in the present study fibrillar $A\beta_{1-42}$ at the concentration of 2 μM did not induce up regulation of the cytokine.

The results obtained also suggest that LPS and Aβ exerted an additive effect on IL-1β concentration inducing a significant increase of cytokine level which was not observed in the case of either treatment alone. In order to be converted into the active form IL-1β requires cleavage of the pro IL-1β form by ICE (for review see Rothwell, 2003). Evidence suggests that LPS *in vitro* exaggerates the response of glial cells to Aβ and it has been suggested that the mechanism by which LPS exerts its action involves increased expression of ICE and the subsequent release of the pro-inflammatory cytokine IL-1β (Lorton et al., 1996; Yao and Johnson, 1997). Data here confirm findings from other laboratories demonstrating that LPS and Aβ in combination were more effective in stimulating IL-1β and TNFα release than either compound alone (Gasic-Milenkovic et al., 2003; Lotz et al., 2005). In this experiment the LPS concentration was chosen on the basis of results from an *in vitro* dose-response study in which very low LPS concentrations were shown to be able to induce MHCII mRNA expression (data not shown) but not IL-1β release. A concentration intermediate between those concentrations and the concentration which induced maximal IL-1β production was considered appropriate to induce priming of microglial cell. In a recent paper it was suggested that pro-inflammatory conditions may promote the toxic effect of APP on neuronal cells (von Bernhardi et al., 2007). The authors showed that treatment of cultured microglia with a combination of APP and LPS+IFNγ compromised the phagocytic activity of the cells and enhanced their reactivity to

APP. It was proposed that these "overstimulated" microglia are responsible for the production of toxic mediators which affect neuronal function and survival. This situation resembles that of the AD brain where activated microglia surround Aβ deposits and, although expressing a pro-inflammatory profile, are not able to phagocytose and clear Aβ (Arends et al., 2000). According to these data, it is suggested that microglia become activated and reactive only in the presence of certain specific inflammatory conditions that can also overcome the modulatory effect of astrocytes on activated microglia. Indeed, it has been shown that Aβ-induced microglial activation may be modulated by astrocytes (von Bernhardi and Eugenin, 2004). However, this modulatory effect was lost in the presence of LPS+IFNγ suggesting that, under specific conditions, microglia do not respond to the regulatory effect of astrocytes and induce an inflammatory response when exposed to Aβ (von Bernhardi and Eugenin, 2004). The additive effect of LPS and Aβ described here is generally consistent with these findings and is supported by previous reports which demonstrated a synergistic action of Aβ and IFNγ on microglial release of NO (Goodwin et al., 1995; Baron et al., 2000).

IFNγ is a potent microglial stimulator and the age-related increased microglial activation has been associated with increased levels of IFNγ concentration (Griffin et al., 2006). Further, incubation of glial cells with IFNγ has been shown to upregulate MHCII mRNA expression and IL-1β concentration (Delgado, 2003; Maher et al., 2006; Moore et al., 2007). In the present study LPS was used because of its ability to stimulate microglial activation and in order to investigate whether an additive effect between endogenous and exogenous stimuli renders microglia more vulnerable to inflammatory stimuli. It has been reported that systemic administration of LPS resulted in compromised APP processing, thereby inducing increased production and accumulation of Aβ (Sheng et al., 2003). Similarly, treatment of cultured glia with LPS resulted in increased APP release (Small et al., 2005). Given that LPS stimulates microglial activation in a similar manner to IFNγ, it was predicted that treatment of cultured glia with a combination of Aβ and LPS would lead to similar response as compared to cells exposed to Aβ and IFNγ in combination. However, further analyses assessing iNOS expression and NO release would be required to support this hypothesis. According to Perry and colleagues (2003) intercurrent infection can lead to exacerbation of neurodegenerative disorders and, although compelling evidence

suggests a role for inflammation in the pathogenesis and progression of AD, it has been reported that a certain degree of inflammation may aid in Aβ clearance. However, mice lacking TLRs have been shown to have increased levels of Aβ in the cerebrospinal fluid and activation of microglia with LPS has been shown to boost their uptake of Aβ (Tahara et al., 2006). Indeed, microglia have been reported to efficiently phagocytose fibrillar and soluble forms of Aβ *in vitro* and *in vitro* although there is contrasting data about their ability to fully degrade it (Shaffer et al., 1995; Paresce et al., 1996; Paresce et al., 1997; Kopec and Carroll, 1998; Weldon et al., 1998).

In conclusion, the data reported confirm the expression of CD200R on glial cells and suggest that CD200/CD200R interaction contributes to the regulation of glial activation. Specifically, while LPS increased microglial activation the evidence suggests it decreased CD200R mRNA and Aβ has been shown to exert somewhat similar effects (Lyons et al., 2007a). The data presented here indicate that LPS and Aβ seemed to exert a certain degree of synergy in inducing IL-1β release. Thus, the findings support the notion that inflammation concurring with the presence of Aβ may result in exaggerated inflammatory response which, in turn, may be responsible for the progression of AD. This increased vulnerability of microglia to inflammatory stimuli may be caused, at least in part, by down-regulation of anti-inflammatory mechanisms such as CD200.

Chapter 4

Analysis of the effect of acute injection of Aβ on rat hippocampus

4.1 Introduction

Growing evidence suggests that the decline in memory which precedes neurodegeneration in AD may be due to a dysfunction of synaptic plasticity. It has been shown that acute exposure to Aβ inhibits LTP, which represents the cellular basis for certain types of memory and learning (Chen et al., 2000; Chen et al., 2002; Wang et al., 2002; Zhao et al., 2004). Although the evidence suggests a role for oligomeric forms of Aβ as a major contributor to impaired memory (Walsh et al., 2002; Klyubin et al. 2005; Yun et al., 2006), recent studies demonstrate that fibrillar aggregates of Aβ exert inhibitory effects on hippocampal LTP when acutely administered to the rat brain (Clarke et al., 2007; Minogue et al., 2007).

It has been shown that acute application of diverse Aβ species induced LTP impairment through mechanisms affecting Ca^{2+} homeostasis suggesting a direct effect of Aβ on neuronal functioning and survival (Chen et al., 2000; Freir et al., 2001; Chen et al., 2002; Wang et al., 2004b). Neurotransmitter release is achieved by fast exocytosis from the presynaptic membrane via a Ca^{2+}-dependent mechanism involving fusion of the synaptic vesicle membrane with the plasma membrane. Formation of complexes between these two membranes is regulated by the SNARE proteins and a further vesicle-associated protein known as synaptophysin (Pyle et al., 2000; Sara et al., 2005; Aravanis et al., 2003; for review see Schweizer and Ryan, 2006). Evidence suggests that LTP maintenance is associated with increased synthesis of proteins involved in neurotransmitter release (Kelly et al., 2000) leading to the hypothesis that inhibition of this mechanism may impact on transmitter release and hence LTP maintenance. In addition, it has been suggested that Aβ-mediated impairment of LTP involves microglial activation which, in turn, results in the production of toxic mediators responsible for neuronal damage (Wang et al., 2004a; Wang et al, 2005; Clarke et al., 2007; Minogue et al., 2007).

4.2 Methods and materials

Young male Wistar rats were injected icv with Aβ at low (18.94 μM and 26.6 μM $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) and high (84 μM and 116 μM, $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) concentration. 4 hours after the injection animals were assessed for their ability to sustain LTP (see section 2.2.7). Injection of Aβ, LTP recording and dissection of tissue was performed in conjuction with the PhD student Anne-Marie Miller. The results from the group treated with the lower concentration of $A\beta_{1-40}$+$A\beta_{1-42}$ (45.5 μM) are the same as those presented in chapter 6. After a period of stabilisation, test shocks (0.033 Hz) were delivered at 30 seconds intervals and recorded for 15 minutes before and 45 minutes after tetanic stimulation (250 Hz for 200 ms). Animals were killed by decapitation and the hippocampi dissected free: an aliquot was snap frozen in liquid nitrogen for RNA extraction while the rest of the tissue was cross-chopped and stored at -80 °C in 10% DMSO Krebs solution.

Analysis of mRNA expression of markers of microglial activation was performed by real time PCR (see section 2.5). Total RNA was extracted from hippocampal tissue using a Nucleospin Kit. The obtained pellets of RNA were resuspended in RNase-free water and analysed for RNA integrity by electrophoresis. Concentration of RNA was measured using a spectrophotometer. Total RNA (1 μg) was reverse transcribed into cDNA using high-capacity cDNA archieve kit according to the protocol provided by the manufacturer. Real-time PCR primers were delivered as "Taqman Gene Expression Assays" for the rat genes listed in Table 2.2. Real-time PCR was performed on Applied Biosystems 7300 Real-time PCR System with Applied Biosystems 7300 System SDS Software 1.3.1. Rat β-actin was used for normalization.

The concentration of synaptic proteins synaptobrevin, SNAP 25 and syntaxin were assessed from crude hippocampal synaptosomal preparation by gel electrophoresis and immunoblotting (see section 2.9).

Analysis of CD86 and ICAM-1 was performed on hippocampal homogenates by western immunoblotting (see section 2.8).

IL-1β protein concentration was assessed by ELISA (see section 2.6) on hippocampal homogenates which were also analysed for caspase-3 activity using

Caspase-3 Drug Discovery Kit, according to the protocol provided by the manufacturer (see section 2.10).

4.3 Results

Acute injection of Aβ attenuates LTP

Evidence from this laboratory suggests that Aβ injection impairs LTP maintenance in the dentate gyrus (Lynch et al et al., 2006; Clarke et al., 2007). However, these studies only analysed the effects of just one Aβ species, either $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$. Given that in the AD brain both species of peptide are found, experiments were performed to study the effects of acute icv injection of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in combination. Results presented in figure 4.1 show an increase in the mean percentage change in population EPSP slope in all groups following a delivery of high frequency train of stimuli to the perforant path (time 0). Analysis of the last 5 minutes of recording post tetanic stimulation with one-way ANOVA showed that acute injection of $A\beta_{1\text{-}40}$+$A\beta_{1\text{-}42}$ significantly attenuated LTP in the dentate gyrus ($F_{(2,29)}$=82.90, $p<0.0001$, n=3-6).

Acute injection of Aβ alters synaptic protein expression

The effect of Aβ treatment on neuronal activity was further investigated by analysis of synaptic proteins. Figure 4.2 (A) shows that acute injection of Aβ at 200 μM induced a significant increase of SNAP-25 protein density compared to control ($F_{(2,24)}$=7.40, $p<0.01$, ANOVA). Treatment with the lower concentration had no effect on SNAP 25 expression. By contrast, the data shown in panel (B) demonstrate a significant decrease (1.5 fold) in syntaxin protein density compared to control due to treatment with Aβ at 45 μM ($F_{(2,22)}$=4.88, $p<0.05$; ANOVA) whereas Aβ at 200 μM exerted no effect. Consistent with this result, panel (C) shows that expression of the synaptic vesicle protein synaptophysin was significantly decreased by treatment with Aβ at 45 μM ($F_{(2,20)}$=6.39, $p<0.01$, ANOVA) while treatment with Aβ at 200 μM had no effect.

Acute injection of Aβ alters mRNA expression of markers of microglial activation

Evidence suggests that Aβ-induced impairment of LTP is associated with an increase of microglial activation which, in turn, may account for neuronal damage and impairment (Clarke et al., 2007). In order to determine whether acute injection of Aβ at different concentrations induced a pro-inflammatory response,

markers of activated microglia were first analysed by real time PCR. Figure 4.3 (A) shows that treatment with Aβ at both concentrations of 45 μM and 200 μM failed to affect MHCII mRNA expression ($F_{(2,23)}$=2.63, p>0.05, ANOVA). By contrast, analysis of CD40 mRNA expression (figure 4.3 (B)) by one-way ANOVA demonstrated that Aβ treatment had a weak, although still significant, effect on CD40 expression ($F_{(2,22)}$=3.53, p<0.05). Neuman-Keuls analysis failed to detect which conditions were significantly different from each other. Data presented in figure 4.3 (C) show that acute injection of Aβ had no effect on CD11b mRNA expression at either the concentrations tested ($F_{(2,22)}$=1.75, p<0.05, ANOVA).

The effect of acute injection of Aβ peptides on mRNA expression of molecules involved in microglial activation is further illustrated in figure 4.4. Panel (A) demonstrates a significant increase of iNOS mRNA expression due to treatment with Aβ at 200 μM ($F_{(2,19)}$=4.95, p<0.05, ANOVA) while injection of peptide at the lower concentration did not affect enzyme mRNA. Given that microglial activation is regulated by CD200/CD200R interaction, CD200 mRNA was also analysed to assess the possibility that Aβ toxicity may be exerted via alteration of inhibitory mechanisms of immune response. Results presented in panel (B) show that treatment with high concentration of Aβ peptides significantly decreased CD200 mRNA expression compared to control ($F_{(2,21)}$=7.67, p<0.01, ANOVA).

Effect of acute injection of Aβ on CD86 and ICAM-1 protein density

In order to further analyse microglial activation in response to Aβ injection, CD86 and ICAM-1 protein density was assessed by western immunoblotting. Results presented in figure 4.5 (A) show the effect of Aβ injection on CD86 expression: analysis of data by one-way ANOVA showed a significant effect of treatment on CD86 protein density ($F_{(2,19)}$=4.48, p<0.05, ANOVA). However, analysis of data with post hoc Neuman-Keuls test failed to detect any difference between conditions. Moreover, treatment with Aβ at the higher concentration induced a significant increase of the mean value of ICAM-1 protein density compared to control, as illustrated in figure 4.5 (B) ($F_{(2,19)}$=7.12, p<0.01,

ANOVA). Acute injection of Aβ at the lower concentration had no effect on ICAM-1 protein expression.

Acute injection of Aβ increases IL-β mRNA expression but not protein concentration

Microglial activation is associated with an increased production of pro-inflammatory cytokines such as IL-1β. Panel (A) shows that treatment with Aβ at 200 μM significantly increased the expression of IL-1β mRNA compared to control ($F_{(2,19)}$=7.55, $p<0.01$, ANOVA) while Aβ at the lower concentration had no effect on cytokine mRNA expression. Surprisingly, results shown in panel (B) demonstrate that treatment with Aβ at both 45 μM and 200 μM concentrations did not affect IL-1β protein concentration.

Acute injection of Aβ stimulates caspase-3 activity

One of the mechanisms involved in neuronal damage and loss includes apoptosis, a process of programmed cell death which neurons undergo in response to stimulation with cytotoxic molecules released by activated microglia during inflammation (Moore et al., 2007). Apoptosis is mediated by caspases, a family of cysteine peptidases, amongst which is caspase-3 that plays an effector role during cell death. Analysis of caspase-3 activity was assessed to further understand the mechanisms involved in Aβ-induced inflammatory response. Data presented in figure 4.7 show that treatment with Aβ at 45.5 μM induced a significant increase in enzyme activity compared to control ($F_{(2,19)}$=11.18, $p<0.001$, ANOVA) whereas injection of the peptide at the higher concentration had no effect.

Figures

Figure 4.1 Acute injection of Aβ attenuates LTP

Analysis of the last 5 minutes of recording post tetanic stimulation with one-way ANOVA showed a significant effect of Aβ on the percentage change in the EPSP slope ($F_{(2,29)}$=82.90, $p<0.0001$, n=3-6).

The mean population EPSP slope evoked by test stimuli delivered at 30 seconds intervals is shown and is expressed as a percentage of the slope recorded in the 5 minutes immediately prior to tetanic stimulation. Values are expressed as means ± SEM. SEM are included for every tenth response and some are so small in some cases as to be obscured.

LTP was recorded by Dr Thelma Cowley.

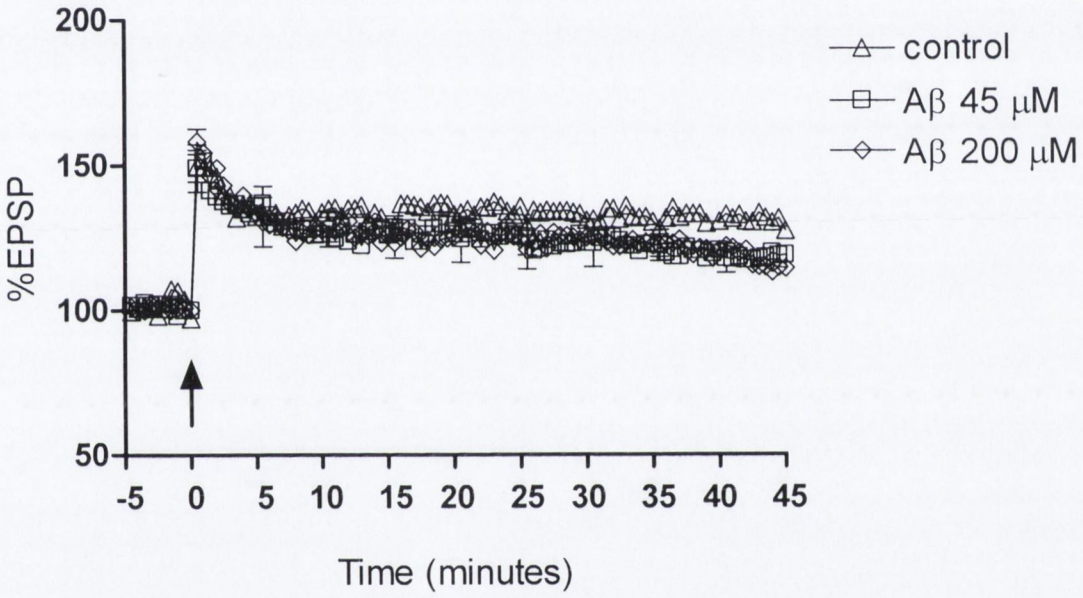

200
150
100
50
%EPSP
-5
0
5
10
15
20
25
30
35
40
45
Time (minutes)
control
Aβ 45 μM
Aβ 200 μM

Figure 4.2 Acute injection of Aβ affects synaptic protein expression

A) A one-way ANOVA showed a significant effect of treatment with Aβ (200 μM) on SNAP-25 protein density ($F_{(2,24)}$=7.39, $p<0.01$, n=5-15). Data are expressed as a ratio of SNAP-25 to actin and are means ± SEM. **$p<0.01$ vs control (Dunnett's test).

B) A one-way ANOVA demonstrated that there was a significant effect of treatment with Aβ (45 μM) on syntaxin protein density ($F_{(2,22)}$=4.88, $p<0.05$, n=6-11). Data are expressed as a ratio of syntaxin to actin and are means ± SEM. *$p<0.05$ vs control (Dunnett's test).

C) A one-way ANOVA showed a significant effect of treatment with Aβ (45 μM) on synaptophysin protein density ($F_{(2,20)}$=6.39, $p<0.01$, n=5-10). Data are expressed as a ratio of synaptophysin to actin and are means ± SEM. **$p<0.01$ vs control (Dunnett's test).

Results for the lower dose Aβ-treated group were generously donated by Fionnuala Cox.

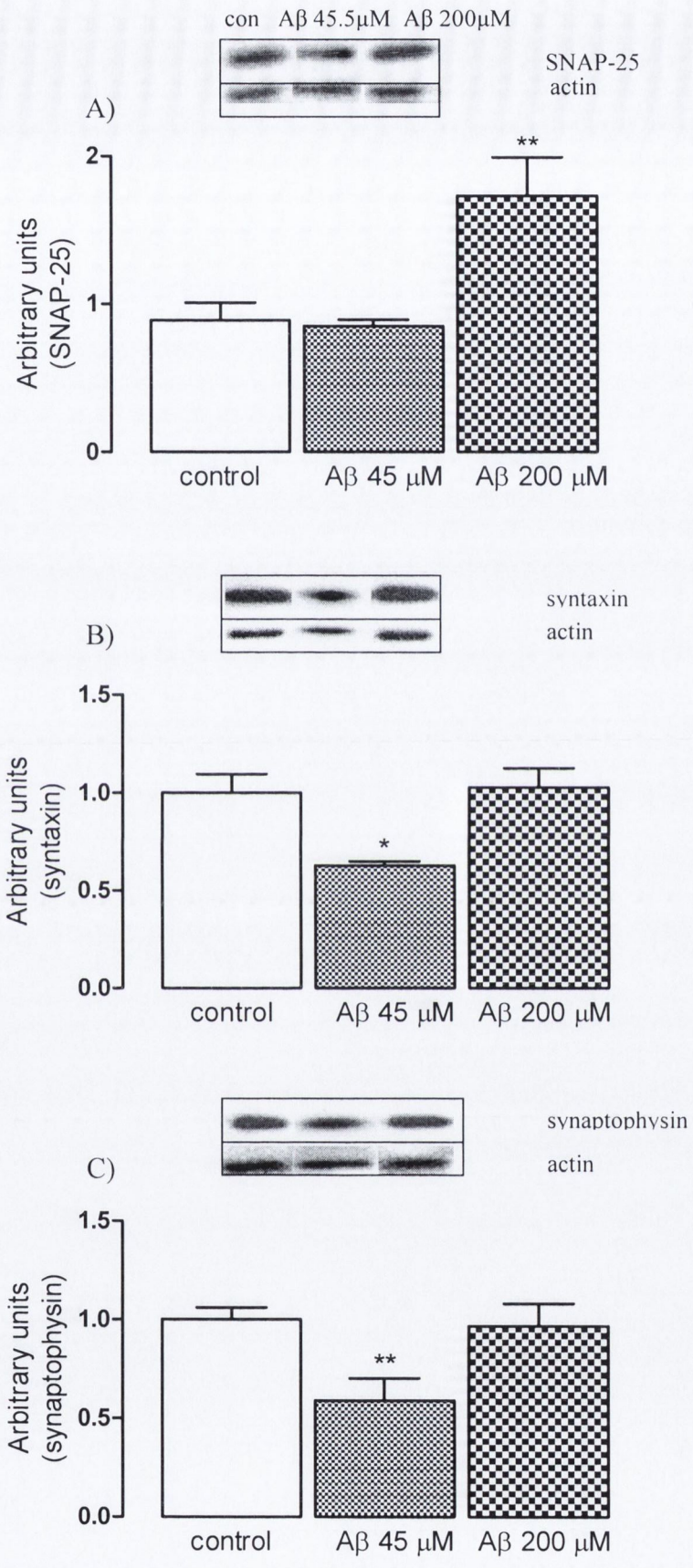

con Aβ 45.5μM Aβ 200μM
SNAP-25
actin
A)
Arbitrary units (SNAP-25)
2
1
0
**
control
Aβ 45 μM
Aβ 200 μM
syntaxin
actin
B)
Arbitrary units (syntaxin)
1.5
1.0
0.5
0.0
*
control
Aβ 45 μM
Aβ 200 μM
synaptophysin
actin
C)
Arbitrary units (synaptophysin)
1.5
1.0
0.5
0.0
**
control
Aβ 45 μM
Aβ 200 μM

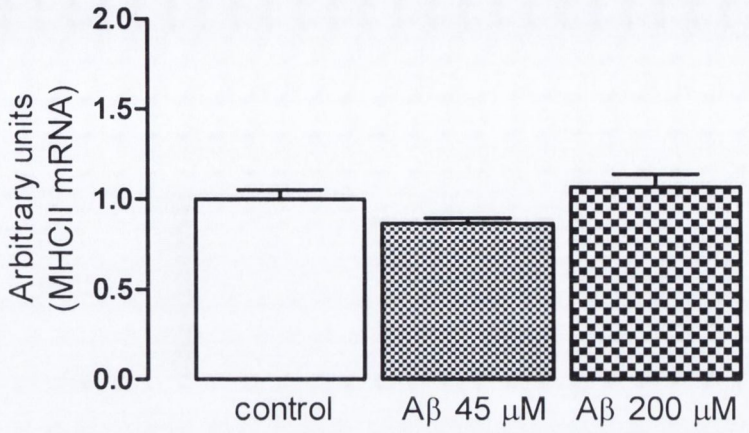
Figure 4.3 Acute injection of Aβ modulates CD40, but not CD11b or MHCII, mRNA expression

A) A one-way ANOVA showed that there was no effect of Aβ on MHCII mRNA expression ($F_{(2,23)}$=2.626, $p>0.05$, n=6-12). Data are expressed as a ratio of MHCII to β-actin and are means ± SEM.

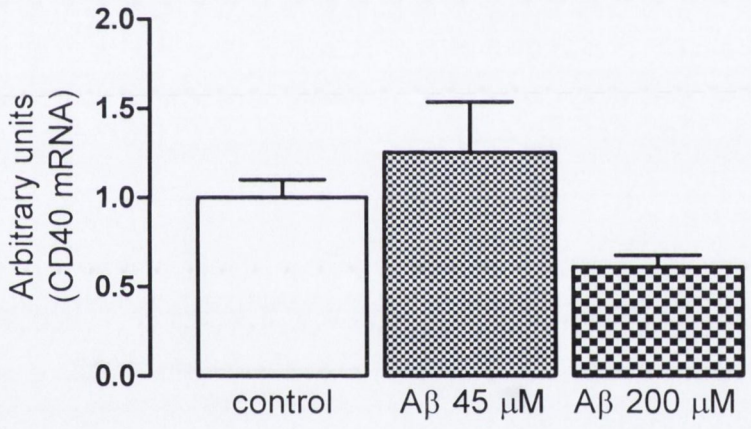
B) A one-way ANOVA showed a significant effect of Aβ on CD40 mRNA expression ($F_{(2,22)}$=3.53, $p<0.05$, n=6-11). However, analysis with post hoc Dunnett's test did not find any difference between conditions. Data are expressed as a ratio of CD40 to β-actin and are means ± SEM.

C) A one-way ANOVA showed that there was no effect of Aβ treatment on CD11b mRNA expression ($F_{(2,22)}$=1.75, $p>0.05$, n=6-11). Data are expressed as a ratio of CD11b to β-actin and are means ± SEM.

A)


Arbitrary units
(MHCII mRNA)
2.0
1.5
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 200 μM

B)


Arbitrary units
(CD40 mRNA)
2.0
1.5
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 200 μM

C)

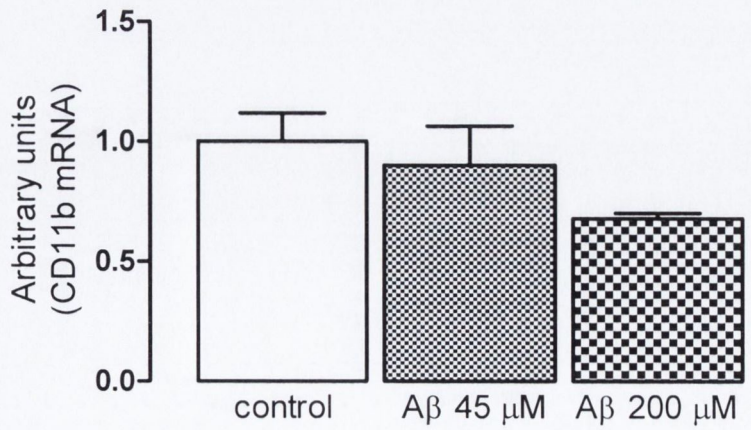
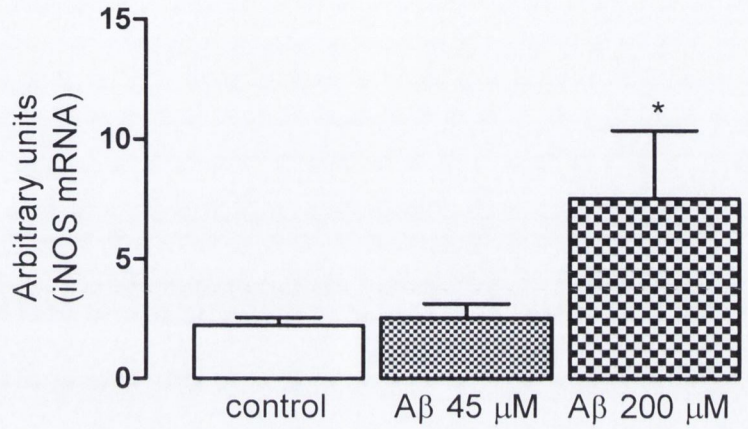

Arbitrary units
(CD11b mRNA)
1.5
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 200 μM

Figure 4.4 Acute injection of Aβ alters iNOS and CD200 mRNA expression

A) A one-way ANOVA demonstrated a significant effect of Aβ (200 μM) on iNOS mRNA expression ($F_{(2,19)}$=4.95, $p<0.05$, n=5-10). Data are expressed as a ratio of iNOS to β-actin and are means ± SEM. *$p<0.05$ vs control (Dunnett's test).

B) A one-way ANOVA demonstrated a significant effect of Aβ (200 μM) on CD200 mRNA expression ($F_{(2,21)}$=7.67, $p<0.01$, n=5-11). Data are expressed as a ratio of CD200 to β-actin and are means ± SEM. **$p<0.01$ vs control (Dunnett's test).

A)


15
10
5
0
Arbitrary units
(iNOS mRNA)
*
control
Aβ 45 μM
Aβ 200 μM

B)

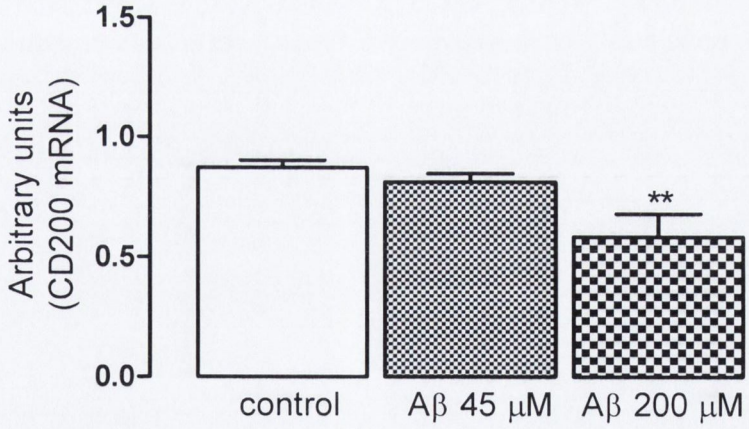

1.5
1.0
0.5
0.0
Arbitrary units
(CD200 mRNA)
**
control
Aβ 45 μM
Aβ 200 μM

Figure 4.5 Effect of acute injection of Aβ on CD86 and ICAM-1 protein density

A) A one-way ANOVA showed a significant effect of Aβ on CD86 protein density ($F_{(2,19)}$=4.48, $p<0.05$, n=5-9). However, analysis with post hoc Dunnett's test did not find any difference between conditions. Data are expressed as a ratio of CD86 to actin and are means ± SEM.

B) A one-way ANOVA demonstrated a significant effect of Aβ (200 μM) on ICAM-1 protein density ($F_{(2,19)}$=7.12, $p<0.01$, n=5-9). Data are expressed as a ratio of ICAM-1 to actin and are means ± SEM. **$p<0.01$ vs control (Dunnett's test).

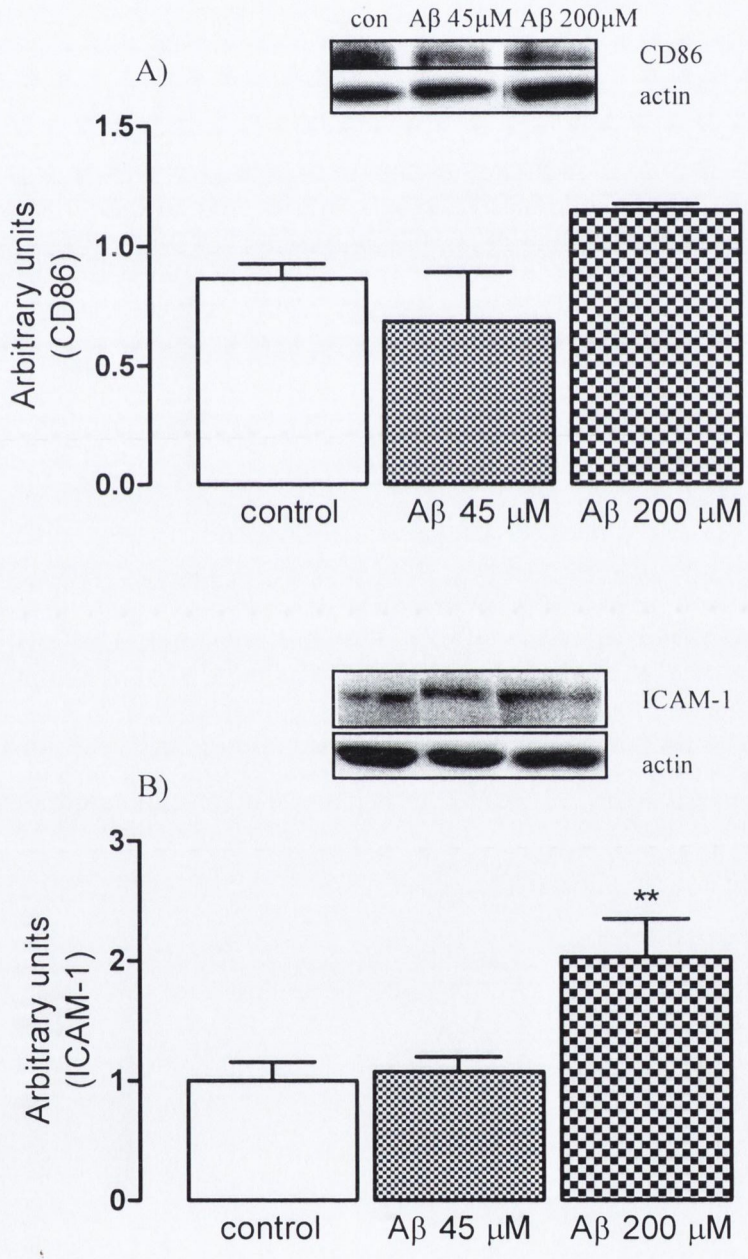
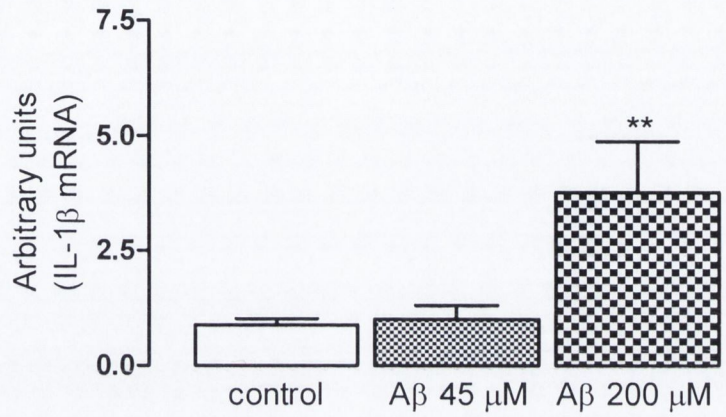

con Aβ 45μM Aβ 200μM
A)
CD86
actin
Arbitrary units (CD86)
1.5
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 200 μM
ICAM-1
actin
B)
Arbitrary units (ICAM-1)
3
2
1
0
**
control
Aβ 45 μM
Aβ 200 μM

Figure 4.6 Acute injection of Aβ increases IL-1β mRNA but not protein

A) A one-way ANOVA showed a significant effect of Aβ (200 μM) on IL-1β mRNA expression ($F_{(2,19)}$=7.55, $p<0.01$, n=6-9). Data are expressed as a ratio of IL-1β to β-actin and are means ± SEM. **$p<0.01$ vs control (Dunnett's test).

B) A one-way ANOVA showed that there was no effect of Aβ on IL-1β protein concentration ($F_{(2,23)}$=3.39, $p>0.05$, n=6-12). Data are expressed as a ratio of IL-1β to protein concentration and are means ± SEM.

A)


7.5
5.0
2.5
0.0
Arbitrary units
(IL-1β mRNA)
**
control
Aβ 45 μM
Aβ 200 μM

B)

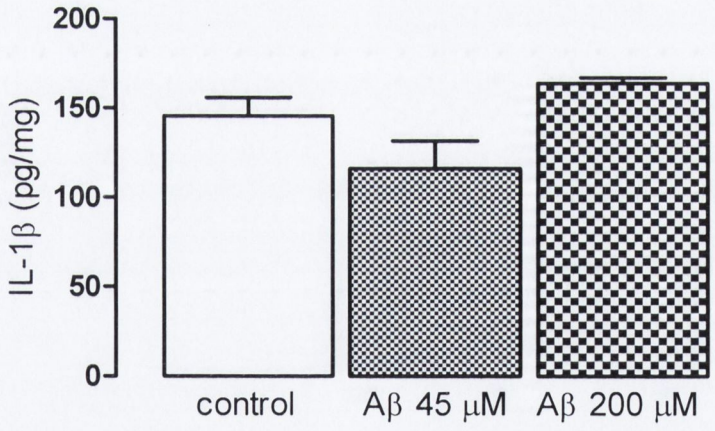
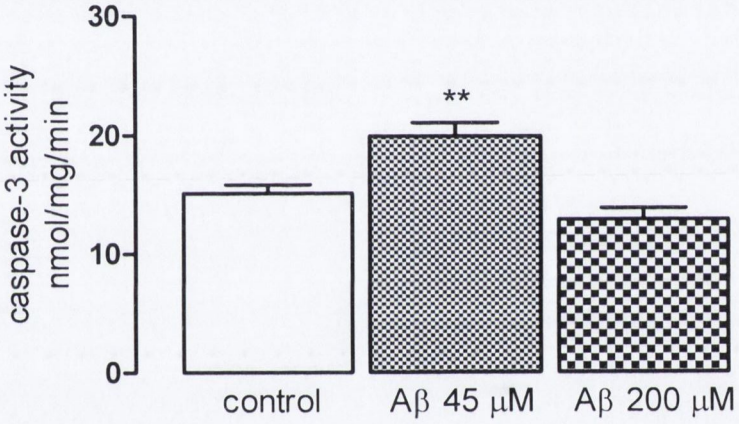

200
150
100
50
0
IL-1β (pg/mg)
control
Aβ 45 μM
Aβ 200 μM

Figure 4.7 Acute injection of Aβ increases caspase-3 activity

A one-way ANOVA demonstrated a significant effect of Aβ (45.5 μM) on caspase-3 activity ($F_{(2,19)}$=11.18, p<0.001, n=4-11). Data are means ± SEM. **P<0.01 vs control (Dunnett's test).


caspase-3 activity
nmol/mg/min
30
20
10
0
**
control
Aβ 45 μM
Aβ 200 μM

4.4 Discussion

The present study was set out to analyse the effect of acute icv injection of $A\beta_{1-40}$ and $A\beta_{1-42}$ in combination, at two different concentration in the rat hippocampus. The results obtained demonstrate that Aβ injection at both the concentrations tested attenuated LTP maintenance: while low Aβ concentration altered the expression of certain synaptic proteins, high Aβ concentration induced expression of markers of microglial activation.

Analysis of LTP data revealed that Aβ injection, at both the concentrations tested, significantly attenuated LTP maintenance. These data are consistent with previous findings obtained in this laboratory demonstrating that acute injection of $A\beta_{1-42}$ induced a significant deficit of LTP in the hippocampus (Clarke et al., 2007; Lyons et al., 2007a). The data also confirm other reports indicating that acute administration of fibrillar $A\beta_{1-40,}$ from the concentration of 60 μM, exerted an inhibitory effect on LTP (Minogue et al., 2003; Minogue et al., 2007). However, it is possible that differences exist between the effects of $A\beta_{1-42}$ alone and the effect of $A\beta_{1-42}$ in combination with $A\beta_{1-40}$ and that different concentration of peptide may account for the different extent to which LTP was attenuated in the diverse studies. Acute injection of $A\beta_{1-40}$ at 20 μM has been shown to exert no effect on the ability of young rats to sustain LTP (Lynch et al., 2006) wherease in the present study a similar concentration of $A\beta_{1-40}$ (26.6 μM) incombination with $A\beta_{1-42}$ (18.94 μM) significantly attenuated LTP. For the purpose of this experiment $A\beta_{1-40}$ and $A\beta_{1-42}$ were also used at the concentrations of 84 μM and 116 μM so that the final concentrations of the two treatments were 45.5 μM and 200 μM respectively.

Along with fibrillar forms of Aβ, naturally secreted oligomers have also been shown to induce deficits in LTP (Walsh et al., 2002) and similar effects have been reported with various Aβ peptide fragments (Freir et al., 2001). Similarly, a deficit in LTP was reported in aged mice overexpressing APP and showing Aβ deposition (Chapman et al., 1999). In light of the fact that both concentrations of Aβ tested here attenuated LTP to the same extent it is arguable that the Aβ-induced effect on LTP may be mediated by the soluble species present in the preparations used rather that the fibrils. Althought Aβ peptides were prepared

according to manufacturer's instruction to obtain fibrils, a certain amount of soluble oligomeric forms were also present, as assessed by gel electrophoresis and Coomassie blue staining. However, because of a lack of testing for molecular sizes of the Aβ preparations used in the present study, it is not possible to compare results obtained in different studies. It is possible that the amount of oligomeric species may differ between Aβ solutions and that this difference may account for the different extent to which LTP is attenuated by Aβ injections. It is thereby suggested that the LTP attenuation seen in the present study is mediated by the oligomeric rather than the fibrillar forms of Aβ. Althought two different concentrations of peptides were used, it is possible that the same amount of oligomers was present in both preparations and this may be responsible for the similar effect on LTP.

Many studies have attempted to understand the mechanisms underlying the detrimental effect of Aβ on synaptic plasticity: it has been suggested that Aβ toxicity may involve interactions with NMDA receptors and disruption of Ca^{2+} homeostasis (Chen et al., 2002; Mattson et al., 1992; Wang et al., 2004b; Kelly and Ferreira, 2006). In addition, induction and expression of LTP depends on the regulated activity of AMPA receptors which have been shown to be modulated by acute exposure to $A\beta_{1-42}$ (Parameshwaran et al., 2007). Consistent with the hypothesis that differences in the structural properties of $A\beta_{1-40}$ and $A\beta_{1-42}$ play a major role in the contrasting biological effects of the two peptides (Bitan et al., 2003), Parameshwaran and colleagues (2007) demonstrated that the shorter peptide, $A\beta_{1-40}$, had no effect on AMPA receptors activity.

$A\beta_{1-42}$, although being produced to a lesser extent than $A\beta_{1-40}$, is the predominant form of peptide found in amyloid plaques in AD (Mann et al., 1996; Awasthi et al., 2005) and it is considered the most amyloidogenic form of peptide. Consistent with this is the evidence that $A\beta_{1-42}$ is more hydrophobic and forms precipitated fibrils faster than $A\beta_{1-40}$. In addition, the rate of degradation of $A\beta_{1-40}$ is greater than that of $A\beta_{1-42}$ (Snyder et al. 1994; Shaffer et al., 1995; Walsh et al., 1997). It has been shown that $A\beta_{1-42}$ has a natural trend to form bigger oligomers that are capable of assembling into insoluble fibrils with β–sheet conformation, whereas $A\beta_{1-40}$ exists as smaller species such as monomers, dimers, trimers and tetramers. Taking into account that Aβ must aggregate into fibrils to induce

microglial activation (Lorton et al., 1996) and that $A\beta_{1-42}$ has a greater tendency to self-aggregate than the shorter peptide, it is predicted that treatment with $A\beta_{1-42}$ would exert higher toxicity than treatment with $A\beta_{1-40}$ and that the higher concentration of $A\beta_{1-40}$+$A\beta_{1-42}$ peptide would be more damaging than the lower one. However, the results show that LTP maintenance was affected to the same extent in the case of both Aβ-treated groups. It has been suggested that $A\beta_{1-40}$ is able to exert a protective role against $A\beta_{1-42}$-induced toxicity (Snyder et al., 1994). Indeed, these authors showed that when the two peptides are mixed together, $A\beta_{1-40}$ retards the aggregation of $A\beta_{1-42}$ in a concentration-dependent manner. Although it must be considered that $A\beta_{1-40}$ retarded aggregation of $A\beta_{1-42}$, Aβ aggregation was not assessed here and it is suggested that oligomers, rather than fibrils are responsible for the Aβ-induced effct on LTP.

Analysis of pre-synaptic protein expression was carried out to investigate the effect of Aβ exposure on synaptic function. The results show that injection of $A\beta_{1-40}$+$A\beta_{1-42}$ at 200 μM induced a significant increase of SNAP-25 protein expression whereas injection of peptides at the lower concentration significantly reduced protein density of syntaxin and synaptophysin. It is possible to suggest that the lower concentration of $A\beta_{1-40}$+$A\beta_{1-42}$ exerted a modulatory effect on neuronal functioning by compromising synaptic transmission and thereby inducing attenuation of LTP. Another interesting aspect is that lack of toxicity by the higher concentration of peptide may be related to the amount of aggregated forms of Aβ and their ability to penetrate neurons. It is plausible that, at higher concentrations of Aβ, aggregation increases due to closer proximity of peptides thereby reducing their ability to penetrate the tissue/cells. Thus, the global effect of higher concentrations of Aβ, as measured by analysis of synaptic protein density, in hippocampal homogenates, is decreased as compared to lower concentrations of Aβ.

It has been suggested that soluble forms of $A\beta_{1-42}$ can selectively bind to neurons and accumulate within the cells (Clifford et al., 2007). Thus, it is possible that soluble Aβ species present in the injected Aβ preparations acted directly on the neurons, by altering expression of the two synaptic proteins syntaxin and synaptophysin and thereby compromising their function. In addition, the changes occurred in response to Aβ confirm data reporting an approximate 30% reduction

in the number and density of synapses in the brain of AD patients (for review see Selkoe, 2002). Post-mortem studies on AD neuropathology have revealed reduced synaptic connectivity in several brain regions including the neocortex and the hippocampus (for review see Small et al., 2001). Synaptophysin is a trans-membrane protein of synaptic vesicles thought to be involved in the processes of docking and fusion of synaptic vesicles (Washbourne et al., 1995). In a recent work, it has been demonstrated that synaptophysin expression was markedly decreased in areas where Aβ oligomers accumulated (Ishibashi et al., 2006). Thus, it is plausible that the synaptic dysfunction typical of early stages of AD may, in part, be due to decreased expression of synaptic proteins. Evidence from the literature suggests a role for oligomeric species of Aβ in the disruption of synaptic function. However, the present work was aimed to evaluate the effect of fibrillar forms of Aβ on the hippocampus: it has been shown that fibril formation is critical for the induction of neuronal dystrophy and that the high degree of β-sheets conformation favours Aβ interaction with transmembrane proteins (Grace et al., 2002). Although the results obtained seem to confirm the data obtained by Grace and colleagues (2002) it is arguable that oligomers rather than aggregated species are responsible for the modulation of synaptic proteins expression leading to compromised neurotransmitter release. Analysis of Aβ immunoreactivity would be interesting to determine whether Aβ deposition occurred in proximity or within neurons and to investigate the pattern of Aβ penetration in the brain tissue.

Analysis of IL-1β mRNA expression revealed that acute injection of $A\beta_{1-40}$+$A\beta_{1-42}$ at the higher concentration increased the cytokine mRNA expression. Surprisingly, this result was not paralleled by MHCII mRNA which was not affected by either of the Aβ concentrations and, similarly, Aβ did not affect CD40 and CD11b mRNA expression. On the other hand, Aβ at the higher concentration significantly increased iNOS mRNA expression. Evidence suggests that NO is an important mediator of Aβ-induced neurotoxicity (Tran et al., 2001; Floden et al., 2005; Jang and Surh, 2005). This notion is reinforced by the finding that up-regulation of iNOS colocalizes with Aβ plaques (Luth et al., 2001) and is stimulated in response to Aβ (Medeiros et al., 2007). Consistent with the Aβ-induced increase in IL-1β and iNOS mRNA expression, acute injection of Aβ at 200 μM induced a significant decrease in CD200 mRNA expression. In a recent

work by Lyons and colleagues (2007a) it has been demonstrated that Aβ treatment decreased CD200 expression *in vivo* and *in vitro*. Further, it has been shown that interaction between CD200 and its receptor, CD200R, modulates microglial activation in response to Aβ (Lyons et al., 2007b). These data, taken together, suggest that Aβ exerts its inflammatory activity at least in part by modulating inhibitory mechanisms involved in maintaining microglia in a quiescent state such as CD200.

Up-regulation of MHCII enables microglia to act as APC, which is a prerequisite for the presentation of processed antigens to infiltrating $CD4^+$ T cells (for review see Aloisi et al, 2000b). It is possible that acute Aβ exposure did not fully activate microglia into APC but rather primed them. Thus, mRNA expression of CD11b, constitutively expressed on the cell surface but rapidly synthesised in response to microglial activation and proliferation (Aloisi et al., 2000a), was not affected by either Aβ concentrations. Similarly, mRNA expression of the co-stimulatory molecule CD40 was not altered by treatment with $A\beta_{1\text{-}40}$+$A\beta_{1\text{-}42}$. It has been suggested that engagement of microglial CD40 represents a mechanism that shifts activated microglia toward antigen presentation, resulting in increased production of pro-inflammatory cytokines (Townsend et al., 2005). Abnormal expression of CD40 and CD40L has been implicated in several diseases including AD: analysis of AD brain has demonstrated that microglial expression of CD40 is upregulated and colocalizes with increased MHCII (Togo et al., 2000). In the present study CD40 expression was analysed only at the level of mRNA, it is possible that analysis of CD40 protein would reveal an Aβ-induced up-regulation as previously described by Minogue and colleagues (2007).

In order to further investigate the effect of acute injection of Aβ on microglial activation, expression of the co-stimulatory molecule CD86 and of the glycoprotein ICAM-1 was analyzed. Both cell surface markers are expressed on cells other than microglia but their expression on microglia is increased when these cells are activated (Wang et al., 1995; Shen et al., 1997). The data reported demonstrate that treatment with $A\beta_{1\text{-}40}$+$A\beta_{1\text{-}42}$ at the higher concentration increased ICAM-1 protein expression although this change was not paralleled by increased CD86 expression. This finding is surprising considering that up-regulation of ICAM-1 has been reported to be coupled with increased expression

of CD86 and also CD80 (Griffin et al., 2006). Further, increased expression of both ICAM-1 and of CD86 in hippocampal tissue has been described following acute injection of $A\beta_{1-42}$ (Clarke et al., 2007). It is possible that the variability in the data (as indicated by the large SEM values) compromised the final result; analysis of a higher number of cases would confirm this result.

In response to pro-inflammatory stimuli, microglia become activated and release pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα (for review see Aloisi, 2001). Surprisingly, analysis of IL-1β concentration revealed that acute injection of $A\beta_{1-40}$+$A\beta_{1-42}$ at either the concentrations of 45.5 μM and 200 μM had no effect on the cytokine concentration. This finding is in contrast with previous results obtained in this laboratory demonstrating that acute injection of Aβ markedly increased levels of hippocampal IL-1β concentration and that attenuation of LTP is often associated with an increased concentration of pro-inflammatory cytokines (Clarke et al., 2007; Lyons et al., 2007b; Lyons et al., 2007a; Minogue et al., 2007). It is possible that the presence of $A\beta_{1-40}$ compromised $A\beta_{1-42}$ toxicity or that Aβ treatment did not induce a profound inflammatory response but rather primed the microglia and consequently did not lead to an increase in IL-1β production.

Apoptosis is a process of programmed cell death which is mediated by the activity of a family of enzymes known as caspases. Among these is the effector enzyme caspase-3: a cysteine endopeptidase responsible for execution of apoptosis (for review see Green and Reed, 1998). It has been reported that IL-1β is one factor which leads to caspase-3 activation by inducing JNK and p38 (O'Donnell et al., 2000; Lynch and Lynch, 2002) and therefore, it was predicted that the lack of effect of Aβ injection on IL-1β concentration would be accompanied by unchanged caspase-3 activity. However, the results demonstrate a significant increase in enzyme activity in response to acute injection of $A\beta_{1-40}$+$A\beta_{1-42}$ at the lower concentration. It is possible that Aβ exerts its toxic effect directly on neurons and that the increased activation of caspase-3 results from neuronal cells, rather that microglia, undergoing stress. Indeed it has been shown that cultured neurons undergo cell death through activation of sphyngomyelinase activity and production of ceramide as a consequence of treatment with Aβ (Jana and Phan, 2004) and evidence from this laboratory suggests that sphyngomyelinase activity/ceramide production may occur irrespective of

microglial activation (Miller et al., unpublished). Further, evidence suggests that oligodendrocytes are vulnerable to Aβ toxicity and undergo apoptosis in response to treatment with the peptide (Lee et al., 2004). Analysis of sections by immunohistochemistry is required to verify whether activated caspase-3 colocalizes with neurons or oligodendrocytes. It would also be interesting to assess whether neurons expressing decreased levels of synaptic proteins colocalize with activated caspase-3.

Although the role of inflammation in the pathogenesis and progression of AD has been well established (Miguel-Hidalgo et al., 2002; for review see McGeer and McGeer, 2001), there is evidence that Aβ can induce neuronal death by directly compromising cell Ca^{2+} homeostasis (Ferreiro et al, 2006). It is also interesting to consider that the lower concentration of peptide exerted more marked effect on caspase-3 activation than the higher concentration. This is consistent with the findings from analysis of synaptic proteins: given that $A\beta_{1-40}$ has been shown to have anti-inflammatory activity (Balboa et al., 2001) it is plausible that variation in the amount of $A\beta_{1-40}$ may account for the difference between the two concentrations of Aβ treatment tested. In addition, as already mentioned, it is possible that oligomeric species, the presence of which has not been assessed, modulated the effect of the two treatments differently.

In conclusion, the present data suggest that acute administration of $A\beta_{1-40}$+$A\beta_{1-42}$ induced priming of microglial cells and neuronal damage in a dose-dependent fashion. It is possible that treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ in combination exerted a less dramatic effect than either the peptides alone, consistent with the hypothesis that $A\beta_{1-40}$ may exert a certain degree of protection from $A\beta_{1-42}$ toxicity. Moreover, it is plausible that microglia express different degree of vulnerability to Aβ than other cell types so that the same concentration of peptides (45.5 μM) has no effect on microglia, but induces stress in oligodendrocytes and neurons. However, further analysis assessing damaged neurons and oligodendrocytes and activated caspase-3 would be required to corroborate this hypothesis.

Variable	Effect of treatment	
	$A\beta_{40+42}$ (45.5 µM)	**$A\beta_{40+42}$ (200 µM)**
LTP (%EPSP)	↓	↓
SNAP-25 (arbitrary units)	no change	↑
Syntaxin (arbitrary units)	↓	no change
Synaptophysin (arbitrary units)	↓	no change
MHCII (arbitrary units)	no change	no change
CD40 (arbitrary units)	no change	no change
CD11b (arbitrary units)	no change	no change
iNOS (arbitrary units)	no change	↑
CD200 (arbitrary units)	no change	↓
CD86 (arbitrary units)	no change	no change
ICAM-1 (arbitrary units)	no change	↑
IL-1β mRNA (arbitrary units)	no change	↑
IL-1β (pg/mg)	no change	no change
Caspase-3 activity (nmol/mg/min)	↑	no change

Table 4.1 Summary of results obtained

Chapter 5

Analysis of the effect of chronic infusion of Aβ on rat hippocampus

5.1 Introduction

It is established that inflammation plays a key role in AD, evidence that treatment with NSAIDs reduces the risk of developing AD further corroborates this notion (for review see in McGeer and McGeer, 2001). The efficacy of NSAIDs in the treatment of AD depends on their action on PPARγ, a transcription factor whose activation has been associated with inhibited production of pro-inflammatory cytokines such as IL-1β and TNFα (Cunard et al., 2002). These data add to the notion that neuroinflammation is a critical component in the development and progression of AD. Moreover, several inflammatory mediators are increased in the brains of AD patients compared to age-matched controls and increased pro-inflammatory activities have been localised to pathologic lesions in AD brains (for review see Akiyama et al., 2000).

Activated microglia exhibiting extended ramified processes are found clustered within and adjacent to the AD brain senile plaques (Tooyama et al., 1990, Verbeek et al., 1994). According to the Aβ hypothesis, progressive accumulation of Aβ, due to either an increased production or a reduced clearance, is the central event in the pathogenesis of AD. However, it has become evident that Aβ plaques are not the most toxic form of peptide and that neurotoxicity is exerted prior to plaque appearance (Hsia et al., 1999). Prolonged glial activation in response to Aβ may account for neuroinflammation in the CNS contributing to the pathogenesis and progression of AD. Increased levels of inflammatory and oxidative molecules produced by chronically activated microglia can lead to neuronal damage and death which, in turn, induce further glial activation in a self-propagating fashion (Craft et al., 2005; for review see Liu and Hong, 2003). Neuronal degeneration as a consequence of chronic inflammation may be responsable for the behavioural and functional deficits of AD (Miguel-Hidalgo et al., 2002).

5.2 Methods and materials

Young male Wistar rats were treated for 28 days with Aβ at low (18.9 μM and 26.6 μM $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) and high concentration (26.9 μM and 36.9 μM $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively). Treatment of the rats and dissection of tissue was performed in conjunction with doctor Antony Lyons (see section 2.2.6). The results from the group treated with the lower concentration of Aβ (45.5 μM) are the same as those presented in chapter 6. Aβ was delivered via osmotic minipumps (6 μl/day). At the end of the treatment animals were killed by decapitation, the hippocampi were dissected free and an aliquot was snap frozen in liquid nitrogen for RNA extraction, while the remaining of tissue was cross-chopped and stored at -80 °C in Krebs solution containing 10% DMSO.

Analysis of iNOS mRNA expression was performed using realt time PCR (see section 2.5). Extraction of total RNA from hippocampal tissue was achieved with a Nucleospin Kit. The obtained RNA was resuspended in RNase-free water and analysed for integrity by electrophoresis. Concentration of RNA was measured due to its ability to adsorb light at 260 nm using a spectrophotometer. Total RNA (1 μg) was reverse transcribed into cDNA using high-capacity cDNA archieve kit according to the protocol provided by the manufacturer. Real-time PCR primers were delivered as "Taqman Gene Expression Assays" for the rat genes listed in Table 2.2. Real-time PCR was performed on Applied Biosystems 7300 Real-time PCR System with Applied Biosystems 7300 System SDS Software 1.3.1 in 96-well format. Rat β-actin was used for normalization.

Analysis of CD86, ICAM-1 and CD200 protein density was performed on hippocampal homogenates by electrophoresis and immunoblotting (see section 2.8).

Caspase-3 activity was carried out using Caspase-3 Drug Discovery Kit in 96 well format, according to the protocol provided by the manufacturer (see section 2.10).

5.3 Results

Chronic infusion of Aβ has no effect on iNOS mRNA expression

The main aim of this study was to investigate and compare the effects of chronic administration of Aβ at different concentrations. Evidence from this laboratory suggests that Aβ is able to induce microglial activation when acutely administered, however, the effects of chronic Aβ infusion have not been analysed. Given that inflammation plays a crucial role in the development of AD, analysis of markers of microglial activation was carried out to investigate the presence of an inflammatory response. Because inflammation often is associated with increased production of NO, analysis of iNOS mRNA was first measured by real time PCR. Figure 5.1 shows that chronic infusion of Aβ for 28 days did not affect iNOS mRNA expression at any of the concentrations tested ($F_{(2,20)}$=0.42, $p>0.05$, ANOVA).

Chronic infusion of Aβ does not alter expression of CD86, ICAM-1 nor CD200

In order to analyse whether chronic infusion of Aβ peptides induced any effect on microglia, expression of adhesion molecules involved in antigen presentation was assessed by western immunoblotting. Results shown in figure 5.2 (A) represent the mean values of CD86 protein density for control and Aβ-treated groups. Analysis of the data with one-way ANOVA failed to detect any significant effect of Aβ infusion on CD86 protein expression ($F_{(2,20)}$=2.51, $p<0.05$). Consistent with this finding, data reported in figure 5.2 (B) show that chronic infusion of Aβ did not exert any effect on ICAM-1 protein density, at either of the two concentrations ($F_{(2,19)}$=0.88, $p<0.05$, ANOVA).

It is known that interaction between CD200, found on neurons and endothelial cells, and its receptor CD200R, mainly expressed within the brain on microglia, is involved in maintaining microglia in a resting state. In order to assess the impact of Aβ on CD200/CD200R interaction, analysis of CD200 protein density was carried out by western immunoblotting. The results shown in figure 5.2 (C), show that there was no effect of chronic administration of Aβ on the protein density, at either of the two concentrations chosen ($F_{(2,19)}$=1.49, $p<0.05$, ANOVA).

Chronic infusion of Aβ does not alter caspase-3activity

Evidence from *in vitro* studies suggests that Aβ may directly affect neuronal function and survival without involvement of activated microglia (Kelly and Ferreira, 2006). Although there was no evidence of microglial activation following 28 days of Aβ infusion into the lateral ventricle, analysis of caspase-3 activity was carried out to assess the possibility of a direct effect of Aβ on neuronal survival. Figure 5.3 represents the mean values of enzyme activity normalised by protein concentration. The results show that chronic infusion of Aβ, at either the concentrations tested, had no effect on caspase-3 activity ($F_{(2,22)}$=2.49, p>0.05, ANOVA).

Figures

Figure 5.1 Chronic infusion of Aβ does not alter iNOS mRNA expression

A one-way ANOVA showed that there was no effect of Aβ treatment on iNOS mRNA expression ($F_{(2,20)}$=0.42, $p>0.05$, n=6-9). Data are expressed as a ratio of iNOS to β-actin and are means ± SEM.

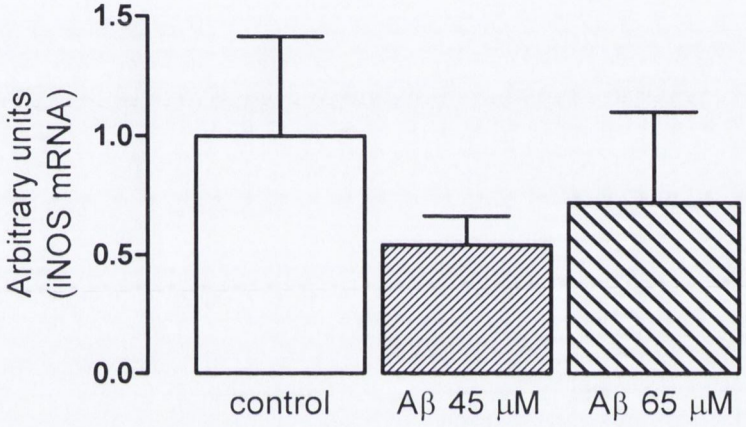

Arbitrary units
(iNOS mRNA)
1.5
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 65 μM

Figure 5.2 Chronic infusion of Aβ does not affect CD86, ICAM-1 and CD200 protein density

A) A one-way ANOVA showed that there was no effect of Aβ treatment on CD86 protein density ($F_{(2,20)}$=2.51, p>0.05, n=5-10). Data are expressed as a ratio of CD86 to actin and are means ± SEM.

B) A one-way ANOVA showed that there was no effect of Aβ treatment on ICAM-1 protein density ($F_{(2,19)}$=0.88, p>0.05, n=6-8). Data are expressed as a ratio of ICAM-1 to actin and are means ± SEM.

C) A one-way ANOVA showed that there was no effect of Aβ treatment on CD200 protein density ($F_{(2,19)}$=1.492, p>0.05, n=5-9). Data are expressed as a ratio of CD200 to actin and are means ± SEM. Results for the higher dose of Aβ have been donated by Dr Antony Lyons

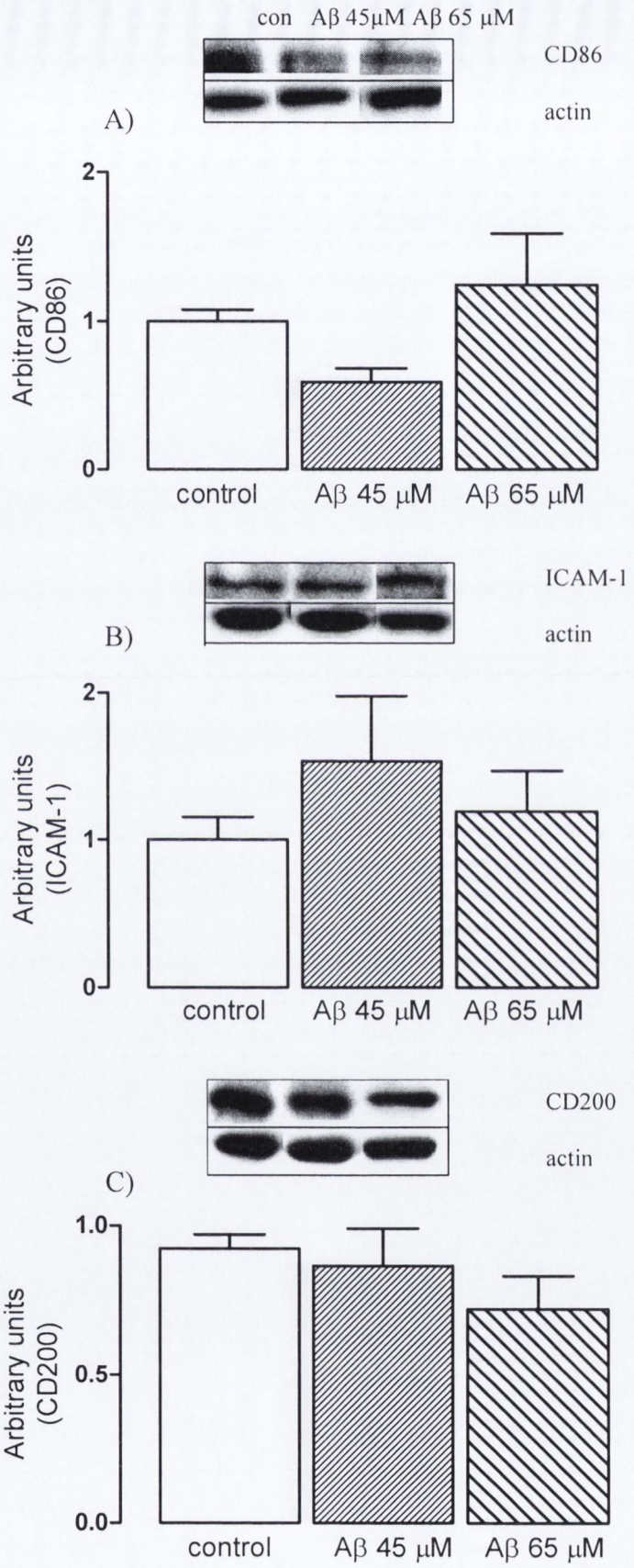
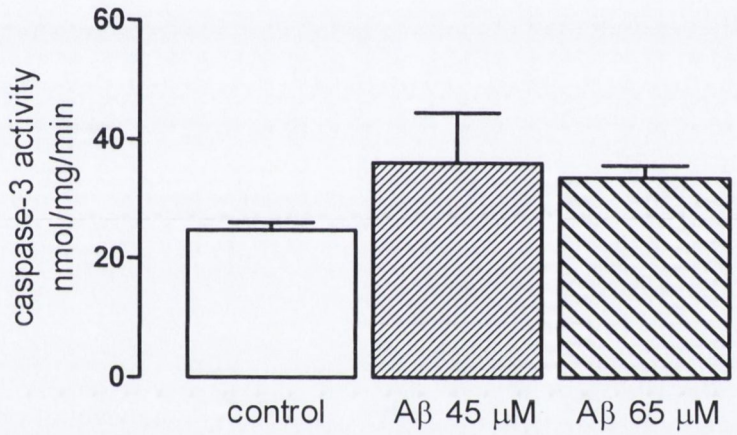

con Aβ 45μM Aβ 65 μM
CD86
actin
A)
Arbitrary units (CD86)
2
1
0
control
Aβ 45 μM
Aβ 65 μM
ICAM-1
actin
B)
Arbitrary units (ICAM-1)
2
1
0
control
Aβ 45 μM
Aβ 65 μM
CD200
actin
C)
Arbitrary units (CD200)
1.0
0.5
0.0
control
Aβ 45 μM
Aβ 65 μM

Figure 5.3 Chronic infusion of Aβ does not alter caspase-3 activity

A one-way ANOVA showed that there was no effect of Aβ treatment on caspase-3 activity ($F_{(2,22)}$=2.49, p>0.05, n=6-11). Data are means ± SEM.


caspase-3 activity
nmol/mg/min
60
40
20
0
control
Aβ 45 μM
Aβ 65 μM

5.4 Discussion

The aim of this study was to investigate the effect of chronic infusion of two different doses of $A\beta_{1-40}$+$A\beta_{1-42}$ on inflammatory/stress markers in the rat hippocampus. The results demonstrate that treatment with $A\beta_{1-40}$+$A\beta_{1-42}$ at both the concentrations tested did not induce an inflammatory response nor altered caspase-3 activity.

Analysis of iNOS by real-time PCR revealed that Aβ infusion had no effect on the enzyme mRNA expression. NO is a messenger molecule playing key role in both physiological and pathological conditions (Bal-Price and Brown, 2001; Steward et al., 2002; for review see Prast and Philippu, 2001). In the brain, NO production relies on the activity of two major NOS isoforms: a constitutive Ca^{2+}-dependent neuronal NOS isoform (nNOS) and an inducible Ca^{2+}-independent NOS isoform (iNOS) which is expressed in glial cells after brain injury or pro-inflammatory stimulation (Vannucchi et al., 2005; for review see Almeida et al., 2005). It has been shown that physiological level of NO prevented the LPS-induced expression of iNOS suggesting a possible inhibitory effect of endogenous NO on iNOS expression (Colasanti et al., 1995). Thus, the balance between activities of nNOS and iNOS may regulate the overall NO concentration thereby determining whether NO will exert a pro- or an anti-inflammatory effect. Although the expression of iNOS has been implicated in the Aβ-induced deficits in cognitive function (Wang et al., 2004a; Medeiros et al., 2007), it has been reported that transgenic mice developing AD-like Aβ deposits showed upregulated nNOS expression in cells surrounding Aβ plaques while there was no significant expression of iNOS (Hartlage-Rubsamen et al., 2001). The present results are apparently in contrast with other reports demonstrating the ability of $A\beta_{1-40}$ and $A\beta_{1-42}$ to stimulate iNOS expression *in vivo* and *in vitro* (Weldon et a., 1998; Combs et al., 2001). Differences in experimental conditions may lead to different pattern of iNOS expression. Variations in the length of treatment may also account for the different results. Indeed, chronic infusion of Aβ has been shown to induce a time-dependent expression of iNOS in the hippocampus which was coupled with spatial memory deficits (Tran et al., 2001). The authors also reported that the peak of iNOS expression was observed at days 3-5 after Aβ infusion and suggested that the delayed effect of the peptide in inducing iNOS

was due to the time required for accumulation of toxic levels of Aβ. In light of these data it is speculated that Aβ infusion induced an increase in iNOS mRNA expression but this returned to basal level by day 28 or else, that Aβ was infused at a concentration too low to reach toxic levels and thereby stimulate iNOS expression.

There was no evidence of changes in CD86 and ICAM-1 expression induced by Aβ. It has been reported that administration of $A\beta_{1-42}$ increased the expression of both CD86 and ICAM concomitantly with microglial activation (Clarke et al., 2007). In light of these data it was predicted that infusion of a cocktail of Aβ peptides would have induced increased expression of both the markers of microglial activation. There are a number of possible explanations for these differences; it must be considered that $A\beta_{1-42}$ used in the present study was at a lower concentration (26.6 μM and 36.9 μM) than that used by Clarke and colleagues (200 μM). Furthermore, variation in the length of treatment, acute vs chronic, may also account for the different results. Data previously obtained from this laboratory show that increased expression of CD86 and ICAM-1, observed after 8 and 20 days of chronic infusion of Aβ, returned to basal levels by day 28 (Miller et al., unpublished). Thus, is it suggested that due to the duration of treatment CD86 and ICAM-1 expression was unaffected by Aβ treatment at day 28. Here, $A\beta_{1-40}$ and $A\beta_{1-42}$ were used in combination wheras in the other reports $A\beta_{1-40}$ was used alone. It has been demonstrated that $A\beta_{1-40}$ exerts a certain degree of protection against $A\beta_{1-42}$-induced cell damage by compromising β-sheet conformation and firbril formation (Zou et al., 2003; Kim e al., 2007b). It is possible that this effect played a part in the present study however, aggregation of Aβ was not assessed. Further, it has been suggested that, in transgenic mice showing increased Aβ burden, microglial activation and cytokines and surface markers expression may be a localised event limited to the Aβ deposits and identifiable only by immohistochemistry (Mehlhorn et al., 2000; Apelt et al., 2002). Thus, although there was no evidence of Aβ deposits in the brains of these rats, as assessed by Congo red staining, it is possible that Aβ infusion induced activation of some microglia but in focussed areas of the brain and that homogenization of the tissue did not allow this to be explored. Analysis of sections with immunostaining would validate this hypothesis However, the possibility remains that the failure of treatment to induce microglial activation is

due to the low concentration of peptides or else, to pour penetration of treatment into the hippocampus.

A further possibility is that the presence of activated astrocytes may be responsible for down-regulation of microglial activation. Indeed, it has been shown that the neurotoxic effect of microglial-conditioned medium was lost in the presence of astrocytes (von Berhardi and Eugenin, 2004). Transgenic mice, overexpressing mutant genes for AD, demonstrated immunoreactivity for IL-10 in addition to IL-1β in reactive astrocytes surrounding Aβ plaques (Apelt and Schliebs, 2001). It is possible that these mechanisms played a part in this study although astrocytic activation and anti-inflammatory cytokines were not assessed.

It is important to consider that different forms of Aβ solution may account for the different results obtained from different laboratories. Due to its highly instable nature, Aβ solubility depends on the preparation protocol, time and presence of carriers such as HDL (Malm et al., 2006). The final concentration and form of Aβ reaching the different brain areas may also represent an important variable accounting for the variation in data. The solution used in the present study contained mostly fibrils and a smaller amount of oligomers and monomers resembling the endogenous Aβ produced in the brain (for review see Mattson, 2004). Given that microglia are capable of phagocytosing Aβ (Shaffer et al., 1995; Paresce et al., 1996) it is speculated that the small amount of Aβ released into the ventricle every day might have been cleared by microglial cells. This hypothesis is supported by evidence that phagocytic microglia can internalise exogenous Aβ in an attempt to clear it from the CNS (Frautschy et al., 1992; Nakagawa et al., 2004). Analysis of cryostat sections prepared from the present study, revealed colocalization of activated microglia with upregulated CD68, a marker of phagocytosis (Grehan, unpublished). Further analysis assessing Aβ immunoreactivity and colocalization with CD68 would validate the hypothesis that chronically infused Aβ was phagocytosed by activated microglia.

Chronic infusion of Aβ at either the concentrations tested had no effect on CD200 expression. CD200 is a membrane glycoprotein found on neurons and endothelial cells: its interaction with the cognate receptor CD200R, found on cells of the myeloid lineage, has been implicated in the regulation of immune cell activity (Chitnis et al., 2007; Jenmalm et al., 2006). A role of CD200, by its interaction with CD200R, in the modulation of microglial activation has been

recently reported by Lyons and colleagues (2007b) who found that Aβ-induced inflammatory response *in vivo* and *in vitro* was associated with decreased expression of the glycoprotein. It should be noted that here, for the first time, CD200 expression was assessed in hippocampal tissue prepared from rats infused with a combination of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$. It is possible that the effect of $A\beta_{1\text{-}42}$ injected acutely may differ from the effect of $A\beta_{1\text{-}42}$ infused with $A\beta_{1\text{-}40}$. Moreover, it is arguable that the Aβ-induced effects depend on the concentration of oligomers and that this may vary between batches, however no consistent quality control of the Aβ solutions used was performed.

It is reported that caspase-3 activity was not affected by treatment with Aβ. Caspase-3 belongs to a family of cysteine endopeptidases that play an important role in the regulation of apoptosis (for review see Green and Reed, 1998). Although the evidence suggests an interaction between increased enzyme activity and cell death (Lynch and Lynch, 2002, Moore et al., 2007), contrasting data demonstrate that cells can be rescued from apoptosis irrespective of enzyme activity (Garnier et al., 2004). Also, it has been shown that Aβ-induced neurotoxicity *in vivo* and *in vitro* is mediated by caspase-3 activity (Minogue et al., 2003; St. John, 2007) thereby consolidating its role in the cognitive decline which accompanies AD. Injection of Aβ into the hippocampus has been shown to induce a significant increase of caspase-3 activity in the ipsilateral hemisphere (Stepanichev et al., 2003). Consistent with this, results from another experiment conducted in this laboratory showed that chronic infusion of $A\beta_{1\text{-}40}$+$A\beta_{1\text{-}42}$ at 63.8 μM induced activation of caspase-3 coupled with activated sphingomyelinase and accumulation of ceramide, in samples prepared from cortical tissue (Miller et al., unpublished). In light of these data it was somewhat surprising that Aβ infusion did not induce a significant increase of caspase-3 activity in the hippocampus. It is possible that the variability in the data may account for the unpredicted result. Analysis of immunoreactivity for Aβ and activated caspase-3 would validate the results obtained. In addition, it is arguable that the lack of effect of Aβ infusion on the parameters analysed is due to blockade of the pump or else interrupted infusion of peptides due to consumption of the solution. Analysis of the content of some pumps after 28 days infusion revealed that the content of the pump is higher than expected suggesting that the delivery rate undergoes a decrease over the

corse of treatment thereby compromising the effectiveness of the treatment protocol adopted.

In conclusion, the present experiment was carried out to assess the effect of chronic infusion of two different concentrations of $A\beta_{1-40}$+$A\beta_{1-42}$ in the rat hippocampus. It is suggested that the concentration of peptides was too low to induce microglial activation but rather, it stimulated their phagocytic activity and subsequent Aβ clearance. At this concentration Aβ treatment had no effect on caspase-3 activity but immunohistochemical analysis would allow further investigation of this, and would enable double labelling to assess whether caspase-3 expression colocalises with a neuronal, microglial or astrocytic marker. Taking into account the results reported in chapter 4 showing that acute injection of Aβ significantly increased caspase-3 activity, it is suggested that cell stress induced by Aβ may be reversed by day 28. However further time-related analysis would be required to consolidate this hypothesis and also to assess whether the presence of $A\beta_{1-40}$ may alter $A\beta_{1-42}$ toxicity over time.

Variable	Effect of treatment	
	$A\beta_{40+42}$ (45.5 μM)	**$A\beta_{40+42}$ (63.8 μM)**
iNOS mRNA (arbitrary units)	no change	no change
CD86 (arbitrary units)	no change	no change
ICAM-1 (arbitrary units)	no change	no change
CD200 (arbitrary units)	no change	no change
Caspase-3 activity (nmol/mg/min)	no change	no change

Table 5.1 Summary of results obtained

Chapter 6

Analysis of the effect of chronic infusion of LPS and Aβ, alone and in combination, on rat brain

6.1 Introduction

LPS is a bacterial toxin widely used experimentally to produce inflammation as a model to study neurodegenerative diseases including AD (Hauss-Wegrzyniak et al., 1998a, Hauss-Wegrzyniak et al., 1998b; Hauss-Wegrzyniak and Wenk, 2002). Activation of immune cells is mediated by the cell surface TLR4 which in the brain is expressed on microglia (Lenhard et al., 2003). Upon ligand binding TLR4 induces activation of different signaling pathways involving several mediators such as JNK and NF-kB thereby stimulating transcription of pro-inflammatory cytokines like IL-1β (Ledeboer et al., 2002; Szcepanik et al., 2001; Barry et al., 2005). These changes, in turn, have been associated with impairment of LTP in the rat hippocampus (Nolan et al., 2004). Evidence that IL-1RI expression is enhanced in response to LPS further emphasises the crucial role of IL-1β in the LPS-mediated inflammatory response (Vereker et al., 2001). Indeed, deficits in LTP have been reported in circumstances in which cytokine concentration is increased including administration of LPS (Vereker et al., 2000; Nolan et al., 2002).

Acute and chronic LPS administration has been shown to induce brain dysfunction associated with increased NO levels (Yamada et al., 1999) thereby validating the notion that LPS-induced neuronal damage is mediated by microglial activation (Szcepanik et al., 1996; Hauss-Wegrzyniak et al., 1999).

Although emerging data suggest a role for LPS-induced inflammation in clearing Aβ deposits, evidence from the literature demonstrates that inflammation may not only increase the expression but also alter the processing of APP to generate larger concentrations of Aβ (DiCarlo et al., 2001; Quinn et al., 2003; Sheng et al., 2003). Pro-inflammatory conditions have been suggested to impair microglial scavenger function thereby enhancing their reactivity against APP (von Bernhardi et al., 2007). In addition, co-administration of LPS and Aβ resulted in a greater activation of microglia primed with IFNγ compared to when either agent was used alone, suggesting an additive effect between the two compounds (Lotz et al., 2005). Similarly, pre-incubation of microglia with Aβ increased the sensitivity of the cells to stimulation by LPS (Gasic-Milenkovic et al., 2003).

6.2 Methods and materials

Young male Wistar rats were treated for 28 days with Aβ (18.9 μM and 26.6 μM, $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) and LPS (0.5 mg/ml) alone and in combination. Chronic infusion of the drugs was conducted by means of osmotic minipumps (6 μl/day) placed on a subcutaneus pocket in the midscapular area (see section 2.2.5).

At the end of the treatment time, animals were assessed for their ability to sustain LTP (see section 2.2.7). After 45 minutes post tetanic stimulation (250 Hz for 200 ms) the rats were decapitated and the brain quickly removed. A longitudinal incision of the brain was made and the dissected quarter was coated in OCT compound and immersed in liquid nitrogen for immunohistochemical analysis. The hippocampus was dissected free and an aliquot was snap frozen in liquid nitrogen for RNA extraction while the remaining tissue was cross-chopped and stored at -80 °C in 10% DMSO Krebs solution.

Analysis of MHCII, IL-1β, IL-4 and IL-10 mRNA expression was performed by RT-PCR (see section 2.4) while iNOS mRNA expression was assessed by real-time PCR (see section 2.5).

MHCII immunostaining was carried out on sections of 10 μm thickness prepared using a cryostat and mounted on gelatine coated glass slides (see section 2.7). Counterstaining was achieved with toluene blue and pictures were taken at 40x magnification.

Measurement of IL-1β protein concentration was performed on hippocampal homogenate by ELISA and the results normalised by protein concentration (see section 2.6). The same homogenates were analysed for caspase-3 activity using Caspase-3 Drug Discovery Kit, according to the protocol provided by the manufacturer (see section 2.10).

Protein density of CD86, ICAM-1 and CD200 was analysed in hippocampal homogenates by electrophoresis and immunoblotting (see section 2.8).

6.3 Results

Chronic infusion of LPS and Aβ, alone and in combination, attenuates LTP

Evidence from the literature indicates that neuroinflammation produced by chronic infusion of LPS attenuates LTP in the dentate gyrus (Hauss-Wegrzyniak et al., 2002) and that acute Aβ exposure can also inhibit LTP (Walsh et al., 2002; Yun et al. 2006). In order to assess the effects of chronic infusion of LPS and Aβ on neuronal function, LTP was assessed after 28 days of treatment with LPS and fibrillar Aβ, alone and in combination. Results presented in figure 6.1 show that delivery of a high frequency train of stimuli to the perforant path (time 0) resulted in an immediate increase of the population EPSP slope in all groups. Analysis of the last 5 minutes of recording post tetanic stimulation with two-way ANOVA showed that chronic infusion of LPS significantly attenuated LTP in the dentate gyrus ($F_{(1,14)}=6.34$, $p<0.05$, n=4-5). No additive effect of Aβ on the LPS-induced attenuation of LTP was detected.

Chronic infusion of LPS and Aβ differentially affects MHCII and iNOS mRNA expression

It is well documented that LPS is a potent immune stimulator which acts by binding to TLR4 and inducing signal transduction by activation of the transcription factor NF-κB (Cario et al., 2000). This, in turn, induces transcription of pro-inflammatory molecules that account for a positive feed-back loop on activated microglia. Aβ, also, has been shown to induce activation of the brain immune cells inducing release of cytotoxic compounds (Tran et al., 2001; Lynch et al., 2007). In order to assess whether chronic infusion of LPS and Aβ, alone and in combination, exerted any effect on microglia, expression of markers of microglial activation was first analysed by RT-PCR. Figure 6.2 (A) shows the mean values of MHCII expression. Analysis of the data with a two-way ANOVA, showed that LPS ($F_{(1,19)}=11.39$, $p<0.001$) and Aβ ($F_{(1,19)}=4.86$, $p<0.05$) exerted a significant effect on MHCII mRNA expression independent of each other. However, post hoc Neuman-Keuls test analysis revealed that mRNA expression was significantly increased in the LPS+Aβ-treated group compared to control, suggesting a significant interactive effect between the two inflammatory stimuli.

Since inflammation is associated with increased production of NO, as a consequence of increased expression of iNOS, mRNA expression of this enzyme was also measured. Figure 6.2 (C) shows that chronic administration of LPS significantly increased the mean mRNA expression value of iNOS compared to control. Surprisingly, co-treatment of LPS with Aβ had no effect of iNOS mRNA expression.

Chronic infusion of LPS induced microglial activation in the cortex

MHCII is a molecule involved in antigen presentation during immune response to pro-inflammatory stimuli and its expression on the cell surface can be regarded as a morphological marker of activated microglia (for review see Aloisi, 2001). In order to further analyse the level of microglial activation in response to chronic infusion of LPS and Aβ, brain slices were immunostained for MHCII, using OX-6 as a marker. Photomicrographs presented in figure 6.3 show a marked increase in the cell surface expression of MHCII in response to treatment with LPS, alone and in combination with Aβ. The photomicrographs were taken at 40x magnification. No Aβ-induced or LPS-induced staining was found within the hippocampus, MHCII expression was detected only within the cortex.

Chronic infusion of LPS and Aβ does not affect adhesion molecules expression

In order to further investigate the effect of chronic infusion of LPS and Aβ on microglial activation, analysis of adhesion molecules expressed on the cell surface of activated microglia and involved in antigen presentation was achieved by western immunoblotting. Data presented in figure 6.4 (A) show that chronic infusion of LPS ($F_{(1,14)}=0.25$, $p>0.05$) and Aβ ($F_{(1,14)}=0.22$, $p>0.05$, ANOVA) did not alter CD86 protein density. Consistent with this finding are the results from analysis of ICAM-1 protein, another adhesion molecule involved in leukocyte adhesion and inflammation. Figure 6.4 (B) demonstrates that ICAM-1 protein density was not affected by chronic treatment with either LPS ($F_{(1,15)}=3.31$, $p>0.05$) nor Aβ ($F_{(1,15)}=1.13$, $p>0.05$, ANOVA).

Chronic treatment with LPS and Aβ does not affect CD200 protein density

The presence of inhibitory factors, intrinsic to the CNS, provides a beneficial mechanism in controlling immune activity within the brain. One of

these factors includes CD200, a membrane protein involved in maintaining microglia in a quiescent state (Lyons et al., 2007b). In an attempt to understand whether LPS and Aβ exert their inflammatory effect by compromising mechanisms involved in the modulation of mictoglial activity, CD200 protein density was analysed. Data presented in figure 6.5 show that chronic infusion of LPS ($F_{(1,15)}$=0.3095, p>0.05) and Aβ ($F_{(1,15)}$=2.511, p>0.05, ANOVA), alone and in combination, did not alter expression of CD200.

Chronic infusion of LPS, alone and with Aβ, increases mRNA expression but not protein concentration of IL-1β

Evidence suggests that increased microglial activation and upregulated production of IL-1β are implicated in the LPS and Aβ-induced attenuation of learning and memory processes (Minogue et al., 2003; Lynch et al., 2004). Data reported in figure 6.6, panel (A), demonstrate that chronic infusion of LPS induced a significant increase of the pro-inflammatory cytokine mRNA expression compared to control ($F_{(1,16)}$=15.74, p<0.001, ANOVA). The increased IL-1β mRNA expression seen in the LPS+Aβ-treated group was due to the presence of LPS since no interactive effect was found between LPS and Aβ. Moreover, chronic infusion of Aβ induced a 4.8 fold increase of the mean value of IL-1β mRNA expression compared to control, however this effect did not reach statistical significance due to variability within the group. This result was not paralleled by results obtained from analysis of IL-1β protein concentration. Panel (B) shows that IL-1β concentration was not affected by chronic treatment with LPS ($F_{(1,20)}$=2, p<0.05) or Aβ ($F_{(1,18)}$=2.84, p<0.05, ANOVA).

Chronic infusion of Aβ alters IL-4 but not IL-10 mRNA expression

It has been proposed that the balance between pro- and anti-inflammatory mediators determines the degree of inflammation in the brain (Maher et al., 2005). Here the expression of the anti-inflammatory cytokines IL-4 and IL-10 mRNA was assessed. Analysis of the data presented in figure 6.7 (A) with a two-way ANOVA showed that chronic infusion of Aβ significantly altered the mean IL-4 mRNA expression and that there was no significant interactive effect between LPS and Aβ ($F_{(1,17)}$=4.83, p<0.05, ANOVA). However, analysis with post hoc Neuman-Keuls test failed to detect which conditions were significantly different

from each other. Figure 6.7 (B) illustrates the results from analysis of IL-10 mRNA and demonstrate that cytokine mRNA expression was not affected by chronic treatment with neither LPS ($F_{(1,18)}$=0.004, $p<0.05$) nor Aβ ($F_{(1,18)}$=0.29, $p<0.05$, ANOVA) alone and in combination.

Chronic infusion of LPS and Aβ does not affect caspase-3 activity

Although analysis of markers of microglial activation revealed that chronic infusion of LPS and Aβ did not induce marked microglial activation, it was argued that LPS and Aβ may exert their toxic effect on neurons by inducing apoptosis. Amongst the molecules involved in the process of programmed cell death is caspase-3 which has been shown to mediate LPS and Aβ-induced apoptosis (Minogue et al., 2003; Nolan et al., 2003). Results illustrated in figure 6.8 show that chronic infusion of LPS ($F_{(1,16)}$=0.19, $p>0.05$) and Aβ ($F_{(1,16)}$=0.36, $p>0.05$, ANOVA) alone or in combination, had no effect on caspase-3 activity.

Figures

Figure 6.1 Chronic infusion of LPS, alone and in combination with Aβ, attenuates LTP

Analysis of the last 5 minutes of recording post tetanic stimulation with two-way ANOVA showed a significant effect of LPS (0.5 mg/ml) on the percentage change in the EPSP slope ($F_{(1,14)}$=6.34, $p<0.05$, n=4-5). No interactive effect between Aβ and LPS was detected.

The mean population EPSP slope evoked by test stimuli delivered at 30 seconds intervals is shown and is expressed as a percentage of the slope recorded in the 5 minutes immediately prior to tetanic stimulation. Values are expressed as means ± SEM. SEM are included for every tenth response and some are so small in some cases as to be obscured.

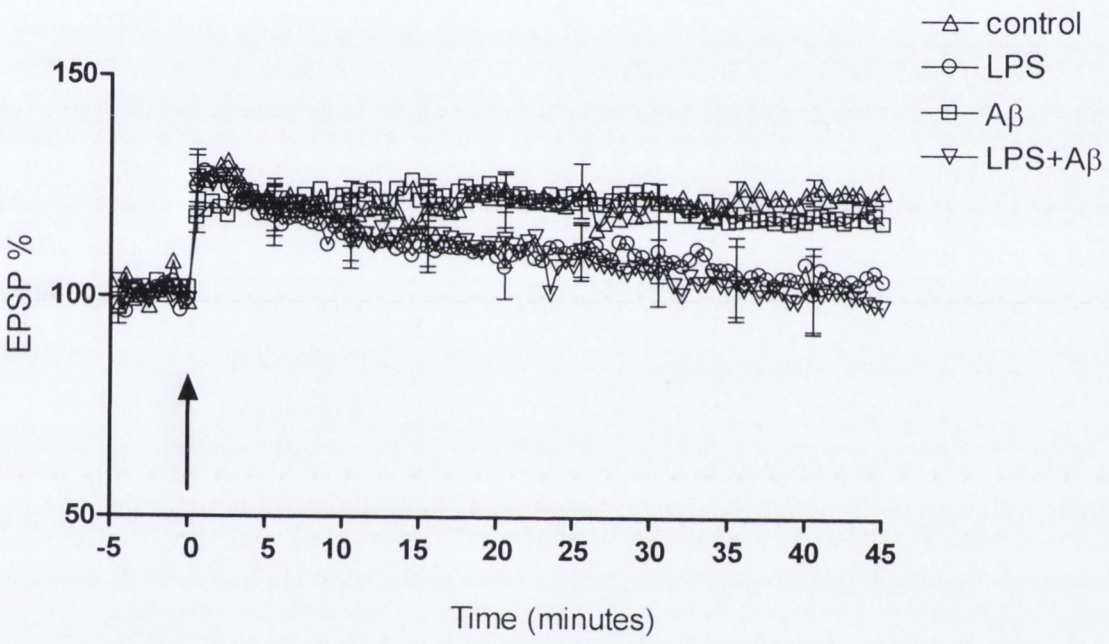

control
LPS
Aβ
LPS+Aβ
150
100
50
EPSP %
-5
0
5
10
15
20
25
30
35
40
45
Time (minutes)

Figure 6.2 Chronic infusion of LPS increases MHCII and iNOS mRNA expression independent of Aβ

A) A two-way ANOVA showed a significant effect of LPS (0.5 mg/ml) ($F_{(1,19)}$=11.39, $p<0.001$, n=5-6) and Aβ (45.5 μM) ($F_{(119)}$=4.86, $p<0.05$) on MHCII mRNA expression. However, analysis with post hoc Newman-Keuls test showed that only LPS and Aβ in combination induced a significant increase of MHCII mRNA expression compared to control. Data are expressed as a ratio of MHCII to β-actin and are means ± SEM. **$p<0.01$ vs control (Newman-Keuls test).

B) A two-way ANOVA showed a significant interactive effect between LPS (0.5 mg/ml) and Aβ (45.5 μM) on iNOS mRNA expression ($F_{(1,16)}$=6.79, $p<0.05$, n=4-6). However, post hoc analysis revealed that LPS alone significantly increased iNOS mRNA expression compared to control. Data are expressed as a ratio of iNOS to β-actin and are means ± SEM. *$p<0.05$ vs control (Newman-Keuls test).

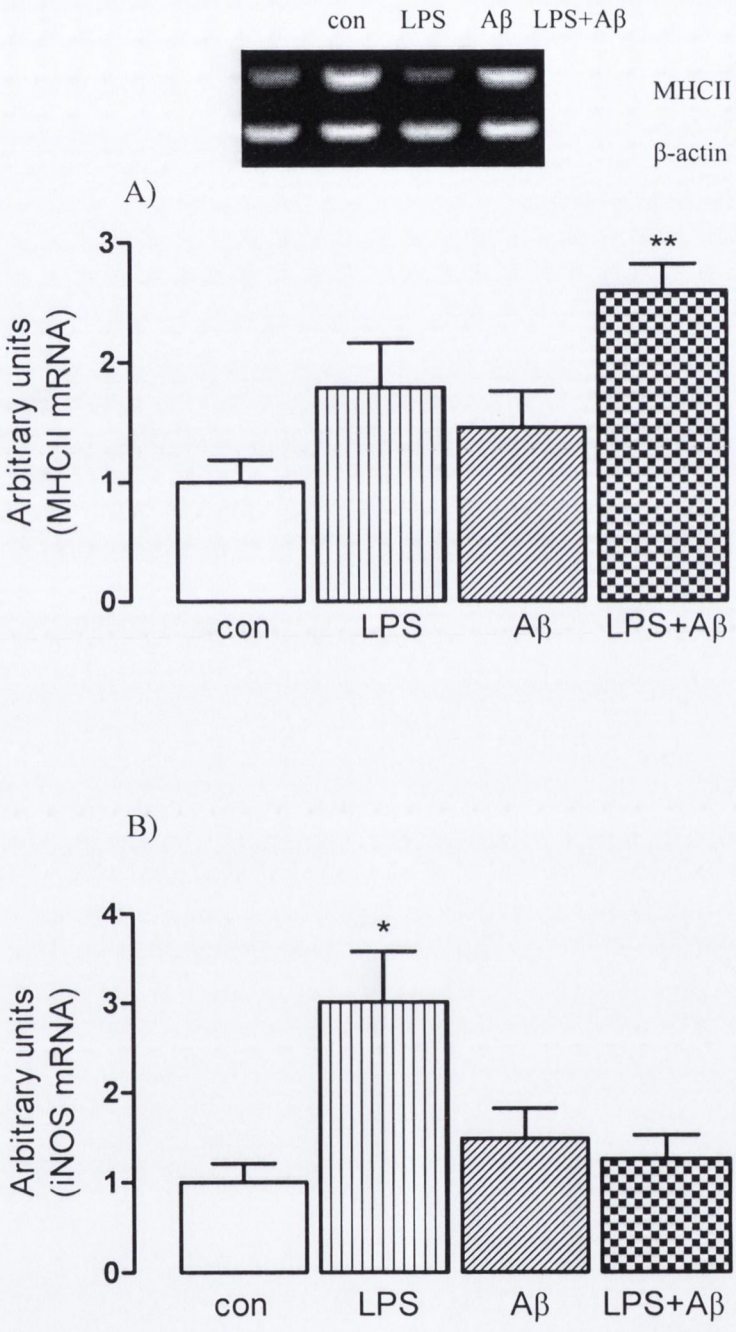

con
LPS
Aβ
LPS+Aβ
MHCII
β-actin
A)
Arbitrary units
(MHCII mRNA)
3
2
1
0
**
con
LPS
Aβ
LPS+Aβ
B)
Arbitrary units
(iNOS mRNA)
4
3
2
1
0
*
con
LPS
Aβ
LPS+Aβ

Figure 6.3 Chronic infusion of LPS increases MHCII expression, independent of Aβ

Microglial activation, assessed by MHCII expression, was increased in cortical slices of LPS (0.5 mg/ml) and LPS+Aβ-treated (0.5 mg/ml and 45.5 μM respectively) rats compared with control, as indicated by the brown staining. MHCII expression was not detectable in cortical slices from control and Aβ-treated rats. Pictures are representative of staining obtained in slices prepared from 6 animals for each treatment group.

Counterstaining was achieved with toluene blue. Magnification 40x, scale bare 100 μm.

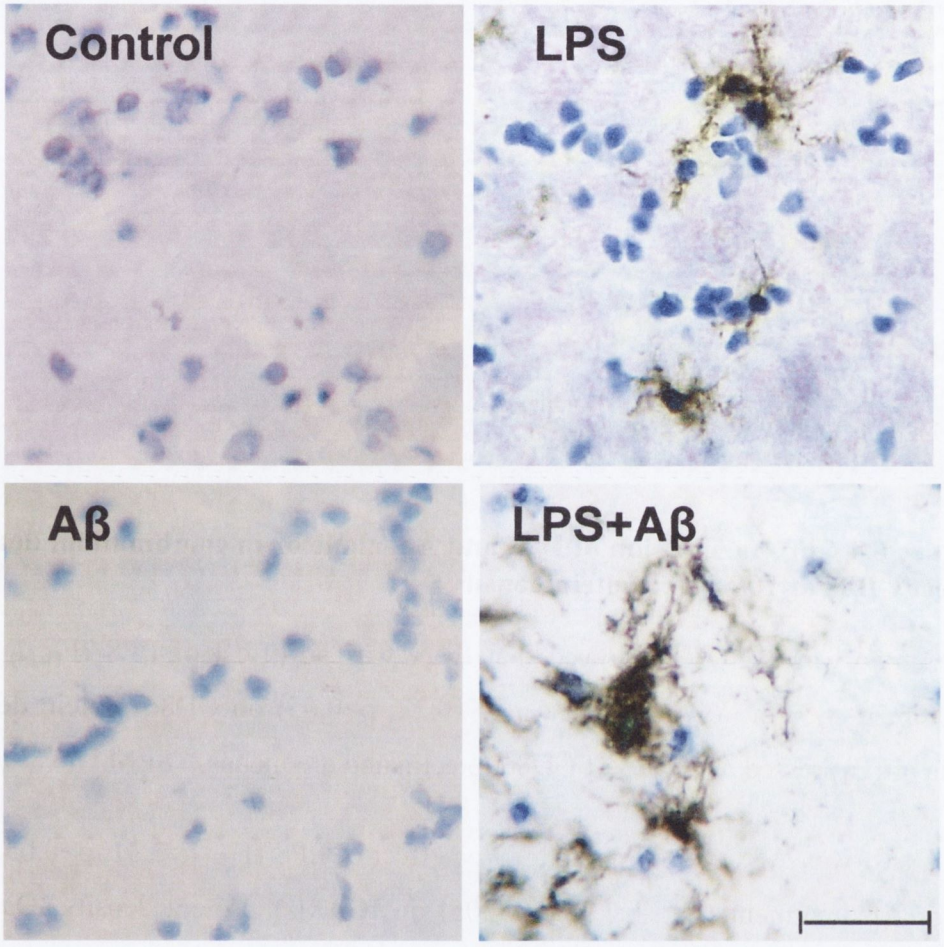

Control
LPS
Aβ
LPS+Aβ

Figure 6.4 Chronic infusion of LPS and Aβ, alone or in combination, does not affect CD86 or ICAM-1 protein density

A) A two-way ANOVA showed that there was no effect of LPS ($F_{(1,14)}$=0.25, p>0.05, n=4-5) or Aβ treatment ($F_{(1,14)}$=0.22, p>0.05) on CD86 protein density. Data are expressed as a ratio of CD86 to actin and are means ± SEM.

B) A two-way ANOVA demonstrated no effect of LPS ($F_{(1,15)}$=3.31, p>0.05, n=4-5) or Aβ treatment ($F_{(1,15)}$=1.13, p>0.05) on ICAM-1 protein density. Data are expressed as a ratio of ICAM-1 to actin and are means ± SEM.

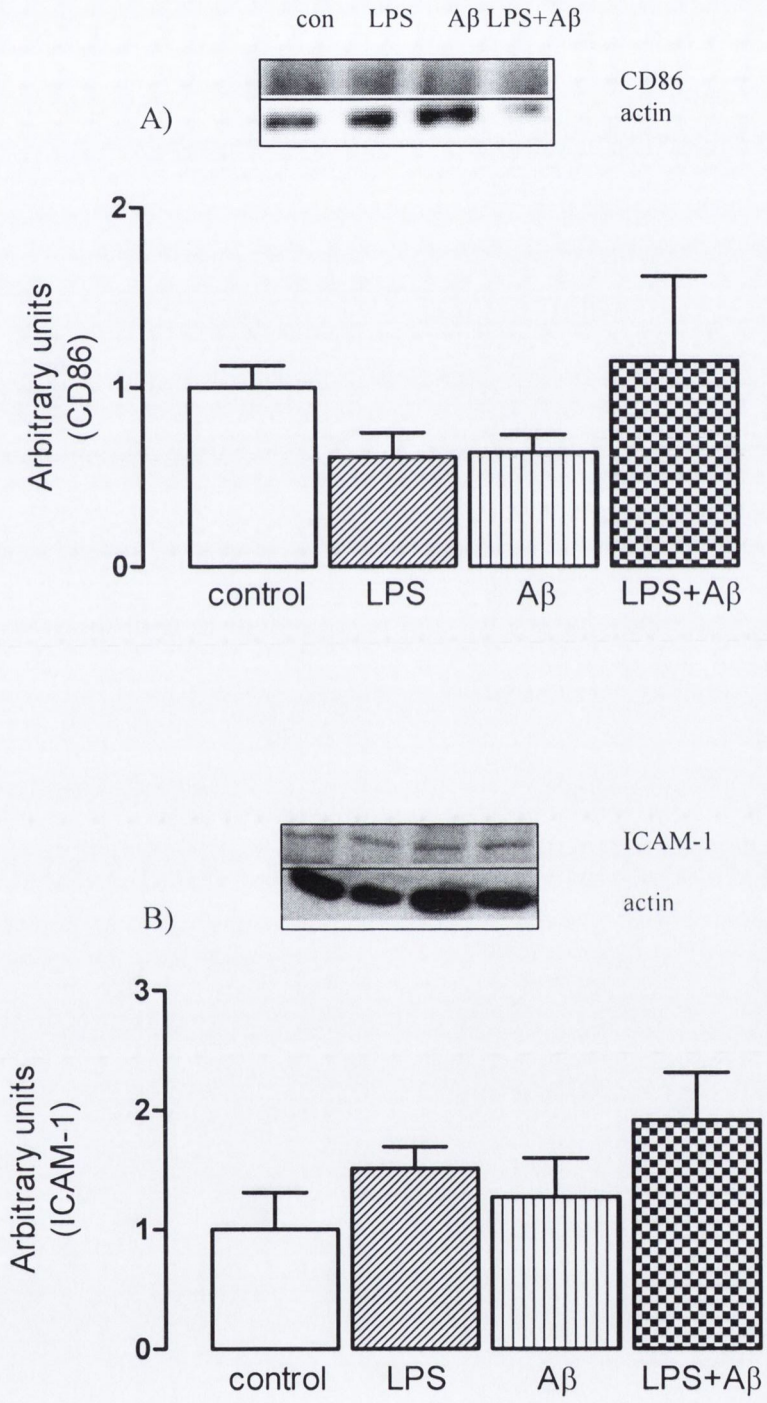

con
LPS
Aβ
LPS+Aβ
A)
CD86
actin
Arbitrary units
(CD86)
2
1
0
control
LPS
Aβ
LPS+Aβ
B)
ICAM-1
actin
Arbitrary units
(ICAM-1)
3
2
1
0
control
LPS
Aβ
LPS+Aβ

Figure 6.5 Chronic infusion of LPS and Aβ, alone or in combination, does not affect CD200 protein density

A two-way ANOVA demonstrated no significant effect of LPS ($F_{(1,15)}$=0.31, $p>0.05$, n=4-5) or Aβ treatment ($F_{(1,15)}$=2.51, $p>0.05$) on CD200 protein density. Data are expressed as a ratio of CD200 to actin and are means ± SEM.

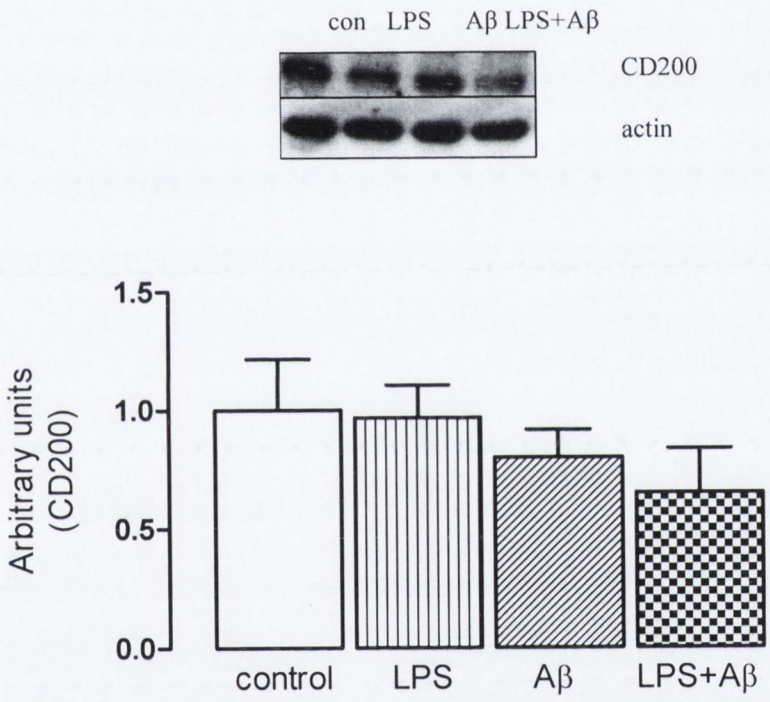

con
LPS
Aβ
LPS+Aβ
CD200
actin
Arbitrary units
(CD200)
1.5
1.0
0.5
0.0
control
LPS
Aβ
LPS+Aβ

Figure 6.6 Chronic infusion of LPS and Aβ, alone or in combination, increases IL-1β mRNA expression but not protein concentration

A) A two-way ANOVA showed a significant effect of LPS (0.5 mg/ml) on IL-1β mRNA ($F_{(1,16)}$=15.74, $p<0.01$, n=4-6). Data are expressed as a ratio of IL-1β to β-actin and are means ± SEM. *$p<0.05$ vs control (Newman-Keuls test).

B) A two-way ANOVA showed no effect of LPS ($F_{(1,20)}$=2, $p>0.05$, n=6) or Aβ treatment ($F_{(1,18)}$=2.84, $p>0.05$) on IL-1β protein concentration. Data are expressed as a ratio of IL-1β to protein concentration and are means ± SEM.

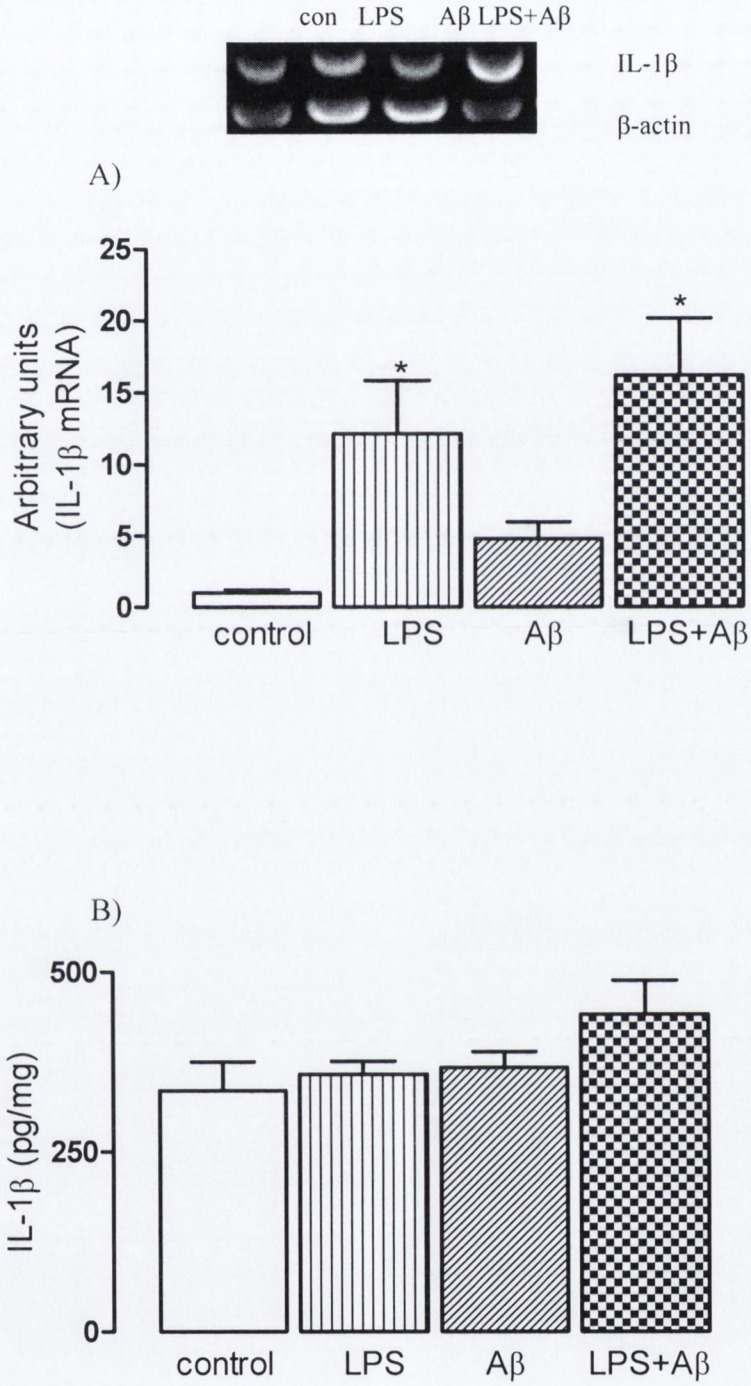

con LPS Aβ LPS+Aβ
IL-1β
β-actin
A)
Arbitrary units
(IL-1β mRNA)
25
20
15
10
5
0
*
*
control
LPS
Aβ
LPS+Aβ
B)
IL-1β (pg/mg)
500
250
0
control
LPS
Aβ
LPS+Aβ

Figure 6.7 Chronic infusion of Aβ alters IL-4 but not IL-10 mRNA expression

A) A two-way ANOVA showed a significant effect of Aβ (45.5 μM) ($F_{(1,17)}$=4.83, $p<0.05$, n=5-6) on IL-4 mRNA expression. However, analysis with Newman-Keuls post hoc test did not find any difference between conditions. Data are expressed as a ratio of IL-4 to 18s and are means ± SEM.

B) A two-way ANOVA showed no effect of LPS ($F_{(1,18)}$=0.004, $p>0.05$, n=5-6) or Aβ treatment ($F_{(1,18)}$=0.29, $p>0.05$) on IL-10 mRNA expression. Data are expressed as a ratio of IL-10 to β-actin and are means ± SEM.

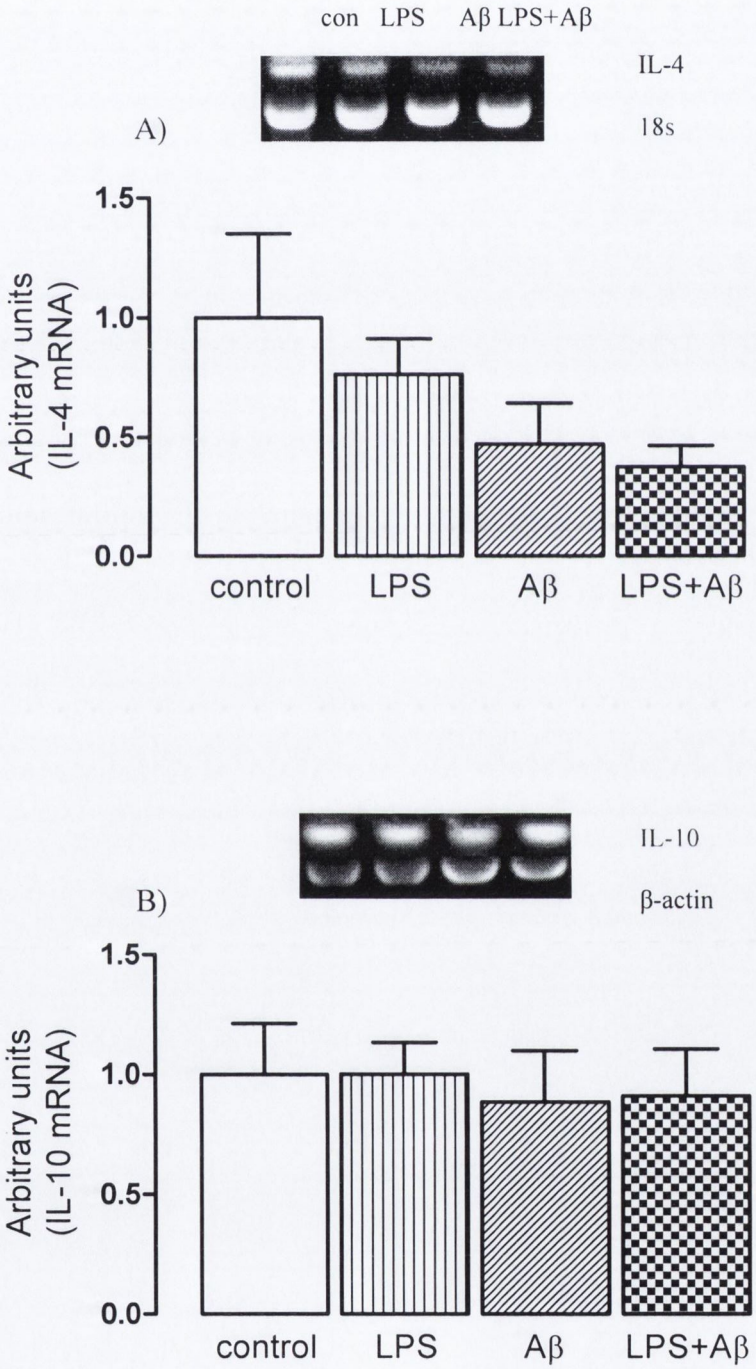
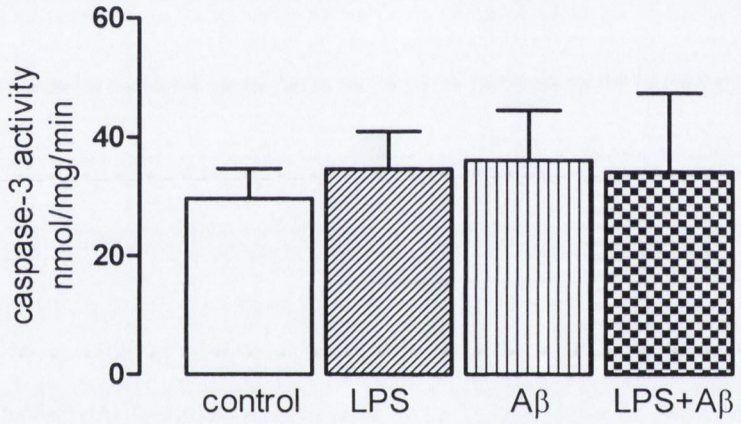

con LPS Aβ LPS+Aβ
IL-4
18s
A)
Arbitrary units
(IL-4 mRNA)
1.5
1.0
0.5
0.0
control
LPS
Aβ
LPS+Aβ
IL-10
β-actin
B)
Arbitrary units
(IL-10 mRNA)
1.5
1.0
0.5
0.0
control
LPS
Aβ
LPS+Aβ

Figure 6.8 Chronic infusion of LPS and Aβ, alone or in combination, had no effect on caspase-3 activity

A two-way ANOVA demonstrated no significant effect of LPS ($F_{(1,16)}$=0.19, p>0.05 , n=4-6) or Aβ ($F_{(1,16)}$=0.36, p>0.05) on caspase-3 activity. Data are means ± SEM.


caspase-3 activity
nmol/mg/min
60
40
20
0
control
LPS
Aβ
LPS+Aβ

6.4 Discussion

The present study was undertaken to assess the effect of chronic infusion of Aβ (18.9 μM and 26.6 μM, $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) and LPS (0.5 mg/ml), alone and in combination, in the rat hippocampus and to analyse whether the agents exert an additive effect inducing an exaggerated immune response. The results obtained demonstrate that infusion of LPS, alone and in combination with Aβ, attenuated LTP and induced a moderate inflammatory response which was independent of Aβ.

Chronic treatment with LPS significantly attenuated LTP compared to controls. This is consistent with results obtained by Hauss-Wegrzyniak and colleagues (2002) who demonstrated that chronic infusion of LPS at a higher concentration (1 mg/ml) inhibited LTP in perforant path-granule cell synapses. It has been suggested that a mechanism implicated in the LPS-induced impairment of learning and memory, as assessed by T-maze task, includes the loss of enthorinal cells and their projections into the hippocampus (Hauss-Wegrzyniak et al., 2000b). Analysis with high-resolution magnetic resonance imaging (MRI) in a rat model of chronic neuroinflammation revealed that chronic infusion of LPS into the fourth ventricle induced enlargement of the ventricles and significantly reduced the size of temporal lobe region and hippocampus (Hauss-Wegrzyniak et al., 2000a). Chronic LPS infusion has been shown to exert detrimental effect on operant behaviour which was reversed by treatment with ibuprofen, suggesting that inflammation is implicated in the LPS-induced behavioural deficit (Richardson et al., 2005). Furthermore, results presented here demonstrate that Aβ infusion had no effect on LTP nor it did exert any additive effect on the LPS-induced impairment in LTP. This finding is in contrast with results obtained from this laboratory demonstrating that chronic infusion of $A\beta_{1-40}$+$A\beta_{1-42}$, although at a higher concentration, induced a significant deficit of LTP (Miller et al., unpublished). In addition, infusion of $A\beta_{1-40}$ alone has been shown to induce severe impairment in cognitive behaviour and LTP (Nitta et al., 1997; Chen et al., 2006b). However, it is arguable that the concentration of peptides used in the present study is too low to significantly attenuate LTP in the hippocampus. A dose-response study with different concentrations of Aβ peptides would address this issue. Also, analysis of Aβ immunoreactivity (Grehan et al., unpublished) in

slices prepared from the brain of Aβ-treated rats showed the presence of immunoreactive peptides mainly within the ventricles. Thus, it is suggestsed that Aβ fibrils did not reach the brain due to access issues and that the oligomers present in the solution tested were at a concentration too low to affect LTP. Indeed, there is compelling evidence for the ability of the oligomeric species to induce deficits of cognitive functions (Walsh et al., 2002; Klyubin et al. 2005; Yun et al., 2006).

The aim of this study was also to assess the possibility that LPS and Aβ may act in synergy to induce an exaggerated response in the rat hippocampus. The results show that the extent to which LTP was impaired in the LPS+Aβ-treated group was similar to that of the LPS-treated group suggesting that LTP impairment was due to the presence of LPS irrespective of Aβ. This is the first time that Aβ has been infused in combination of LPS. Evidence from the literature suggests that transient systemic inflammatory events enhance susceptibility of the brain to Aβ neurotoxicity or lead to brain protection, depending on the duration, time and severity of inflammation (Cakala et al., 2007). Here, it is reported that LPS alone induced a profound neuronal impairment which was not exacerbated by Aβ co-infusion due to low concentration of peptides.

One mechanism involved in the loss of pyramidal neurons as well as enthorinal cells is enhanced inflammatory response as a consequence of microglial activation (Hauss-Wegrzyniak et al., 1998b). In the present study microglial activation was assessed by analysis of MHCII. The results obtained indicate that MHCII mRNA expression was increased only in the presence of both LPS and Aβ. These data lead to suggest that LPS and Aβ exerted a synergistic effect on MHCII mRNA expression at a transcriptional level. This hypothesis is validated in part by a recent report implicating the transcription factor NF-κB as a regulator of MHCII expression (Liu et al., 2007) and by the notion that both LPS and Aβ are capable of activating NF-κB (Cario et al., 2000; Valerio et al., 2006). It is thereby speculated that the increased MHCII mRNA expression reported here was triggered by concomitant activation of NF-κB by LPS and Aβ. This hypothesis is supported by a previous report demonstrating that cotreatment with Aβ and LPS caused greater microglial activation than with either compound alone (Lotz et al., 2005). Similarly, small fibrillar Aβ peptides, although being relatively weak inflammatory stimulators alone, have been shown to enhance the effect of

pro-inflammatory signals such as LPS or IFNγ (Gasic-Milenkovic et al., 2003). The additive effect of endogenous and exogenous stimuli has been described by several reports demonstrating that pro-inflammatory stimulation of an organism with primed glial cells leads to an exaggerate response as compared to that obtained from non primed cells (Tateda et al., 1996; Combrinck et al., 2002; Holmes at al., 2003; for review see Perry et al., 2003). It should be noted that there was marked variation between the observations (as indicated by the high SEM values) and this may account for the lack of effect of treatment with LPS alone on MHCII mRNA expression.

Microglial activation was also assessed by analysis of iNOS mRNA. The results show that infusion of LPS induced a significant increase in the expression of the enzyme. This is consistent with previous data demonstrating that LPS exposure induced iNOS expression *in vivo* and *in vitro* (Molina-Holdago et al., 2001; Takagi et al., 2007). It has been suggested that a mechanism involved in the LPS-induced expression of iNOS includes activation of MAPKs leading to induction of the transcription factor NF-κB (Jung et al., 2007). Given that also Aβ-induced signalling involves MAPKs activity and subsequent activation of NF-κB, it was predicted that cotreatment with LPS and Aβ might exert an additive effect on iNOS mRNA expression. Surprisingly, the effect of LPS on iNOS expression was lost when LPS was infused in combination with Aβ. Indeed, it has been shown that $A\beta_{1\text{-}40}$ *in vitro* reversed the LPS-induced increase in prostaglandin E2 (PGE_2) concentration thereby preventing termination of microglial activation and subsequent release of pro-inflammatory mediators (Balboa et al., 2001). An explanation for this unpredicted result remains to be established.

Further analysis of activated microglia by immunohistochemistry, using OX-6 as a marker, revealed that LPS, alone and in combination with Aβ, induced a marked increase in OX-6 reactivity in the cortex. This is consistent with previous results showing increased expression of the cell surface marker in response to chronic administration of LPS (Hauss-Wegrzyniak et al., 1998b; Richardson et al., 2005). The data reported here are also consistant with the general notion that inflammation is involved in the development and progression of AD (for review see Sastre et al., 2006). Indeed several inflammatory mediators are increased in the brains of AD patients as compared to age-matched controls

(for review see McGeer and McGeer, 1999; Akiyama et al., 2000). Analysis with fluorescent immunohistochemistry of slices prepared from the rats infused with Aβ showed that treatment with peptides alone induced an increased immunoreactivity for OX-6 in the hippocampus, compared to controls. It is possible that infusion with Aβ and LPS, alone and in combination, induced a weak increase of MHCII expression in the hippocampus and that the technique for immunostaining used in the present study was not sensitive enough to detect low levels of OX-6 reactivity. Analysis of slices prepared from the LPS- and LPS+Aβ-treated groups with fluorescence staining would be required to assess whether LPS induced microglial activation in the hippocampus. The evidence also indicates a marked increase in OX-6 positive reactive microglia in the cortex of rats which were infused with a combination of LPS and Aβ. Although LPS alone and LPS+Aβ induced microglial activation to a similar extent, it should be noted that the processes appear shorter and less defined in the LPS+Aβ-treatd group than those in the LPS-treated group suggesting a phagocytic morphology. Confocal microscopy would allow establish whether activated microglia, as assessed by analysis of OX-6 expression, colocalise with CD68, a marker for phagocytosis, and validate this hypothesis.

The data demonstrate that chronic infusion of LPS and Aβ, alone and in combination, had no effect on the expression of CD86, ICAM-1 and CD200. This is apparently in contrast with other works showing that Aβ injection induced increased expression of both CD86 and ICAM-1 (Clarke et al., 2007) and that Aβ-induced microglial activation, as indicated by increased IL-1β concentration, was associated with a marked decrease in CD200 expression *in vivo* and *in vitro* (Lyons et al., 2007b). An inverse correlation has been described between microglial CD86 and neuronal CD200 expression (Downer et al., submitted). It must be noted that in those studies $A\beta_{1-42}$ was administered alone and acutely wheras in the present study $A\beta_{1-42}$ was infused in combination with $A\beta_{1-40}$ for 28 days. It is possible that variations in the protocol of Aβ administration and preparation may account for the different results. Up-regulation of CD86 and ICAM-1 has been associated with activated microglia *in vivo* and *in vitro* (Carson et al., 1998; Clarke et al., 2007; Griffin et al., 2006; Kim et al., 2006), the results presented demonstrate that infusion of LPS and Aβ, alone and in combination, did not induce activation of microglia. It has been reported that microglia are capable

to phagocytose Aβ (Shaffer et al., 1995; Paresce et al., 1997) and that a certain degree of inflammation aids to Aβ clearance (Quinn et al., 2003). It is suggested that infused Aβ might have been cleared by LPS-primed microglia. However, further analysis assessing Aβ immunoreactivity and phagocityc microglia, by CD68 analysis, would be required to assess this.

Chronic infusion of LPS, alone and in combination with Aβ, induced a significant increase in IL-1β mRNA expression which, surprisingly, was not paralleled by an increase in IL-1β concentration. This finding is not consistent with previous works conducted in this laboratory demonstrating increased levels of IL-1β concentration and induced signaling in respone to LPS (Curran et al., 2003; Kelly et al., 2003; Lonergan et al., 2004; Barry et al., 2005). Although the evidence has suggested that IL-1β exerts an inhibitory effect of LTP, the results presented indicate that the LPS-induced attenuation of LTP was not associated with increased levels of IL-1β. It is suggested that the marked variations between observations may account for the different results. It is also possible that the presence of high lipids content in the homogenates compromised the reading of the assay accounting for the high levels of IL-1β observed in all the groups. Further analysis assessing IL-1β immunostaining will be required to confim the results obtained. The importance of IL-1β in the Aβ-induced signaling has been highlighted by evidence that mice lacking IL-1Ra showed enhanced microglial activation and exaggerated neuronal damage in response to Aβ infusion (Craft et al., 2005). Also, increased microglial activation, as indicated by F4/80 immunoreactivity, and IL-1β concentration have been reported following chronic infusion of Aβ (Craft et al., 2005). According to Snyder and colleagues (1994), $A\beta_{1\text{-}40}$ inhibits aggregation of $A\beta_{1\text{-}42}$ thereby compromising its toxicity. It is possible that this effect may account for attenuated toxicity of the Aβ solution used in this study; however, Aβ aggregation was not assessed. It is also suggested that the concentration of peptides used in the present study was too low to induce microglial activation and subsequent IL-1β production.

Maher and colleagues (2005) reported that the age-associated deficit in LTP was coupled with a decrease of the anti-inflammatory cytokine IL-4. It was therefore considered that LPS and Aβ might exert their effect also by decreasing the expression of IL-4 and IL-10. The results obtained analysing cytokines mRNA expression reveal that chronic infusion of LPS and Aβ, alone and in combination,

had no effect on the expression of IL-4 and IL-10 mRNA. Evidence suggests that the balance between pro- and anti-inflammatory cytokines is one determinant for regulating glial cells activation. Indeed, increased pro-inflammatory activities have been associated with down-regulation of mechanisms involved in maintaining microglia in a quiescent state such as IL-10, CD200 and IL-4 (Maher et al., 2004; Frank et al., 2006; Maher et al., 2005). Moreover, it has been shown that LPS exerts its inflammatory action by decreasing levels of IL-10 and IL-4 concentration (Kavanagh et al., 2004) which have been shown to exert protective effect against the LPS- and Aβ-induced inflammatory changes (Molina-Holgado et al., 2001; Szczepanik et al., 2001; Lynch et al., 2004; Lyons et al., 2007b).

There was no evidence that LPS, Aβ or LPS+Aβ affected caspase-3 activity. Activation of caspase-3 has been considered as a marker of cell stress, however, it has been reported that activation of caspase-3 is not always associated with cell death (Nolan et al., 2003; Garnier et al., 2004; for review see Green and Reed, 1998). It has been shown that hypoxia in the gerbil brain induced marked caspase-3 gene expression which was not associated with cells death (Garnier et al., 2004). The interaction between inflammation and increased caspase-3 activity has been investigated previously in this laboratory and the data have demonstrated that the age-associated deficit in LTP was coupled with increased IL-1β concentration and increased activity of caspase-3 in hippocampal homogenate (Lynch and Lynch, 2002). The ability of IL-1β to induce activation of caspase-3 has been confirmed in a recent report which showed that IL-1β increased active caspase-3 immunoreactivity in cultured neurons (Moore et al., 2007). Similarly, LPS has been shown to induce caspase-3 activation (Barry et al., 2005). Although it is possible that the different procedure of LPS treatment, acute vs chronic and ip vs icv injection, may accout for the different result it must be noted that there was high variation between observations and this also impacts on the final result. Aβ also has been shown to induce caspase-3 activation in cultured neurons (Minogue et al., 2003), and chronically infused $A\beta_{1\text{-}40}$ +$A\beta_{1\text{-}42}$ induced a marked increase of the enzyme activity in cortical tissue (Miller et al., unpublished). Thus, it is suggested that Aβ concentration was too low to induce activation of caspase-3.

In conclusion the results demonstrate that chronic infusion of LPS and Aβ, alone and in combination, did not induce marked microglial activation although LPS significantly attenuated LTP. It is possible that the concentration of LPS and

Aβ were too low to induce a marked inflammatory response but enough to compromise neuronal function. Analysis of synaptic proteins expression would be required to investigate the mechanisms underlying the LPS-induced attenuation in LTP. It is also suggested LPS infusion might have stimulated microglial phagocytosis of Aβ, however, further analysis of Aβ immunoreactivity and phagocytic microglia would validate this hypothesis.

Variable	**Effect of treatment**		
	LPS (0.5mg/ml)	**$A\beta_{40+42}$ (45.5 μM)**	**LPS+ $A\beta_{40+42}$**
LTP (%EPSP)	↓	no change	↓
MHCII mRNA (arbitrary units)	no change	no change	↑
iNOS mRNA (arbitrary units)	↑	no change	no change
CD86 (arbitrary units)	no change	no change	no change
ICAM-1 (arbitrary units)	no change	no change	no change
CD200 (arbitrary units)	no change	no change	no change
IL-1β mRNA (arbitrary units)	↑	no change	↑
IL-1β (pg/mg)	no change	no change	no change
IL-4 mRNA (arbitrary units)	no change	no change	no change
IL-10 mRNA (arbitrary units)	no change	no change	no change
Caspase-3 activity (nmol/mg/min)	no change	no change	no change

Table 6.1 Summary of results obtained

Chapter 7

Analysis of the effect of chronic infusion and intra-hippocampal injection of Aβ on young and aged rat hippocampus

7.1 Introduction

Brain aging is associated with a progressive decline of cognitive and memory functions accompanied by an overall decrease of brain volume (for review see Anderton, 2002). These changes are partly the result of neuronal damage and loss which are though to be caused by the progressive accumulation of ROS. Increased level of pro-inflammatory cytokines has also been implicated in the changes observed in the aged brain (Lynch and Lynch, 2002; Moore et al., 2003; for review see Squier, 2001). In support to this, is the evidence that the age-related impairment in LTP has been correlated with increased levels of IL-1β concentration (Murray et al., 1997; Vereker et al., 2000; Kelly et al., 2001; Vereker et al., 2001; Kelly et al., 2003).

Activated microglia, which represent the main source of IL-1β, may account for the age-related increase in cytokine concentration. Indeed, activated microglia have been reported in the aging brain and have been characterised by upregulated expression of, among others, MHCII and costimulatory molecules (Griffin et al., 2006, Moore et al., 2007). It has been suggested that the age-dependent shift toward a pro-inflammatory microenvironment may be a consequence of an imbalance between pro-and anti-inflammatory processes (Maher et al., 2004; Frank et al., 2006; Maher et al., 2005). Thus, up-regulation of glial inflammatory cytokines with aging may lower the threshold for the development of neurodegenerative diseases like AD (Yu et al., 2002). It has been suggested that microglia in the healthy aging brain are primed (Godbout et al., 2005) and this may account for the increased vulnerability of the aged brain to inflammatory stimuli (Long et al., 1998; Deng et al., 2006; Lecanu et al., 2006; Liang et al., 2007). Evidence that older rats were more susceptible to the effects of Aβ than younger animals further corroborates this notion (Lynch et al., 2007; Minogue et al., 2007).

7.2 Methods and materials

The first experiment was carried out on young and aged rats which were chronically infused with a combination of 26.6 μM $A\beta_{1-40}$ and 36.9 μM $A\beta_{1-42}$ using osmotic minipumps (see section 2.2.5). This study was carried out in conjunction with Dr Anthony Lyons. The results from the young Aβ- treated groups are the same as those presented in chapter 5. After 28 days treatment period, rats were sacrificed and their brains dissected.

In the second experiment young and aged rats received a single intra-hippocampal injection of $A\beta_{1-42}$ (200 μM) as described in section 2.2.5 and 7 days post-treatment were assessed for their ability to sustain LTP (see section 2.2.7). After a period of stabilisation test shocks (0.033 Hz) were delivered at 30 seconds intervals and recorded for 15 minutes before and 45 minutes after tetanic stimulation (250 Hz for 200 ms). At the end of the test animals were decapitated and the hippocampi dissected free to obtain an aliquot for RNA extraction and cross chopped tissue stored in 10% DMSO Krebs.

Analysis of IL-1β protein concentration and caspase-3 activity was performed on hippocampal homogenates using ELISA (see sections 2.3) and a Caspase-3 Drug Discovery Kit respectively (see section 2.10).

Real-time PCR was used to assess mRNA expression of MHCII, CD11b, CD40, IL-1β, iNOS and CD200. Total RNA was purified from hippocampal tissue with a Nucleospin Kit (see section 2.5.1). RNA was reverse transcribed into cDNA using high-capacity cDNA archieve kit according to the protocol provided by the manufacturer (see section 2.5.3). Real-time PCR was performed on Applied Biosystems 7300 Real-time PCR System with Applied Biosystems 7300 System SDS Software 1.3.1 in 96-well format as described in section 2.5.4. Rat β-actin was used for normalization.

7.3 Results

Chronic infusion of Aβ does not exacerbate the age-related increase of MHCII, CD40 and CD11b mRNA expression

It is thought that the age-related increase in inflammation may render the brain more vulnerable to inflammatory stimuli and to assess this, young and aged rats were treated with chronic infusion of $A\beta_{1-40}$+$A\beta_{1-42}$ (63.8 μM). Figure 7.1 (A) illustrates the results from analysis of MHCII mRNA using real time PCR. Analysis of data with a two-way ANOVA demonstrated a significant interactive effect of age and Aβ treatment ($F_{(1,19)}$=5.44, $p<0.05$) on MHCII mRNA expression. Post hoc Neuman-Keuls test analysis demonstrated that statistical significance for MHCII mRNA expression lay between the aged control-treated group and the young control-treated group. Consistent with this finding, figure 7.1 (B) shows that CD40 mRNA expression was significantly increased in the aged control-treated group compared to young controls ($F_{(1,17)}$=5.5, $p<0.05$, ANOVA). A two-way ANOVA analysis also revealed a significant interactive effect of age and Aβ treatment on CD40 mRNA ($F_{(1,17)}$=4.96, $p<0.05$). However, analysis with Newman-Keuls test indicated that statistical significance for CD40 mRNA expression lay between the aged control- treated group and the aged Aβ-treated group.

It is known that gliosis is associated with cell division; the integrin CD11b is normally expressed on the surface of quiescent microglia but its expression is readily upregulated during inflammation (Alosi et al., 2000). Figure 7.1 (C) shows the effects of chronic infusion of Aβ on CD11b mRNA expression; analysis of data with two-way ANOVA showed that expression was significantly increased in the hippocampus of aged rats compared with young controls ($F_{(1,20)}$=15.85, $p<0.001$). Post hoc Neuman-Keuls test analysis showed that Aβ treatment reversed the age-related increase of CD11b mRNA expression so that there was no difference between the aged Aβ-treated group and the young controls.

Age and chronic infusion of Aβ do not alter iNOS and CD200 mRNA expression

Microglial was further investigated by analysis of iNOS mRNA expression. Figure 7.2 (A) represents the mean values for mRNA expression from

hippocampal tissue of young and aged rats which were treated for 28 days with Aβ at 65 μM. Results show that neither age ($F_{(1,16)}$=2.28, p>0.05) nor treatment ($F_{(1,16)}$=1.12, p>0.05, ANOVA) altered enzyme mRNA expression. Evidence from this laboratory suggests that age-related microglial activation is associated with a decreased expression of CD200 (Lyons et al., 2007b). Here the expression of CD200 was assessed by measure of mRNA. Data reported in figure 7.2 (B) show that neither age ($F_{(1,19)}$=0.76, p>0.05) nor treatment with Aβ ($F_{(1,19)}$=1.23, p>0.05, ANOVA) altered CD200 mRNA expression.

Age and Aβ treatment do not affect CD86 and ICAM-1 protein expression

Activation of microglia is characterised by different stages during which morphological and functional changes are observed. One stage of microglial activation involves antigen presentation associated with up-regulation of adhesion molecules like ICAM-1 (for review see Aloisi, 2000). Expression of CD86 and ICAM-1 was analysed by western immunoblotting and the results presented in figure 7.3 (A) show that there was no increase of CD86 protein density associated with age ($F_{(1,20)}$=3.93, p=0.06). Chronic infusion of Aβ also failed to modulate CD86 in the young and in the aged group ($F_{(1,20)}$=0.27, p>0.05, ANOVA). Figure 7.3 (B) demonstrates that ICAM-1 protein density was not affected by age ($F_{(1,20)}$=0.38, p>0.05) and Aβ treatment ($F_{(1,20)}$=0.38, p>0.05, ANOVA).

Age and chronic infusion of Aβ significantly affect IL-1β mRNA and protein

Microglial activation in response to chronic treatment with Aβ was further investigated by analysis of IL-1β mRNA expression and protein concentration in hippocampal tissue. Analysis of the data by two-way ANOVA showed that IL-1β mRNA expression was significantly altered due to an interactive effect of age and Aβ treatment ($F_{(1,18)}$=5.13, p<0.05). However, post hoc Neuman-Keuls test analysis failed to find any difference of IL-1β mRNA expression between conditions (see figure 7.4 (A)).

The data shown in panel (B) illustrate the effects of Aβ on IL-1β protein concentration. Analysis of data with a two-way ANOVA showed that age had a significant effect on IL-1β protein concentration and that cytokine levels were significantly altered due to an interaction between age and Aβ ($F_{(1.19)}$=5.97, p<0.05). However, analysis with post hoc test Neuman-Keuls revealed that Aβ

treatment significantly increased IL-1β concentration in the young rats but not in the aged rats and that there was no difference of mean IL-1β levels due to age.

Age and Aβ treatment increase caspase-3 activity

Figure 7.5 illustrates mean values of caspase-3 activity on hippocampus of young and aged rats infused with Aβ. Analysis of data with a two-way ANOVA demonstrated a significant effect of Aβ treatment on caspase-3 activity ($F_{(1,20)}$=6.14, $p<0.05$). However, analysis with Neuman-Keuls post hoc test did not find any difference between conditions. There was no interactive effect between age and Aβ treatment.

LTP is altered by age and intra-hippocampal injection of Aβ

To further investigate the effects of Aβ on hippocampal function, young and aged rats were injected intra-hippocampally with $Aβ_{1-42}$ at 200 μM and assessed for their ability to sustain LTP 7 days after the injection. Figure 7.6 shows an increase in the mean percentage change in population EPSP slope in all groups following stimulation with train of high frequency stimuli to the perforant path (time 0). Analysis of the last 5 minutes of recording post tetanic stimulation with two-way ANOVA showed a significant effect of Aβ on the percentage change in the EPSP slope ($F_{(1,15)}$=5.115, $p<0.05$, n=3-6). A significant interactive effect between age and Aβ was also detected ($F_{(1,14)}$=19.94, $p<0.001$, n=3-6). Analysis with Neuman-Keuls post hoc test revealed that statistical significance lay between the aged control-treated group and the young control-treated one and between the aged Aβ-treated group and the aged control-treated one.

Intra-hippocampal injection of Aβ does not alter mRNA expression of markers of microglial activation

Results presented in figure 7.7 (A) show that MHCII mRNA expression was not affected by age ($F_{(1,20)}$=0.12, $p>0.05$) or Aβ ($F_{(1,20)}$=0.04, $p>0.05$, ANOVA). Figure 7.7 (B) illustrates that CD40 mRNA expression was also not affected by treatment ($F_{(1,17}<0.0001$, $p>0.05$) and age ($F_{(1,17)}$=0.43, $p>0.05$, ANOVA). Another molecule associated with microglial activation is CD11b which has been shown to be upregulated as a consequence of cell proliferation, characteristic of

microglial activation. However, results of analysis of CD11b mRNA expression, illustrated in figure 7.7 (C), show that neither age ($F_{(1,18)}$=0.52, p>0.05) nor Aβ injection ($F_{(1,18)}$=1.73, p>0.05, ANOVA) significantly altered integrin mRNA expression.

In order to further analyse the possibility of microglial activation in response to Aβ injection, iNOS mRNA expression was assessed. Results presented in figure 7.8 (A) show that neither age ($F_{(1,20)}$=0.002, p>0.05) nor Aβ ($F_{(1,20)}$=0.008, p>0.05, ANOVA) exerted any significant effect on iNOS mRNA expression. Consistent with these results, analysis of CD200 mRNA revealed that mean values were not affected by age ($F_{(1,20)}$=0.002, p>0.05) or intra-hippocampal injection of Aβ peptide (figure 7.8 B) ($F_{(1,20)}$=0.008, p>0.05, ANOVA). Data presented in figure 7.8 (C) show that mean IL-1β mRNA expression was not affected by age ($F_{(1,17)}$=0.07, p>0.05) or administration of Aβ ($F_{(1,17)}$=0.04, p>0.05, ANOVA).

Figures

Figure 7.1 Age increases MHCII, CD40 and CD11b mRNA expression

A) A two-way ANOVA showed that there was a significant interactive effect between age and Aβ (63.8 μM) on MHCII mRNA expression ($F_{(1,19)}$=5.44, $p<0.05$, n=5-6). Analysis with Neuman-Keuls test showed that statistical significance for MHCII mRNA expression lay between the aged control-treated group and the young control-treated group. Data are expressed as a ratio of MHCII to β-actin and are means ± SEM. **P<0.01 vs young control.

B) A two-way ANOVA showed that there was a significant interactive effect between age and Aβ (63.8 μM) on CD40 mRNA expression. ($F_{(1,17)}$=4.96, $p<0.05$, n=4-6). However, analysis with Neuman-Keuls test showed that statistical significance for CD40 mRNA expression lay between the aged control-treated group and the young control-treated group. Data are expressed as a ratio of CD40 to β-actin and are means ± SEM. *P<0.05 vs young control

C) A two-way ANOVA showed that there was a significant effect of age on CD11b mRNA expression ($F_{(1,20)}$=15.85, $p<0.001$, n=6). Data are expressed as a ratio of CD11b to β-actin and are means ± SEM. **P<0.01 vs young control (Neuman-Keuls test).

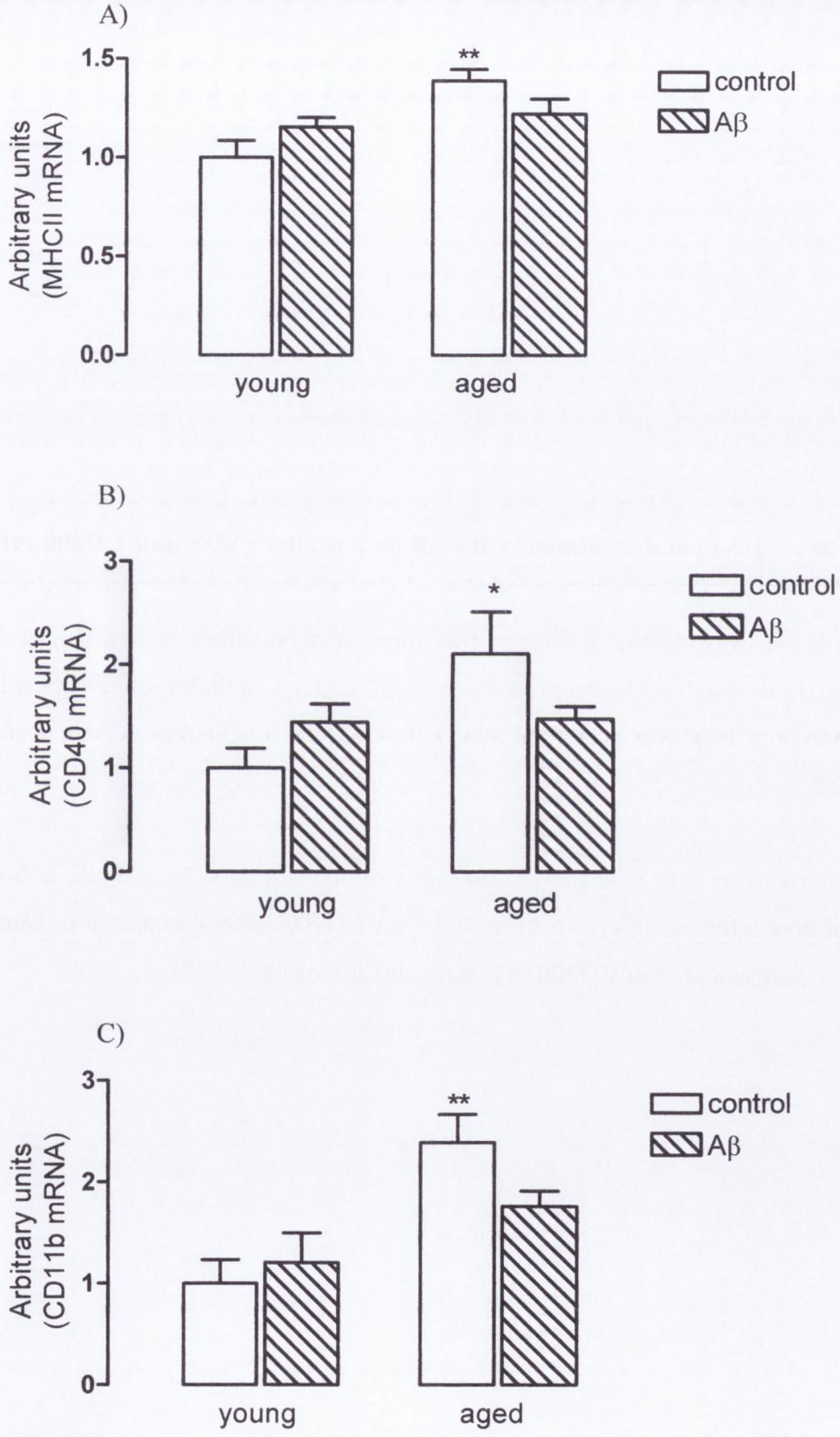

A)
Arbitrary units
(MHCII mRNA)
1.5
1.0
0.5
0.0
**
control
Aβ
young
aged
B)
Arbitrary units
(CD40 mRNA)
3
2
1
0
*
control
Aβ
young
aged
C)
Arbitrary units
(CD11b mRNA)
3
2
1
0
**
control
Aβ
young
aged

Figure 7.2 Age and treatment with Aβ do not alter iNOS and CD200 mRNA expression

A) A two-way ANOVA showed that there was no effect of age ($F_{(1,16)}$=2.28, p>0.05, n=3-6) or treatment with Aβ ($F_{(1,16)}$=1.12, p>0.05) on iNOS mRNA expression. Data are expressed as a ratio of iNOS to β-actin and are means ± SEM.

B) A two-way ANOVA showed no effect of age ($F_{(1,19)}$=0.76, p>0.05, n=5-6) or treatment with Aβ ($F_{(1,19)}$=1.23, p>0.05) on CD200 mRNA expression. Data are expressed as a ratio of CD200 to β-actin and are means ± SEM.


A)
Arbitrary units
(iNOS mRNA)
3
2
1
0
young
aged
control
Aβ

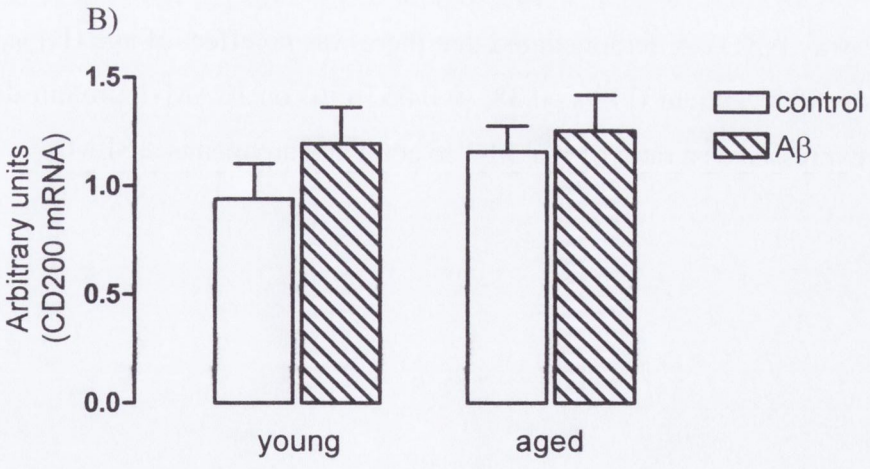

B)
Arbitrary units
(CD200 mRNA)
1.5
1.0
0.5
0.0
young
aged
control
Aβ

Figure 7.3 Age and treatment with Aβ do not alter CD86 and ICAM-1 protein density

A) A two-way ANOVA showed that there was no effect of age ($F_{(1,19)}$=4.26, p=0.0529, n=5-6) or Aβ treatment ($F_{(1,19)}$=0.37, p>0.05) on CD86 protein density. Data are expressed as a ratio of CD86 to actin and are means ± SEM.

B) A two-way ANOVA demonstrated that there was no effect of age ($F_{(1,20)}$=0.38, p>0.05) or Aβ treatment ($F_{(1,20)}$=0.38, p>0.05, n=6) on ICAM-1 protein density. Data are expressed as a ratio of ICAM-1 to actin and are means ± SEM.

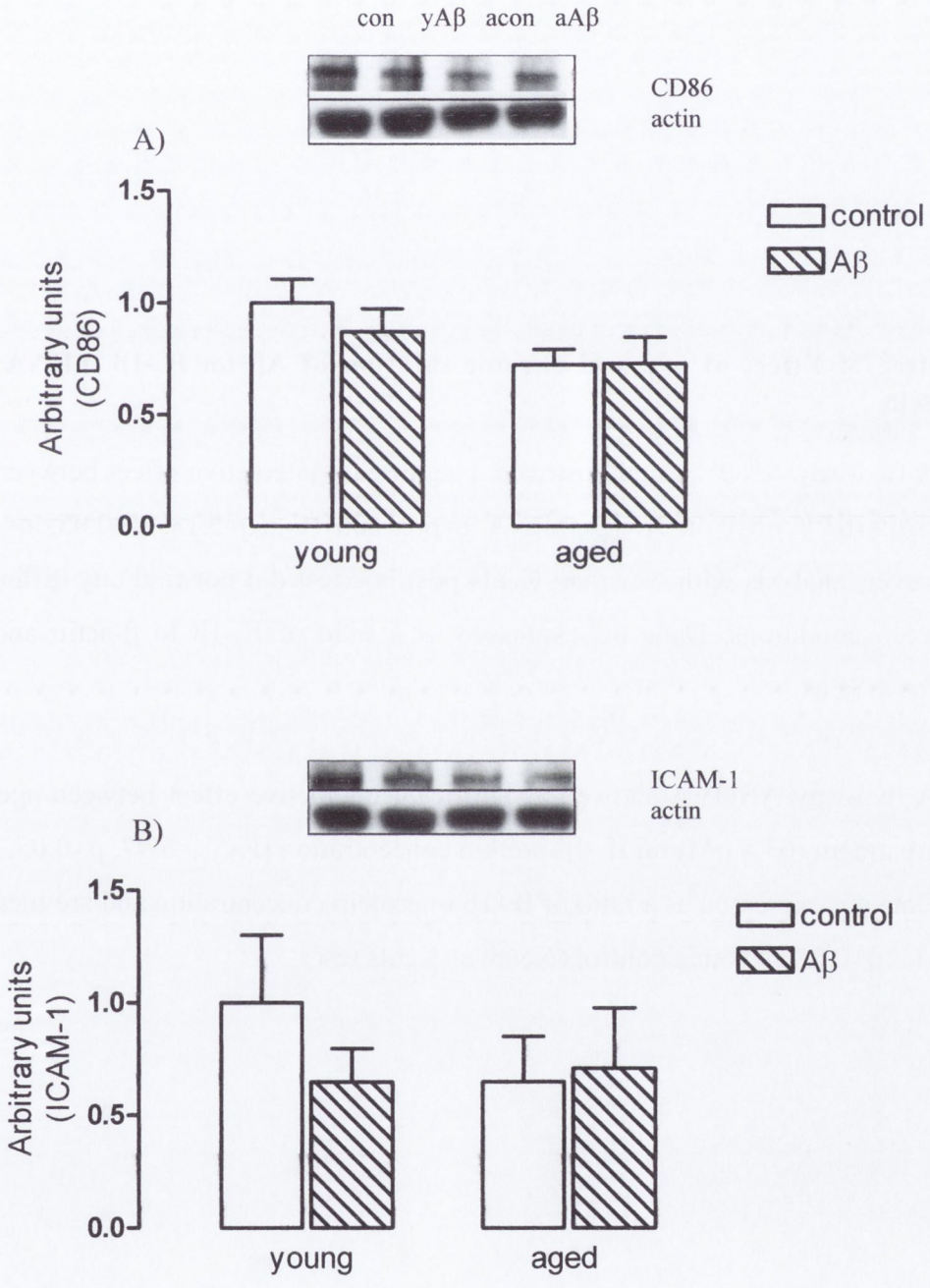
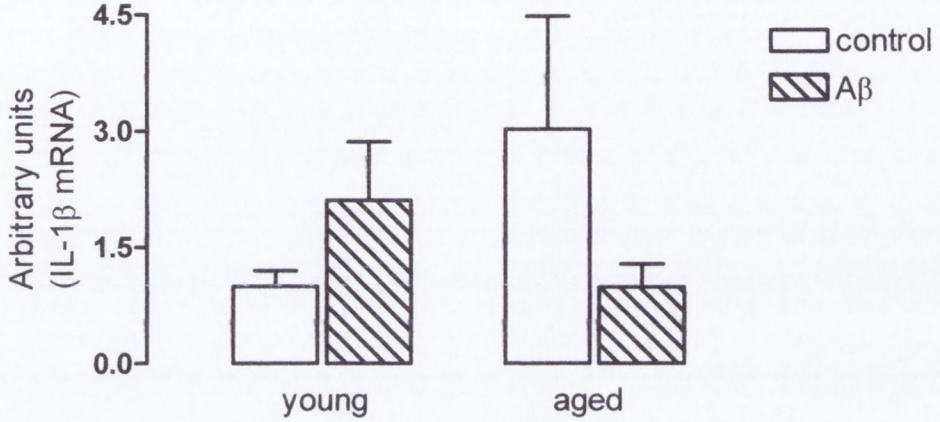

con yAβ acon aAβ
CD86
actin
A)
Arbitrary units (CD86)
1.5
1.0
0.5
0.0
control
Aβ
young
aged
ICAM-1
actin
B)
Arbitrary units (ICAM-1)
1.5
1.0
0.5
0.0
control
Aβ
young
aged

Figure 7.4 Effect of age and chronic infusion of Aβ on IL-1β mRNA and protein

A) A two-way ANOVA demonstrated a significant interactive effect between age and Aβ (63.8 μM) on IL-1β mRNA expression ($F_{(1,18)}$=5.13, $p<0.05$, n=4-6). However, analysis with Newman-Keuls post hoc test did not find any difference between conditions. Data are expressed as a ratio of IL-1β to β-actin and are means ± SEM.

B) A two-way ANOVA showed a significant interactive effect between age and Aβ treatment (63.8 μM) on IL-1β protein concentration ($F_{(1,19)}$=5.97, $p<0.05$, n=5-6). Data are expressed as a ratio of IL-1β to protein concentration and are means ± SEM. *$p<0.05$ vs young control (Neuman-Keuls test).


4.5
3.0
1.5
0.0
Arbitrary units
(IL-1β mRNA)
control
Aβ
young
aged

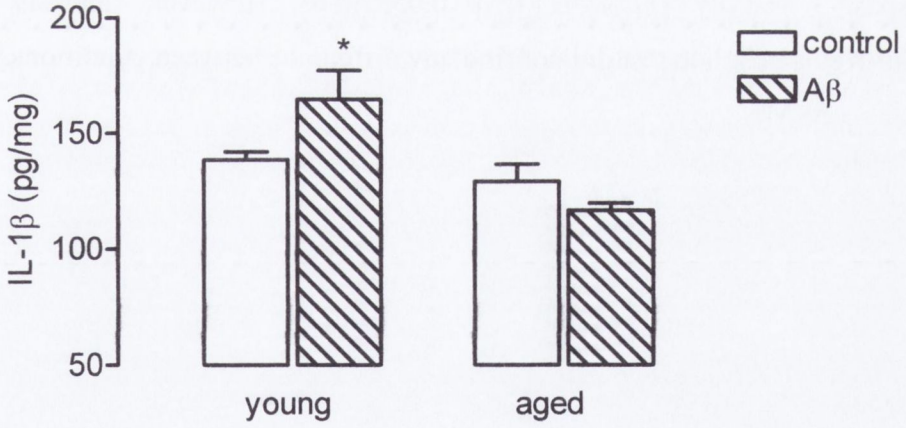

200
150
100
50
IL-1β (pg/mg)
*
control
Aβ
young
aged

Figure 7.5 Chronic infusion of Aβ and age do not affect caspase-3 activity

A two-way ANOVA demonstrated a significant effect of Aβ treatment (63.8 μM) on caspase-3 activity ($F_{(1,20)}$=6.14, $p<0.05$, n=6). However, analysis with Newman-Keuls post hoc test did not find any difference between conditions. Data are means ± SEM.

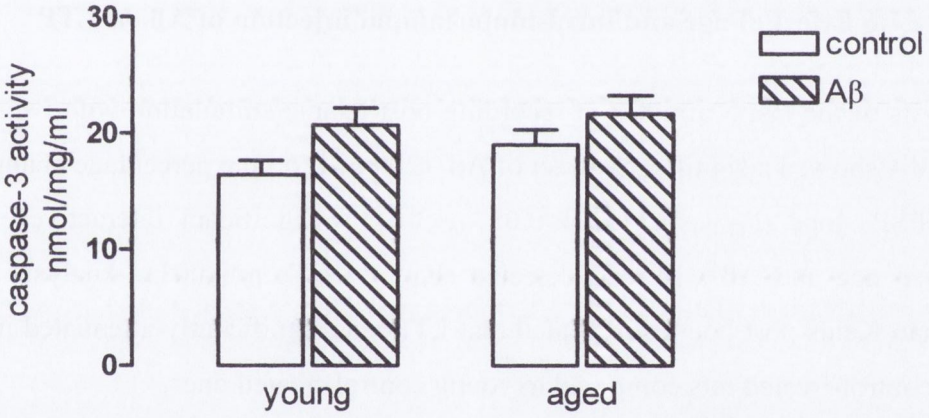

caspase-3 activity
nmol/mg/ml
30
20
10
0
young
aged
control
Aβ

Figure 7.6 Effect of age and intra-hippocampal injection of Aβ on LTP

Analysis of the last 5 minutes of recording post tetanic stimulation with two-way ANOVA showed a significant effect of Aβ (200 μM) on the percentage change in the EPSP slope ($F_{(1,15)}$=5.115, $p<0.05$, n=3-6). A significant interactive effect between age and Aβ was also detected ($F_{(1,14)}$=19.94, $p<0.001$). Analysis with Neuman-Keuls post hoc test revealed that LTP was significantly attenuated in the aged control-treated rats compared to young control-treated ones.

The mean population EPSP slope evoked by test stimuli delivered at 30 seconds intervals is shown and is expressed as a percentage of the slope recorded in the 5 minutes immediately prior to tetanic stimulation. Values are expressed as means ± SEM. SEM are included for every tenth response and some are so small in some cases as to be obscured.

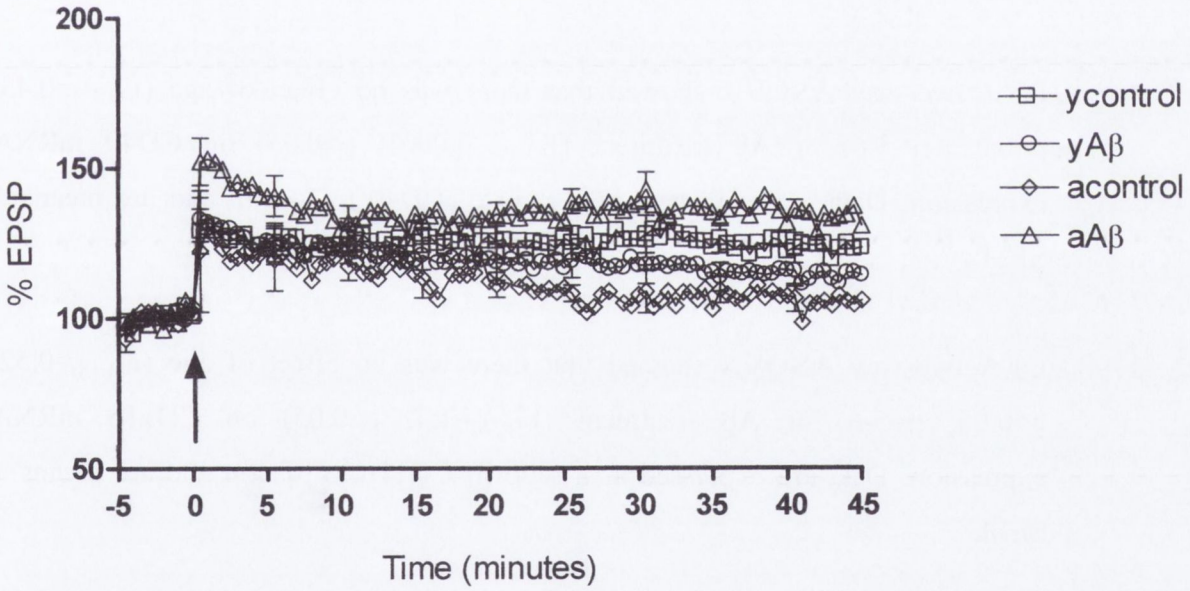

200
150
100
50
% EPSP
-5
0
5
10
15
20
25
30
35
40
45
Time (minutes)
ycontrol
yAβ
acontrol
aAβ

Figure 7.7 Intra-hippocampal injection of Aβ does not alter MHCII, CD40 and CD11b mRNA expression

A) A two-way ANOVA showed that there was no effect of age ($F_{(1,20)}$=0.12, p>0.05, n=6) or Aβ treatment ($F_{(1,20)}$=0.04, p>0.05) on MHCII mRNA expression. Data are expressed as a ratio of MHCII to β-actin and are means ± SEM.

B) A two-way ANOVA showed that there was no effect of age ($F_{(1,17)}$=0.43, p>0.05, n=5-6) or Aβ treatment ($F_{(1,17)}$<0.0001, p>0.05) on CD40 mRNA expression. Data are expressed as a ratio of CD40 to β-actin and are means ± SEM.

C) A two-way ANOVA showed that there was no effect of age ($F_{(1,18)}$=0.52, p>0.05, n=5-6) or Aβ treatment ($F_{(1,18)}$=1.7, p>0.05) on CD11b mRNA expression. Data are expressed as a ratio of CD11b to β-actin and are means ± SEM.

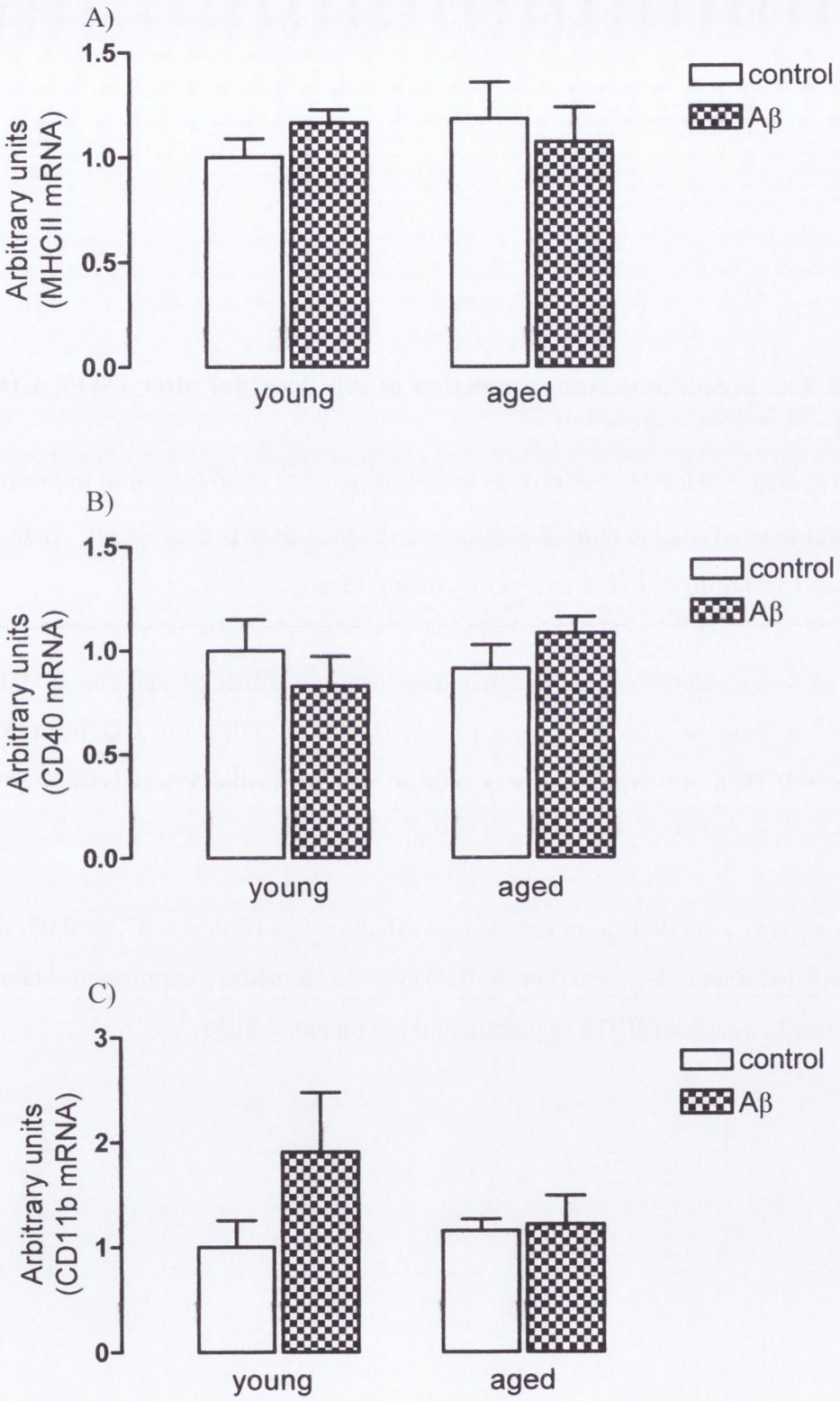

A)
Arbitrary units
(MHCII mRNA)
1.5
1.0
0.5
0.0
young
aged
control
Aβ
B)
Arbitrary units
(CD40 mRNA)
1.5
1.0
0.5
0.0
young
aged
control
Aβ
C)
Arbitrary units
(CD11b mRNA)
3
2
1
0
young
aged
control
Aβ

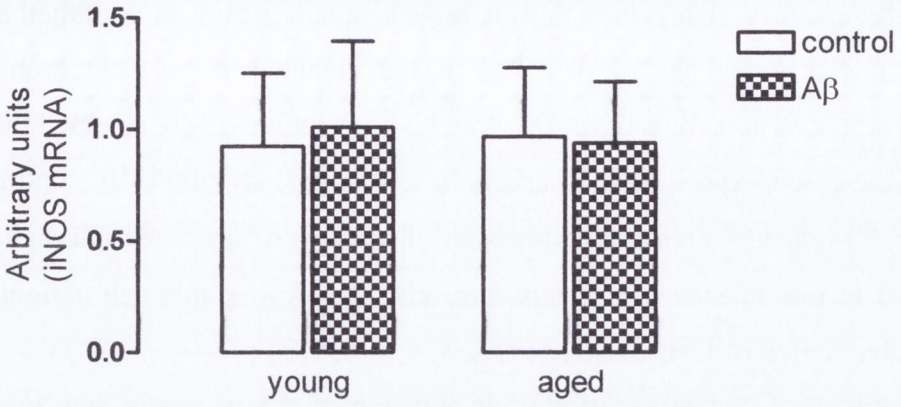
Figure 7.8 Intra-hippocampal injection of Aβ does not alter iNOS, CD200 and IL-1β mRNA expression

A) A two-way ANOVA showed no effect of age ($F_{(1,20)}$=0.002, p>0.05, n=6) or Aβ treatment ($F_{(1,20)}$=0.008, p>0.05) on iNOS mRNA expression. Data are expressed as a ratio of iNOS to β-actin and are means ± SEM.

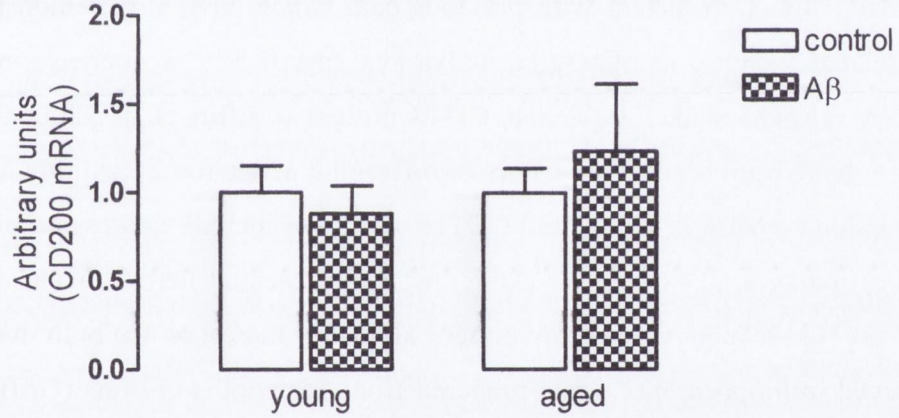
B) A two-way ANOVA showed that there was no effect of age ($F_{(1,20)}$=0.002, p>0.05, n=5-6) or Aβ treatment ($F_{(1,20)}$=0.008, p>0.05) on CD200 mRNA expression. Data are expressed as a ratio of CD200 to β-actin and are means ± SEM.

C) A two-way ANOVA demonstrated no effect of age ($F_{(1,17)}$=0.07, p>0.05, n=5-6) or Aβ treatment ($F_{(1,17)}$=0.034, p>0.05) on IL-1β mRNA expression. Data are expressed as a ratio of IL-1β to β-actin and are means ± SEM.

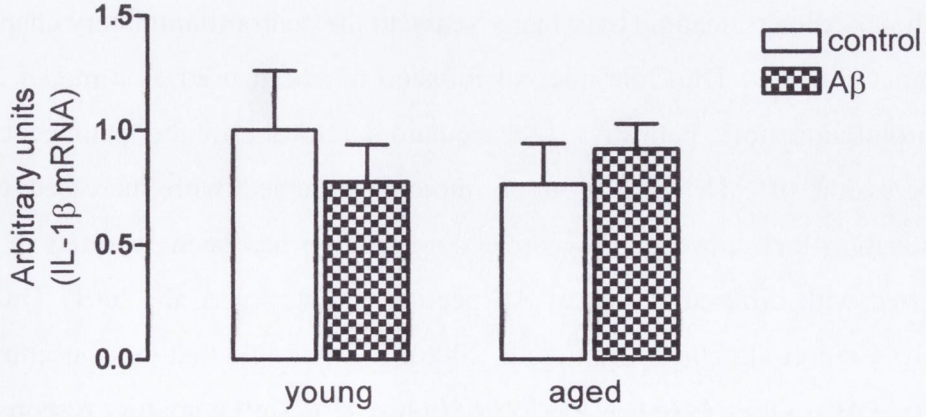
A)


Arbitrary units
(iNOS mRNA)
1.5
1.0
0.5
0.0
young
aged
control
Aβ

B)


Arbitrary units
(CD200 mRNA)
2.0
1.5
1.0
0.5
0.0
young
aged
control
Aβ

C)


Arbitrary units
(IL-1β mRNA)
1.5
1.0
0.5
0.0
young
aged
control
Aβ

7.4 Discussion

The present study was established to assess whether aging was associated with inflammatory changes in the rat hippocampus and to assess whether age exacerbates the response to Aβ. The results obtained show that chronically-infused Aβ, used as a cocktail of $A\beta_{1-40}$ and $A\beta_{1-42}$, paradoxically reversed the age-related increased expression of markers of microglial activation. By contrast, no age- or Aβ-related increase in expression of markers of activated microglia was observed in rats injected intra-hippocampally with $A\beta_{1-42}$ although a significant age-related deficit of LTP was observed.

Analysis of the effect of chronic infusion of Aβ in young and aged rats revealed that there was an age-related increase in mRNA expression of three cell surface markers which are indicative of microglial activation, MHCII, CD40 and CD11b. This is consistent with previous data which have also demonstrated an age-related increase in microglial activation, specifically an increase in MHCII mRNA (Lynch et al., 2007) and CD40 protein (Griffin et al., 2006). Several indices have been used as indicators of microglial activation in addition to mRNA expression of MHCII, CD40 and CD11b and these include expression of the cell surface markers CD86 and ICAM-1; the data presented here indicate that there was no age-related change in either, although increases in both have been observed in hippocampal tissue prepared from 15 months-old rats (Griffin et al., 2006). Activated microglia and increased cytokine expression have been described at sites of pathology in the AD brain (Verbeek et al., 1994; Apelt et al., 2001; Apelt et al., 2002) suggesting that inflammatory mediators might interact with Aβ deposits, leading over many years, to the neuroinflammatory changes that characterise AD. Thus, chronic Aβ infusion has been used as a model to study neuroinflammatory pathways and mediators relevant to the pathogenesis and progression of AD. Activation of microglia coupled with increased cytokine production and subsequent synaptic degeneration has been reported in rodents infused with different forms of Aβ peptide (Frautschy et al., 2001; Tran et al., 2001; Craft et al., 2004; Craft et al., 2006). It is possible that the concentration of $A\beta_{1-40}$+$A\beta_{1-42}$ used here was too low to induce a pro-inflammatory response in the young rats. It has been shown that $A\beta_{1-40}$ *in vitro* reversed the LPS-induced increase in PGE_2, thereby suggesting that the peptide may have anti-inflammatory

properties (Balboa et al., 2001). In light of this finding, it is suggested that the presence of $A\beta_{1-40}$ might have overcome the toxic effect of $A\beta_{1-42}$ and exerted a protective role reversing the age-related increase in MHCII, CD11b and CD40 mRNA expression. Another possible explanation for the unexpected result is based on the notion that microglial cells undergo senescence (Streit et al., 2004). The evidence has indicated that microglia in the healthy aged brain express increased susceptibility to pro-inflammatory stimuli (Tateda et al., 1996; Combrinck et al., 2002; Barrientos et al., 2006). It is arguable that Aβ treatment in the aged rats induced cell death and this accounts for the Aβ-induced decrease in expression of the measures analysed. Thus, decreased expression of markers of microglial activation is related to an overall decrease in cell number: quantification of microglia by immunohistochemical analysis would confirm this hypothesis.

Consistent with the hypothesis that Aβ concentration was too low to induce marked microglial activation is the finding that neither CD86 nor ICAM-1 expression were affected by the treatment. Indeed up-regulation of CD86 and ICAM-1 has been validated as a marker of activated microglia (Griffin et al., 2006; Clarke et al., 2007) and chronic infusion of Aβ has been shown to induce glial activation (Craft et al., 2006). However, it is possible that differences between diverse molecular weight species of Aβ may also account for the different results. In addition, it is possible that presence of both $A\beta_{1-40}$ and $A\beta_{1-42}$ may be decisive in producing an effect different than that produced by either peptide alone. Both CD86 and ICAM-1 are involved in the functional activation of T cells by mediating their interaction with APCs and subsequent intracellular signal transduction (for review see Aloisi et al., 2000b; Dietrich, 2002). Thus, it is possible that primed cells in the aged brain are not involved in antigen presentation activities thereby accounting for the unchanged expression of CD86 and ICAM-1 expression. The evidence has indicated that interaction between CD200 ligand and its receptor plays an important role in modulating microglial activation (Barclay et al., 2002; Lyons et al., 2007b), and there is an inverse correlation between microglial activation (as indicated by CD86 expression) and CD200 expression on neurons (Downer et al., submitted). Here the data show that

neither CD86 nor CD200 changed with age or Aβ treatment although other markers of microglial activation were upregulated with age.

The finding that some, but not all, markers of microglial activation increase highlight the fact that there are likely to be several stages of cell activation (Perry et al., 2003; Cunningham et al., 2005; Perry, 2007) and that it is not yet clear which markers identify particular activation states. During CNS inflammation, microglia express adhesion/costimulatory molecules like CD40, CD86 and CD80 which are necessary for antigen presentation function and upregulate the expression of MHCII and CD11b (for review see Aloisi, 2001). However, it has been demonstrated that activated microglia, following spinal cord lesions, showed increased expression of MHCII which was not paralleled by increased expression of CD86 (Schmitt et al., 2000). Thus, it is possible that activated microglia express different surface markers depending on the nature, strength and duration of the inflammatory stimulus which is also decisive for the type of activity undertaken by the activated microglia. It has been suggested that microglial cells are prone to undergo senescent changes (Streit et al., 2004) and it has been shown that microglia from aging donor brains exhibited an amoeboid-like phenotype and expressed markers typical of activated state like MHCII (Rozovsky et al., 1998). Microglial cells in the healthy aging brain have been described as primed (Godbout et al., 2005). Further activation of primed glia by LPS induces an exaggerated response compared to that obtained from non primed cells (Tateda et al., 1996; Combrinck et al., 2002; Barrientos et al., 2006). Although primed microglia show alterations of morphology and expression of surface markers typical of the activated state, it has been shown that they do not produce pro-inflammatory mediators (Cunningham et al., 2005). The data presented here, indicating that the relative increase in MHCII was not accompanied by increase in IL-1β, are consistent with this.

In this study there was no evidence that Aβ exerted any significant effect on microglial activation in young rats. The lack of effect of Aβ on these measures highlights differences between the effect of Aβ species and the variation in response to different modes of delivery of Aβ. It was previously shown that acute administration of $A\beta_{1-40}$ increased CD40 protein in a dose dependent manner (Minogue et al., 2007) wherase chronic administration of fibrillar Aβ exerted no significant effect on CD40 mRNA. Acute injection of $A\beta_{1-42}$ has been shown to

increase MHCII mRNA (Lyons et al., 2007a) as well as ICAM-1 and CD86 protein (Clarke et al., 2007), while no effect of chronic administration of $A\beta_{1-42}$ was observed here. It is possible that differences exist between the effect of $A\beta_{1-42}$ and $A\beta_{1-40}$ in combination and the effect of either peptide alone. It should also be noted that variation in the duration of treatment, chronic vs acute, may also account for the different results.

Analysis of hippocampal tissue from rats infused with Aβ showed that there was no age-related increase of IL-1β mRNA expression and protein concentration. This is in contrast with previous reports demonstrating an age-related increase in IL-1β protein and induced signaling (Lynch and Lynch, 2002; Martin et al., 2002; Maher et al., 2004; Moore et al., 2005; Griffin et al., 2006; Moore et al., 2007). The increase in IL-1β levels reported in the aged brain has been implicated in the synaptic dysfunction responsible for the LTP impairment observed in the dentate gyrus of aged rats (Murray and Lynch, 1998a; Maher et al., 2005). The age-related increase in IL-1β, as well as other pro-inflammatory cytokines, including IL-18 (Griffin et al., 2006), suggests that microglial activation increases with age thereby rendering the aged brain more susceptible to pro-inflammatory stimuli (Minogue et al., 2007). Moreover, Aβ treatment induced a significant increase in IL-1β concentration in the young rats which was not observed in the case of the aged Aβ-treated animals. The Aβ-induced increase in IL-1β in the young rats confirms a previous report implicating IL-1β in the Aβ-induced signalling and inflammatory response (Craft et al., 2005). It was predicted that Aβ might amplify the age-related increase in microglial activation, but the evidence indicates that administration of Aβ to aged rats exerted no significant effect on these measures. It has been shown that chronic infusion of LPS into the fourth ventricle of aged rats did not significantly increase the number of activated microglia (Hauss-Wegrzyniak et al., 1999). Given that the process of microgliosis is characterised by proliferation of microglial cells (Beyer et al., 2000), the authors suggested that the presence of already activated microglia in the aged brain would account for unchanged number of cells in response to LPS.

The results presented show that caspase-3 activity was unchanged with age and Aβ although evidence from the literature has indicated that it may be increased with age and coupled with microglial activation (Lynch and Lynch,

2002; Maher et al., 2005; Moore et al., 2007). An interaction between Aβ, neuronal stress and caspase-3 activity has also been demonstrated (Minogue et al., 2003). Also, injection of Aβ into the hippocampus has been shown to induce a significant increase in caspase-3 activity in the ipsilateral hemisphere, irrespective of microglial activation (Stepanichev et al., 2003) while the same concentration of $A\beta_{1-40}$+$A\beta_{1-42}$ used here (63.8 μM) induced an increase in caspase-3 activity in cortical tissue of young rats (Miller et al., unpublished).

One significant finding in this study was that there were no age-related or Aβ-induced changes in mRNA expression of MHCII, CD40 and CD11b in the study in which young and aged rats were treated intrahippocampally with Aβ or control peptide. This is in contrast with the data from the experiment in which the effects of chronic infusion were assessed. One possible reason for the apparent lack of effect in aged rats is that the injection induced inflammation in the hippocampus of young rats increasing the expression of MHCII, CD40 and CD11b and consequently an appropriate comparison (ie against a background of low or no microglial activation) was not possible. Interestingly, a similar lack of effect of intra-hippocampal injection of Aβ was observed in mice (McQuillan et al., unpublished). However, the results demonstrate that there was a significant age-related deficit in LTP and that Aβ injection induced a significant increase in the mean population EPSP slope in the aged animals compared to controls. Control-treated aged rats did not sustain LTP. This is consistent with previous reports from this laboratory demonstrating a marked deficit of LTP with age (O'Donnell et al., 2000; Martin et al., 2002; Griffin et al., 2006). Reports demonstrate that injection of Aβ into the hippocampus induced impairment of different forms of learning and memory 7 days after peptide administration. It has been shown that Aβ injection into the hippocampus induced marked gliosis and subsequent neuronal death (Ryu et al., 2004). Thus, given that LTP is likely to represent the cellular correlate for learning and memory, it was predicted that Aβ injection into the hippocampus would induce a deficit in LTP. However, paradoxically, Aβ treatment in the aged rats was associated with maintenance of LTP. An explanation for this remains to be established. It is possible that a resolution of the pro-inflammatory response occurred during the 7 days following injection.

It is possible that excitotoxicity may account for the increased mean population EPSP slope observed in the aged animals treated with Aβ. Excitotoxicity is the process by which nerve cells can be damaged or killed by excessive activation of receptors for excitatory amino acids. Activation of glutamate receptors, for instance, has been reported to induce increased Ca^{2+} influx which eventually induces cell death (Mantha et al., 2005) and it has been reported that Aβ *in vitro* compromised neuronal Ca^{2+} homeostasis and potentiated glutamate toxicity by increasing Ca^{2+} fluxes (Mattson et al., 1992; Chen et al., 2000; Freir et al., 2001; Chen et al., 2002). Another mechanism involved in Aβ toxicity includes rapid impairment of mitochondrial transport (Rui et al., 2006) which can lead to increased ROS which has been correlated with age-associated neurodegenerative changes (Murray and Lynch, 1998b; Lynch et al., 2007). In light of the fact that the aged brain is characterised by disruption of Ca^{2+} homeostasis (for review see Foster, 2007) and accumulation of ROS (for review see Squier, 2001), it is proposed that it is more vulnerable to Aβ-induced cytotoxicity.

Analysis of markers of microglial activation revealed a lack of effect of Aβ. It is possible that within 7 days post Aβ injection, the peptide was cleared up by phagocytic microglia and that any inflammation had subsided by the time tissue from these rats was assessed. Evidence from the literature suggets that microglia are capable of phagocytosing Aβ (Shaffer et al., 1995; Paresce et al., 1997; Kopec and Carroll, 1998). Moreover, activated microglia expressing the phagocytic marker CD68 have been reported in proximity to Aβ deposits in the brain of rats which received an intra-hippocampal injection of $A\beta_{1-42}$ (Takata et al., 2007). It is surprising, however, that there was no evidence of microglial activation in the hippocampus of the aged rats. It is plausible that the shift toward a pro-inflammatory environment that characterises the aged brain is not an inevitable process (Maher et al., 2005). The data here suggest that there is no correlation between attenuation of LTP and microglial activation and also hippocampal IL-1β concentration (data not shown). These findings are in contrast with evidence from the literature demonstrating an important role of microglial activation in the age-related deficit of LTP (Griffin et al., 2006; Maher et al., 2006). It is possible that Aβ injection induced a time-dependent expression of markers of microglial activation, which might have returned to basal level by day 7 after the treatment

(Tran et al., 2001). Similarly, as previous work from this laboratory demonstrated, acute administration of Aβ induced microglial activation (Clarke et al., 2007) and the results presented here show that there were no Aβ-induced changes at day 7 after injection, it is speculated that microglial activation was triggered shortly after Aβ injection but was restored by day 7. Analysis of Aβ-induced changes at different time points would allow better understanding of this process.

In conclusion, the data obtained from chronic infusion of Aβ failed to support the hypothesis that $A\beta_{1-40}$+$A\beta_{1-42}$ induced a more profound effect in aged compared to young rats. Similarly, intra-hippocampal injection of fibrillar $A\beta_{1-42}$ did not lead to activation of microglia, as assessed by analysis of MHCII, CD40, CD11b, iNOS and IL-1β mRNA. However, Aβ injection paradoxically enhanced LTP in the aged rats although the mechanism underling this effect has yet to be established.

Variable (Units)	**Effect of treatment**	
	age	**Chronic icv $A\beta_{40+42}$ (63.8 μM)**
MHCII mRNA (arbitrary units)	↑	no change
CD40 mRNA (arbitrary units)	↑	no change
CD11b (arbitrary units)	↑	no change
iNOS (arbitrary units)	no change	no change
CD200 (arbitrary units)	no change	no change
CD86 mRNA (arbitrary units)	no change	no change
ICAM-1 mRNA (arbitrary units)	no change	no change
IL-1β mRNA (arbitrary units)	no change	no change
IL-1β (pg/mg)	no change	↑
Caspase-3 activity (nmol/mg/min)	no change	no change

Table 7.1 Summary of results obtained from chronic Aβ study on young and aged rats

Variable (Units)	Eeffect of treatment	
	age	$A\beta_{1-42}$ (200 µM)
LTP (%EPSP)	no change	↑ in aged rats
MHCII mRNA (arbitrary units)	no change	no change
CD40 mRNA (arbitrary units)	no change	no change
CD11b mRNA (arbitrary units)	no change	no change
iNOS mRNA (arbitrary units)	no change	no change
CD200 mRNA (arbitrary units)	no change	no change
IL-1β mRNA (arbitrary units)	no change	no change

Table 7.2 Summary of results obtained from intra-hippocampal injection of Aβ study on young and aged rats.

General discussion

8.1 Discussion

This study was prompted by a number of previous observations which indicated (1) that Aβ–induced neurotoxicity is exerted prior to the appearance of extracellular senile plaques (Hsia et al., 1999), (2) that inflammation is involved in the pathogenesis and development of AD (for review see McGeer and McGeer, 2001), and (3) that the response of primed glia to a pro-inflammatory stimulus is exaggerated compared to that obtained from non primed cells (Tateda et al., 1996; Combrinck et al., 2002; Barrientos et al., 2006; Frank et al., 2007). In light of these observations, it has been hypothesised that the progressive up-regulation of glial inflammatory cytokines with aging may raise the vulnerability for the development of neurodegenerative diseases like AD in response to other genetic and environmental insults (Yu et al., 2002). In order to investigate the cellular mechanisms relevant to AD and validate the previous observations, different species of Aβ were administered to young and aged rats according to different protocols. The results, overall, fail to support the hypothesis that Aβ, irrespective of species, induces microglial activation and also fail to support the contention that age and LPS exacerbate the response to Aβ.

Acute icv injection of $A\beta_{1-40}$+$A\beta_{1-42}$ induced a significant decrease (compared with control-treated animals) in population EPSP slope in the last 5 minutes of recording. This effect was less marked in the animals that received an intra-hippocampal injection of $A\beta_{1-42}$ and absent in the case of rats chronically infused with $A\beta_{1-40}$+$A\beta_{1-42}$. The evidence that acute icv injection of Aβ attenuates LTP in the rat dentate gyrus supports previous findings obtained in this laboratory which demonstrate that acute administration of fibrillar $A\beta_{1-42}$ or $A\beta_{1-40}$ exerted a profound inhibitory effect on LTP (Minogue et al., 2003; Clarke et al., 2007; Lyons et al., 2007a; Minogue et al., 2007). A comparison of the data show that differences exist between the effects of $A\beta_{1-42}$ alone and the effects of $A\beta_{1-40}$+ $A\beta_{1-42}$ and it is likely that different concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ may also account for the different extent to which LTP was attenuated in the different studies. Acute injection of 200 μM $A\beta_{1-42}$ and 60 μM $A\beta_{1-40}$ both induced deficits in LTP in young rats (Clarke et al., 2007; Minogue et al., 2007). The present results indicate a less profound effect on LTP in the rats which received an acute injection of 18.94 μM $A\beta_{1-40}$+26.6 μM $A\beta_{1-42}$ or 84 μM $A\beta_{1-40}$+116 μM $A\beta_{1-42}$.

Infusion of $A\beta_{1-40}$ has been shown to induce severe impairment in cognitive behaviour and LTP (Nitta et al., 1997; Chen et al., 2006b), an effect which was not observed in the case of rats infused with $A\beta_{1-40}$ and $A\beta_{1-42}$ in combination. It is important to emphasize that $A\beta_{1-40}$ has been shown to have anti-inflammatory properties (Balboa et al., 2001) and to compromise $A\beta_{1-42}$ toxicity by retarding its aggregation (Snyder et al., 1994). It is possible that this effect played a part in this study although Aβ aggregation was not assessed here.

It is important to consider that different species of Aβ peptides may account for the variation in results obtained from different experiments. Here, for the first time, LTP was assessed in animals which were treated with a combination of fibrillar $A\beta_{1-40}$ and $A\beta_{1-42}$. The solution used in the present study contained mostly fibrils, however, a smaller amount of oligomers and monomers was also present. Oligomeric forms of Aβ have been shown to exert an inhibitory effect on hippocampal LTP *in vivo* and *in vitro* (Walsh et al., 2002; Klyubin et al. 2005; Yun et al., 2006) and similar effects have been reported with various Aβ peptide fragments (Freir et al., 2001). Similarly it has been reported that soluble forms of Aβ induce deficits in LTP (Zhao et al., 2004; Cakala et al., 2007). The interaction between Aβ oligomers and the cognitive impairment associated with AD has been highlighted by evidence that immunization using antibodies against Aβ oligomers prevented LTP inhibition *in vivo* (Rowan et al., 2005). Here, it is argued that the Aβ-induced changes in LTP are likely to be mediated by the soluble, oligomeric species rather than by the fibrillar forms of Aβ peptides. Results from analysis of immunoreactive Aβ by fluorescent staining, showing presence of Aβ focussed in the ventricles, further support this hypothesis and suggest that Aβ fibrils may not reach the hippocampus due to access issues. The different concentration of oligomers present in the preparation used may then account for the extent to which LTP is attenuated in the different experiments. However, Aβ solution was not analysed consistently in each study. In addition to the variation in response to different Aβ species, it must be considered that the preparation protocol and presence of carriers such as HDL in the vehicle impact on Aβ solubility and its toxicity, and consequently impact on results.

An important feature in the cognitive decline which characterises AD is the strong influence of aging. In animals, cognitive decline has been suggested by the age-related deficit in LTP (Mc Gahon et a., 1999; O'Donnell et al., 2000; Martin

et al., 2002; Nolan et al., 2005; Watson et al., 2006) and by age-related memory impairments (Chang and Gold, 2007). The aging brain is characterised by increased expression of pro-inflammatory cytokines accompanied by activated astrocytes and microglia (Deng et al., 2006; Lynch et al., 2006; Stichel and Luebbert, 2006; Hayakawa et al., 2007) which have been implicated in the age-related deficit in LTP (Griffin et al., 2006). Similarly to age, LPS has been shown to induce LTP deficits (Hauss-Wegrzyniak et al., 2002; Kelly et al., 2003; Kavanagh et al., 2004; Barry et al., 2005) and memory impairment (Arai et al., 2001) and these changes are also coupled with increased microglial activation.

One of the objectives of the present work was to investigate the role of microglial activation in response to Aβ. The results fail to support the hypothesis that Aβ induces marked microglial activation and subsequent release of pro-inflammatory mediators. It has been shown that upon stimulation by pro-inflammatory stimuli, microglia undergo a process of gradual maturation (ie activation) that is characterised by proliferation and formation of multinucleated giant cells (Beyer et al., 2000). It is generally accepted that activated microglia undergo morphological alterations, changing from resting ramified cells to activated amoeboid cells, and produce a variety of pro-inflammatory mediators which are toxic for neurons (Combs et al., 2001; Walker et al., 2001; Butovsky et al., 2005). Surface molecules, like MHCII, CD86, CD80 and CD40 which are necessary for acquisition of APC function, are also upregulated when microglia are activated (Clarke et al, 2007; Griffin et al., 2006; for review see Aloisi, 2001). In the present work microglial activation was assessed by analysing mRNA and protein expression of the cell surface markers MHCII, CD11b, CD40, CD86, ICAM-1. Up-regulation of some of these molecules has been described in the aged brain (Griffin et al., 2006) as well as in response to Aβ (Clarke et al., 2007) and LPS (Hauss-Wegrzyniak et al., 1998). Activation of microglial cells was further investigated by analysis of transcription of iNOS and IL-1β which may lead to release of NO and IL-1β respectively, and by measuring IL-1β concentration in hippocampal tissue. The results indicate that although some of these markers were increased with Aβ, age or LPS, parallel changes in all the markers of microglial activation were not observed. This supports the view that there are likely to be several stages of microglial activation each with different phenotypes (Perry et al., 2003). Indeed, it has been reported that primed

microglia, which show characteristic alterations of morphology and surface markers expression typical of the activated state, do not produce pro-inflammatory mediators (Cunningham et al., 2005). Similarly, although MHCII expression has been generally referred to as a marker of APC function, Schmitt and colleague (2000) demonstrated that increased MHCII expression was not paralleled by increased expression of the costimulatory molecule CD86. It seems likely that microglial reactivity to inflammatory stimuli may vary depending on the nature, strength and duration of the stimulus (Perry, 2007). In addition, it should be noted that none of the markers analysed in the present study to measure microglial activation are specific to microglial cells. It is thereby possible that analysis of more specific markers of activated microglia or else colocalisation between microglial markers and markers of microglial activation would allow more accurate interpretation of the data.

In order to further investigate the effects of Aβ administration in the rat hippocampus, cell stress and neuronal function were assessed by analysis of caspase-3 activity and synaptic proteins expression in hippocampal homogenates. The results demonstrate that acute icv injection of $A\beta_{1-40}$+$A\beta_{1-42}$ at 45.5 μM induced a decrease in syntaxin and synaptophysin expression and an increase in caspase-3 activity. These findings support the previous observation that caspase-3 is likely to contribute to Aβ-induced neurotoxicity (Minogue et al., 2003; St. John et al., 2007) and are consistent with the hypothesis that the synaptic dysfunction typical of early stages of AD may, in part, be due to decreased expression of synaptic proteins. Indeed, decreased expression of synaptophysin has been described in areas where Aβ oligomers accumulated (Ishibashi et al., 2006).

However, caspase-3 activity was not significantly increased after 28 days of chronic Aβ infusion and it might be suggested that the Aβ-induced changes are time-related. In addition, the fact that there was no evidence that acute injection of $A\beta_{1-40}$+$A\beta_{1-42}$-induced microglial activation suggests that increased caspase-3 activity and microglial activation may be two separate events. It is generally accepted that inflammation plays an important role in the development of AD (for review see in McGeer and McGeer, 2001) however, it has been reported that Aβ-induced neurotoxicity can occour irrespective of microglial activation (Malm et al., 2006).

Given that the aged brain has been characterised by increased levels of pro-inflammatory cytokines expression (Vereker et al., 2000; Lynch and Lynch, 2002; Martin et al, 2002) and that primed glial have been described in the aged brain (Godbout et al., 2005) I considered that age, and similarly LPS, may exacerbate the response to Aβ. However, the results demonstrate that neither age nor LPS induced an exaggerated response to Aβ. Surprisingly, there was no evidence of marked age-related microglial activation, although a deficit of LTP was observed in these aged rats. The finding that increased expression of MHCII, CD40 and CD11b mRNA was not paralleled by increased expression of CD86 and ICAM-1 may indicate that primed microglia in the aged brain are not involved in antigen presentation. Although the evidence has indicated that microglia are capable of phagocytosing Aβ (Shaffer et al., 1995; Paresce et al., 1997; Kopec and Carroll, 1998) and that a certain degree of inflammation aids to Aβ clearance (DiCarlo et al., 2001; Quinn et al., 2003), microglial phagocytosis of Aβ was not assessed here. The possibility remains that LPS promoted Aβ clearance and this was responsible for the lack of an additional effect between Aβ and LPS. Moreover, it is arguable that the concentrations of Aβ tested were too low to induce marked effect in the hippocampus or else that the Aβ-induced changes were restored by the time the animals were sacrificed and the tissue dissected for analysis. It is also possible that the lack of effect of Aβ treatment lays on the inability of the peptides to penetrate the brain and accumulate at levels high enough to induce an inflammatory response.

In conclusion, the results suggest that variation in the protocol, preparation and duration of Aβ treatment impact on the effects exerted by Aβ and that microglial activation is likely to involve different states, each characterised by the expression of different markers. The data presented do not support the hypothesis that Aβ treatment induces marked microglial activation and that age or LPS exaggerate the response to Aβ. Although treatment with Aβ is a useful model to study the cellular mechanisms involved in AD, caution must be used when interpreting and comparing data from different experiments.

8.2 Future work

- Analysis of Aβ immunoreactivity in tissue prepared from each treatment schedule would be interesting to determine whether Aβ deposition occurred in the brain.
- The issue of whether infused Aβ was phagocytosed by microglia warrants investigation, immunohistochemical analysis of colocalisation between phagocytic microglia and Aβ would be required to assess this.
- The data suggest that LTP impairment was associated with activated caspase-3. It would be of interest to establish whether this was associated with neurons, astrocytes or microglia and confocal microscopy should be carried out on tissue prepared from treated and untreated rats.
- I have mentioned that $A\beta_{1-40}$ has been shown to retard the aggregation of $A\beta_{1-42}$ and this may be responsible for the attenuated effect of $A\beta_{1-40}$+$A\beta_{1-42}$ on LTP and microglial activation. Analysis of the effect of $A\beta_{1-40}$ on $A\beta_{1-42}$ aggregation and toxicity would address this issue.
- Considering the effect of chronic infusion of Aβ on the expression of markers of microglial activation and caspase-3 activity, as compared to its acute effects, it would be interesting to investigate time-related changes in response to Aβ.
- The treatment protocol used in this study included acute and chronic icv administration of $A\beta_{1-40}$ and $A\beta_{1-42}$ in combination, it would be interesting to compare the effect of this combination with the effect of treatment with either peptide alone. Similarly it would be interesting to assess the effect of either peptide alone in aged and LPS-treated rats.

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VII. Appendix I. Mean data

Variable (Units)	- Neurons		+ Neurons	
	control	LPS (100 ng/ml)	control	LPS (100 ng/ml)
MHCII mRNA (arbitrary units)	1 ± 0.67	4.12 ± 0.8	1.86 ± .037	3.23 ±0.91
CD200R mRNA (arbitrary units)	1.05 ± 1.16	0.30 ± 0.60		
IL-1β mRNA (arbitrary units)	1 ± 0.40	3.12 ± 1.00	0.98 ± .027	1.46
IL-1β (pg/ml)	140.67 ± 8.67	1130.17 ± 189.52	158.35 ± 20.46	320.57 ± 67. 16
IL-18 (pg/ml)	208.43 ± 60.23	853.34 ± 68.69	129.11 ± 16.76	456.26

Table 1.1 Raw data from study in which glia were pre-treated with neurons. Values are expressed as means ± SEM.

Variable (Units)	Control	LPS (5 ng/ml)	LPS (20 ng/ml)	LPS (50 ng/ml)	LPS (100ng/ml)
IL-1β (pg/ml)	96.33 ± 17.30	106.08 ± 25.99	279 ± 17.39	285.96 ± 42.49	300.9 ± 63.47

Table 1.2 Raw data from LPS dose response study. Values are expressed as means ± SEM.

Variable (Units)	DMEM		$A\beta_{1-42}$ (2 μM)	
	control	LPS (10 ng/ml)	control	LPS (10 ng/ml)
MHCII mRNA (arbitrary units)	1 ± 0.37	3.58 ± 0.51	1.32 ± .025	4.54 ±0.75
IL-1β (pg/ml)	62.42 ± 10.33	101.32 ± 22.96	122.35 ± 20.98	194.92 ± 35.66

Table 1.3 Raw data from LPS+Aβ *in vitro* study. Values are expressed as means ± SEM.

Variable (Units)	Control	$A\beta_{40+42}$ (45.5 µM)	$A\beta_{40+42}$ (200 µM)
LTP (%EPSP)	127.80 ± 0.61	118.10 ± 0.27	116.40 ± 0.95
SNAP-25 (arbitrary units)	0.89 ± 0.12	0.85 ± 0.04	1.73 ± 0.26
Syntaxin (arbitrary units)	1 ± 0.10	0.63 ± 0.02	1.03 ± 0.09
Synaptophysin (arbitrary units)	1 ± 1.13	0.59 ± 0.11	0.96 ± 0.11
MHCII (arbitrary units)	1 ± 0.05	0.86 ± 0.03	1.07 ± 0.07
CD40 (arbitrary units)	1 ± 0.09	1.25 ± 0.28	0.61 ± 0.07
CD11b (arbitrary units)	1 ± 0.12	0.90 ± 0.16	0.68 ± 0.02
iNOS (arbitrary units)	2.25 ± 0.32	2.56 ± 0.59	7.56 ± 2.82
CD200 (arbitrary units)	0.87 ± 0.03	0.81 ± 0.04	0.58 ± 0.09
CD86 (arbitrary units)	0.87 ± 0.06	0.68 ± 0.21	1.15 ± 0.03
ICAM-1 (arbitrary units)	1 ± 0.16	1.07 ± 0.123	2.03 ± 0.32
IL-1β mRNA (arbitrary units)	0.89 ± 0.15	1.01 ± 0.30	3.77 ± 1.11
IL-1β (pg/mg)	145.4 ± 10.39	116 ± 15.53	163.8 ± 3.13
Caspase-3 activity (nmol/mg/min)	15.35 ± 0.73	19.91 ± 1.10	16.59 ± 3.79

Table 1.4 Raw data from acute icv study with Aβ. Values are expressed as means ± SEM.

Variable (Units)	Control	$A\beta_{40+42}$ (45.5 μM)	$A\beta_{40+42}$ (63.8 μM)
iNOS mRNA (arbitrary units)	1 ± 0.41	0.54 ± 0.12	0.72 ± 0.38
CD86 (arbitrary units)	1 ± 0.08	0.59 ± 0.09	1.25 ± 0.34
ICAM-1 (arbitrary units)	1 ± 0.156	1.53 ± 0.44	1.19 ± 0.27
CD200 (arbitrary units)	0.92 ± 0.048	0.86 ± 0.12	0.72 ± 0.11
Caspase-3 activity (nmol/mg/min)	24.73 ± 1.32	36.13 ± 8.41	33.67 ± 2.16

Table 1.5 Raw data from chronic icv study with Aβ. Values are expressed as means ± SEM.

Variable (Units)	Control		$A\beta_{40+42}$ (45.5 μM)	
	control	LPS (0.5mg/ml)	control	LPS (0.5mg/ml)
LTP (%EPSP)	120.00 ± 0.33	104.20 ± 0.50	118.40 ± 0.28	100.40 ± 0.44
MHCII mRNA (arbitrary units)	1 ± 0.18	1.79 ± 0.37	1.45 ± 0.3	2.59 ± 0.22
iNOS mRNA (arbitrary units)	1 ± 0.21	3.01 ± 0.56	1.48 ± 0.33	1,25 ± 0.26
CD86 (arbitrary units)	1 ± 0.12	0.61 ± 0.13	0.64 ± 0.1	1.15 ± 0.27
ICAM-1 (arbitrary units)	1 ± 0.31	1.512 ± 0.18	1.272 ± 0.33	1.913 ± 0.40
CD200 (arbitrary units)	1 ± 0.21	0.97 ± 0.14	0.70 ± 0.11	0.64 ± 0.19
IL-1β mRNA (arbitrary units)	1 ± 0.22	12.13 ± 3.71	4.79 ± 1.19	16.18 ± 3.96
IL-1β (pg/mg)	334.8 ± 40.17	357.9 ± 17.92	367.1 ± 21.53	440.5 ± 47.49

IL-4 mRNA (arbitrary units)	1 ± 0.35	0.76 ± 0.15	0.47 ± 0.17	0.38 ± 0.09
IL-10 mRNA (arbitrary units)	1 ± 0.21	1 ± 0.13	0.89 ± 0.21	0.91 ± 0.20
Caspase-3 activity (nmol/mg/min)	29.64 ± 5.05	34.61 ± 6.36	36.13 ± 8.42	34.14 ± 13.30

Table 1.6 Raw data from chronic LPS+Aβ study. Values are expressed as means ± SEM.

Variable (Units)	**Young**		**Aged**	
	control	**$A\beta_{40+42}$ (63.8 μM)**	**control**	**$A\beta_{40+42}$ (63.8 μM)**
MHCII mRNA (arbitrary units)	1 ± 0.085	1.15 ± 0.05	1.39 ± 0.06	1.22 ± 0.07
CD40 mRNA (arbitrary units)	1 ± 0.19	1.45 ± 0.17	2.11 ± 0.40	1.48 ±0.12
CD11b (arbitrary units)	1 ± 0.23	1.21 ± 0.29	2.39 ± 0.28	1.76 ± 0.15
iNOS (arbitrary units)	1 ± 0.45	0.48 ± 0.25	1.69 ± 0.99	0.79 ± 0.39
CD200 (arbitrary units)	0.94 ± 0.16	1.19 ± 0.17	1.16 ± 0.12	1.25 ±0.17
CD86 mRNA (arbitrary units)	1 ± 0.11	0.87 ± 0.10	0.72 ± 0.07	0.73 ± 0.12
ICAM-1 mRNA (arbitrary units)	1 ± 0.30	0.65 ± 0.15	0.65 ±0.20	0.71 ± 0.27
IL-1β mRNA (arbitrary units)	1 ± 0.20	2.11 ± 0.75	3.03 ± 1.46	0.99 ±0.30
IL-1β (pg/mg)	138.4 ± 3.45	164.5 ± 12.50	129.1 ± 7.29	116.4 ± 3.20
Caspase-3 activity (nmol/mg/min)	16.42 ± 1.23	20.72 ± 1.45	19.02 ± 1.30	21.67 ± 1.60

Table 1.7 Raw data from chronic Aβ study on young and aged rats. Values are expressed as means ± SEM

Variable (Units)	Young		Aged	
	control	$A\beta_{1-42}$ (200 μM)	control	$A\beta_{1-42}$ (200 μM)
LTP (%EPSP)	123.30 ± 0.49	113.70 ± 0.51	104.60 ± 0.78	133.80 ± 0.84
MHCII mRNA (arbitrary units)	1 ± 0.09	1.16 ± 0.06	1.18 ± 0.17	1.073 ± 0.16
CD40 mRNA (arbitrary units)	1 ± 0.14	0.83 ± 0.14	0.91 ± 0.11	1.08 ± 0.08
CD11b mRNA (arbitrary units)	1 ± 0.26	1.91 ± 0.57	1.16 ± 0.11	1.22 ± 0.27
iNOS mRNA (arbitrary units)	0.93 ± 0.33	1.01 ± 0.38	0.97 ± 0.31	0.94 ±0.27
CD200 mRNA (arbitrary units)	1 ± 0.15	0.88 ± 0.16	1 ± 0.17	1.19 ± 0.31
IL-1β mRNA (arbitrary units)	1 ± 0.26	0.77 ± 0.16	0.76 ±0.18	0.92 ± 0.11

Table 1.8 Raw data from intra-hippocampal injection of Aβ study on young and aged rats. Values are expressed as means ± SEM

VIII. Appendix II: company addresses

Abcam	Abcam Inc 1 Kendall Square Ste 341 Cambridge MA 02139-1517 USA
Alzet	DURECT Corporation PO Box 530 Cupertino CA 95015-0530 USA
Amersham	Amersham Biosciences Ltd Amersham Place Little Chalfont Buckinghamshire HPA 9NA UK
Applied Biosystems	Applied Biosystems 850 Lincoln Centre Drive Foster City, CA 94404 USA
Biomol	Biomol International LP Palatine House Matford Court Exeter EX2 8NL UK
Bio Rad	BioRad Laboratories Ltd Bio-Rad House Maylands Avenue

	Hemel Hempstead Hertfordshire HP2 7TD UK
Biosource	Biosource Internationals 542 Flyn Road Camarillo CA 93012 USA
Beckman Coulter	Beckman Coulter Canada, Inc. 6755 Mississauga Road, Suite 600 Mississauga, Ontario L5N 7Y2 USA
Chemicon	Chemicon International Inc 28820 Single Oak Drive Temecula CA 92590 USA
Clark Electromedical Instruments	Clark Electromedical Instruments Fircroftway Edenbrige Kent TN8 6HE UK
Cruinn	Cruinn Diagnostics Ltd Unit 5b Hume Centre Park West Industrial Estate Dublin 12 Ireland

DAKO	Dako Corporation California Inc 6392 Via Real Carpintera CA 93013 USA
Gibco	Gibco Ltd 3 Fountain Drive Linchinnan Drive Paisley PA4 9RF UK
GraphPad Software	GraphPad Software Inc 11452 E1 Camino Real San Diego CA 92130 USA
Invitrogen	Invitrogen Ltd 3 Fountain Drive Linchinnan Drive Paisley PA4 9RF UK
Jencons	Jencons Scientific Ltd Cherrycourt Way Stanbridge Road Leighton Buzzard Bedforshire LU7 4UA UK
Lennox	Lennox Laboratoriy Supplies Jonh F. Kennedy Drive Nass Road

	Dublin 12 Ireland
Meyer	Meyer Instruments Inc 1304 Langham Creek Suite 235 Huston TX 77084 USA
Mickle Laboratories Engineering	Mickle Laboratories Engineering Guildford Surrey UK
NUNC	NUNC A/S Kamstrupvej 90 PO box 280 DK-4000 Roskilde Denmark
Pierce	Pierce Biotechnologies 3747 N. Meridian Road PO Box 117 Rockford IL 61105 USA
Promega	Promega Corporation 2800 Wood Hollow Road Madison WI 53711 USA
R&D System	R&D System 614 McKinley Place NE

	Minneapolis MN 55413 USA
Santa Cruz	Canta Cruz Biotechnologies 2161 Delaware Avenue Santa Cruz CA 95060 USA
Sarsdetd	Sarsdetd Ltd Sinnottstown Lane Drinagh Wexford Ireland
Serotec	Serotec Ltd 22 Bankside South Approach Kidlington Oxford OX5 1JE UK
Sigma	Sigma-Aldrich Company Ltd Fancy Road Poole Dorset BH12 4gH UK
Ultra-Violet Products	Ultra-Violet Products Ltd Unit 1 Trinity Hall Farm Estate Hullfield Road Cambridge CB41 ITG

	UK
Vector	Vector Laboratories Inc 30 Ingol Road BurlingameCA 94010 USA
Whatman	Whatman Plc Whatman House St Leonard's Road Maidstone Kent ME16 OLS UK

IX. Appendix III: solutions

Artificial cerebrospinal fluid (aCSF)

Solution A

NaCl 150 mM

KCl 3 mM

$CaCl_2$ 0.19 mM

$MgCl_2$ 0.8 Mm

Solution B

NA_2HPO_4 0.8 mM

NAH_2PO_4 0.2 mM

Krebs solution containing $CaCl_2$

NaCl 136 mM

KCl 2.54 mM

KH_2PO_4 1.18 mM

$MgSO_4$ 1.18 mM

$NaHCO_3$ 16 mM

glucose 10 mM

$CaCl_2$ 2 mM

Lysis buffer, pH 7.4

sucrose 320mM

HEPES 5mM

Phosphate buffered saline (PBS), pH 7.3

NaCl 137 mM

KCl 207 mM

Na_2HPO_4 8.1 mM

KH_2PO_4 1.5 mM

Tris-Buffered Saline (TBS), pH 7.4

Tris HCl 20 mM

NaCl 150 mM