β_2-adrenoceptor modulation of the inflammatory response induced by thrombin and amyloid-β in glial cells

by

Dana Kilroy

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

Thesis submitted May 2010

Department of Physiology
Trinity College Institute of Neuroscience
Trinity College
Dublin 2.

Declaration

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin, Trinity College and has not been submitted to any other university as an exercise for a degree. I declare that this thesis is entirely my own work and I give permission to the library to lend or copy this thesis upon request

Dana Kilroy

Dana Kilroy

Contents

Summary

Thrombin is a blood protein that leaks into the brain parenchyma when the blood-brain barrier is damaged, and has been implicated in the neuropathology associated with stroke, traumatic brain injury and Parkinson's disease (PD). Amyloid-β (Aβ) is a protein which aggregates to form senile plaques during Alzheimer's disease (AD) and is thought to mediate the neuronal loss observed in this disorder. Both molecules can elicit neuroinflammation which contributes to the neurodegenerative process. Evidence suggests that the monoamine neurotransmitter noradrenaline (NA) elicits anti-inflammatory actions in the brain, and consequently may play an endogenous neuroprotective role. Studies indicate that the anti-inflammatory actions of NA occur via activation of β_2-adrenoceptors (ARs) on microglia and astrocytes, which increases intracellular cAMP concentrations and subsequent downstream activation of PKA. Experiments in this thesis were conducted on rat primary cortical glial or neuronal cultures. Cell viability was assessed using an alamar blue assay or LDH assay, mRNA expression of inflammatory and neurotrophic molecules was measured using RT-PCR, and protein concentration of inflammatory molecules was determined using ELISA.

This study examined the ability of thrombin and Aβ to induce an inflammatory response in glial cells and the ability of NA interventions to inhibit this response. In mixed glial, astrocyte, and microglia cultures, thrombin induced expression of the pro-inflammatory cytokines IL-1β and TNFα, the co-stimulatory molecule CD40, the enzyme iNOS, the pro-inflammatory chemokine IP-10, and the cell adhesion molecule VCAM-1. Aβ induced a more restricted inflammatory response characterised by expression of IL-1β, TNFα, and CD40. NA, the β-AR agonist salbutamol, the β_2-AR agonist salmeterol, the cAMP analogue db-cAMP, and the PKA activator BNZ attenuated the thrombin- and Aβ-induced inflammatory response in terms of TNFα, CD40, IP-10, and VCAM-1 expression. Interestingly, salbutamol and the β_1-AR agonist xamoterol augmented the thrombin- and Aβ-induced inflammatory response in IL-1β.

This study also examined the effect of thrombin, Aβ, and β-AR agonists on neurons. It was expected that media from glial cells treated with thrombin and Aβ would induce cell death in neuronal cultures. However, media from thrombin-activated glial cells increased

neuronal viability, and it was hypothesised that this was due to the finding that thrombin induced expression of the neurotrophic factors GDNF and NGF, and the anti-inflammatory cytokines IL-10 and TGFβ1 from mixed glial and astrocyte cultures. Salbutamol, salmeterol, and db-cAMP augmented the increase in neuronal viability induced by media from thrombin-activated glial cells, and this was paralleled by an augmentation of thrombin-induced GDNF, NGF, IL-10, and TGFβ1 expression. In neuronal cultures, salbutamol, salmeterol, and db-cAMP induced the expression of the anti-inflammatory IL-1β receptor (IL-1R2) and the anti-apoptotic TNFα receptor (TNFR2), indicating that β-AR activation can have a direct positive effect on neurons. Thrombin also induced neuronal expression of IL-1R2 and TNFR2. Media from Aβ-activated glial cells did not alter neuronal viability, and Aβ did not alter glial production of neurotrophic or anti-inflammatory factors, or neuronal cytokine receptor expression.

Overall, this project has yielded significant insights into the inflammatory response induced by thrombin and Aβ, and how NA and subsequent β-AR activation exert an anti-inflammatory phenotype *in vitro*. It has also shown that thrombin can induce an anti-inflammatory/neurotrophic response in glial cells, and that β-AR activation is capable of augmenting this, resulting in increased neuronal viability.

Acknowledgements

Firstly I would like to thank the Health Research Board (HRB) for providing the funding to allow me to do this Ph.D.

I would also like to thank the people who helped me with the actual process of doing this thesis for all the help and encouragement they gave me: my supervisor Dr. Tom Connor and everybody in the Connor and Harkin lab groups for making this process more enjoyable than it otherwise would have been.

Finally thank you to my family and friends for all their support and understanding. They were fantastic and I couldn't have done it without them.

Dana Kilroy

1. Introduction

1.1 Central Nervous System

The nervous system is divided into the central nervous system (CNS), comprised of the brain and spinal cord, and the peripheral nervous system, consisting of the somatic, the autonomic, and the enteric nervous systems. Although neurons are the most well known cell in the brain, they are in fact outnumbered ten-fold by supportive cells called glia (glia means glue in Greek; Figure 1.1) (Stevens, 2003). Glial cells form a structural and functional network with complex cell-cell interactions and communications that facilitate fast and slow signalling between other glial cells and neurons. They have a regulatory influence on normal synaptic transmission and are thought to have the ability to alter it in disease (Banati, 2003). There are three major classes of glia based on their unique morphology and biochemistry: macroglia, subdivided into astrocytes and oligodendrocytes; and microglia. Oligodendrocytes are the myelinating glia; they provide insulating layers of myelin membrane around neuronal axons, allowing nerve impulses to transmit rapidly.

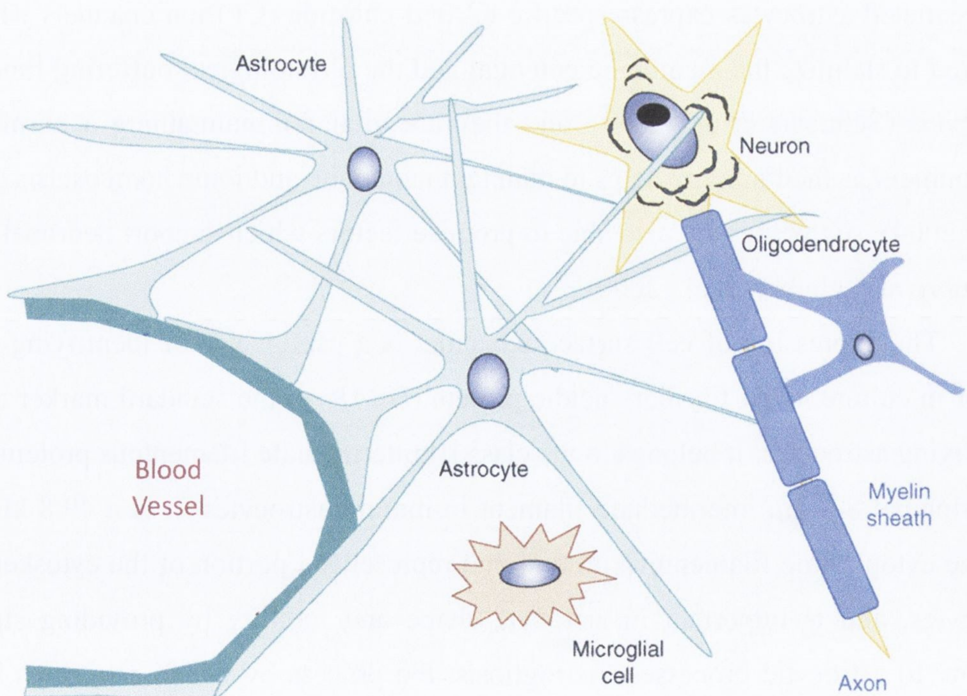


Figure 1.1: Cells of the CNS (Farina et al., 2007)

In the CNS, the neurons are surrounded by glia (astrocytes, microglia, and oligodenrocytes). The astrocytes also surround the blood vessels forming part of the blood-brain barrier.

1.1.1 Astrocytes

Astrocytes are the most numerous type of glia within the CNS. They are large, star-shaped cells, with elongated processes tipped with end-feet. They are separated into two subgroups on the basis of their morphology, function, and location: Protoplasmic astrocytes are found in grey matter and are closely associated with synapses, while fibrillary astrocytes are found in white matter and contact the nodes of ranvier on neurons (Stevens, 2003). Astrocytes are primarily support cells providing structural and nutritional support for the neurons. They store glycogen, export lactate, regulate the supply of glucose to neurons, remove neurotransmitters such as glutamate from the synaptic cleft, and help regulate homeostasis through uptake of extracellular potassium (K^+). They also surround blood vessels where they are responsible for generating and maintaining a tight blood-brain barrier (BBB) by inducing tight-junctions between endothelial cells (Schubert et al., 2000, Jessen, 2004, Farina et al., 2007). Another important function of astrocytes is that they, like neurons, can elicit a quiescent effect on neighbouring microglia. These effects are mediated via cell-cell interactions and the production of soluble factors (Vincent et al., 1997, Zietlow et al., 1999). Mature differentiated astrocytes express specific K^+ and chloride (Cl^-) ion channels which are required to stabilize the membrane potential and the physiological buffering function of astrocytes (Schubert et al., 2000), and they are vital for maintaining a normal CNS environment as their activity helps to maintain metabolic and ionic homeostasis (Liberto et al., 2004). Astrocytes are also able to produce factors which support neuronal growth and survival (Schubert et al., 2000).

The expression of cell surface receptors is a useful way of identifying cells *in situ* or in culture. Glial fibrillary acidic protein (GFAP) is the standard marker used for identifying astrocytes. It belongs to the class III intermediate filamentous proteins and is the principal 8-9 nm intermediate filament in mature astrocytes. It is a 49.8 kDa non-soluble cytoplasmic filamentous protein that represents a portion of the cytoskeleton in astrocytes, and is important in astrocyte shape and motility by providing structural stability to astrocytic processes. Astrogliosis, the process by which astrocytes become reactive and responsive, is characterized by rapid synthesis of GFAP (Eng et al., 2000).

1.1.2 Microglia

Microglia are the resident immune cells within the CNS. They respond to, as well as secrete, immunoregulatory factors to control the proliferation, death, or differentiation of neighbouring cells thereby regulating the structure and function of neural tissues during development and in response to injury (Giulian and Tapscott, 1988). Originally there was controversy about whether or not these cells were simply invading macrophages from the blood, however an in depth study by Giulian and colleagues (1995) showed that microglia are in fact a separate class of macrophages unique to the CNS, differing from macrophages of the spleen, bone, blood, and liver in such categories as proliferative capacity, patterns of differentiation, surface morphologies, responses to cytokines, and interactions with astrocytes. They accurately described the different cell surface morphology of the microglia and showed that microglia differ in appearance depending on developmental stage, functional state, and anatomical location. These differences can be clearly viewed with scanning electron microscopy. Amoeboid microglia are developing microglia in the CNS. Mature and resting microglia have a ramified morphology; they have twenty or more spines projecting from the cell bodies and the spines are more than three times the diameter of the cell body. Cellular ramification is a useful anatomic marker for microglial differentiation as it is strikingly different to the smooth or ruffled appearances of macrophages from spleen, liver, or bone. Upon stimulation the spines retract and microglia transform into reactive microglia capable of an immune response including secretion of cytokines and neural growth factors, antigen presentation, and phagocytosis. Afterwards microglia return to a quiescent state displaying reduced secretions, reduction in cell surface receptors, and loss of phagocytic activity. They also showed that, unlike other macrophages, contact with astrocytes is necessary for proper microglial development and differentiation. The astrocytes help maintain microglia in a differentiated, dormant state in order to restrict negative actions of microglia such as synaptic stripping and release of neurotoxins. Resting microglia are present throughout the CNS where they form a complex network of immuno-effector cells (Streit et al., 2004).

Microglia express many different cell surface receptors depending on their level of maturity and activation state. Expression of cell surface receptors is also a useful way of distinguishing between microglia and non-microglial macrophages. Microglia show the phenotype CD45low CD11b/c$^+$ whereas non-microglial but CNS-associated macrophages are CD45high CD11b/c$^+$ (Ford et al., 1995). Resting microglia express CD11a, CD11b, CD11c, CD18, leukocyte common antigen, and immunoglobulin receptor Fc gamma RI (Akiyama and McGeer, 1990). The expression of all of these molecules, as well as the expression of MHC-class-II and the associated CD40 and B7 molecules, is up-regulated when the microglia are activated (O'Keefe et al., 2002) making them useful phenotypic marker for microglial activation (Wei and Jonakait, 1999, Twig et al., 2005).

1.2 Inflammation

Inflammation is a reaction of living tissues to irritation or injury. There are two types of inflammation; acute and chronic (Streit et al., 2004). For many years the brain was thought to be an 'immune privileged' organ that was mostly unaffected by the body's inflammatory responses and was not susceptible to inflammation. However, it is now known that the brain does elicit an immune response, although it differs considerably from other tissues in its responses to pathogenic challenges. For example, the recruitment of leukocytes, which tends to be rapid in systemic organs, occurs later and to a lesser extent in the brain (Lucas et al., 2006). Leukocytes are also known as white blood cells and are the immune cells of the periphery, such as monocytes, macrophages, lymphocytes (B cells, T cells, and natural killer (NK) cells), neutrophils, basophils, and eosinophils.

1.2.1 Acute inflammation

Acute inflammation is the immediate and early response to a harmful agent and is a defensive response that paves the way for repair of the damaged site. In the periphery it is mediated by leukocyte infiltration of tissues by polymorphonuclear cells (neutrophils). In the CNS it leads to reactive gliosis, the immediate accumulation of enlarged glial cells at the site of injury. Minor neuronal damage triggers glial cell activation without breakdown of the BBB. In these cases the site of injury is repaired with no further damage to the surrounding tissue. However if the BBB is damaged it results in leukocyte infiltration which produces a more aggressive immune response causing activated glial cells to release factors that act on and produce responses in target cells similar to that in the periphery (Streit et al., 2004).

1.2.2 Chronic inflammation

Chronic inflammation results from stimuli that are persistent. It is characterized by activation of mononuclear cells; macrophages, lymphocytes, and plasma cells in the periphery, and microglia in the CNS. It was long suspected that there is a role for immune responses, including antigen presentation and pro-inflammatory cytokines, in neurodegenerative diseases. However the term neuroinflammation only came into use in 1995. Since then it has come to mean "chronic, CNS-specific, inflammation-like glial responses that do not reproduce the classic characteristics of inflammation in the periphery but that may engender neurodegenerative events; including plaque formation, dystrophic neurite growth, and excessive tau phosphorylation" (Streit et al., 2004). Neuroinflammation applies to chronic, persistent cycles of injury and reaction, in which the increasing ill effects of immunological microglial and astrocytic activation increase the initial neurodestructive effects, so maintaining and worsening the disease process through their actions. The activated microglia and astrocytes of the innate immune response, but not the leukocyte actions of the adaptive immune response, are considered to be the potentially pathogenic factor in a number of CNS diseases. Chronic microglial activation contributes to neuronal dysfunction, injury, and loss, and consequently to disease progression. This microglia-driven neuroinflammatory response is implicated in many chronic CNS disorders from multiple sclerosis (MS), prion diseases, and human immunodeficiency virus (HIV), to severe neurodegenerative diseases particularly Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (Streit et al., 2004).

Acute inflammation is necessary and indeed beneficial directly following an immune stimulation, however, if this stimulation is not quickly resolved then the inflammation becomes chronic and increasingly detrimental. Streit and colleagues (2004) put it perfectly when they said "The idea that neuroinflammation is detrimental implies that glial cell activation precedes and causes neuronal degeneration, a sequence of events that appears to be at odds with experimental models of neurodegeneration in which glial cell activation occurs secondary to neuronal damage. What is missing from this simple linear model is the understanding that chronic neurological diseases are just that – chronic, and that this chronicity introduces complex interactions and feedback loops between neurons and glia that render attempts to construct simple, linear cascades of cause and effect inelegant."

1.3 Inflammatory molecules

Key features of CNS inflammation are glial activation, expression of major histocompatibility complex (MHC), synthesis of inflammatory mediators such as cytokines, chemokines, free radicals, and prostaglandins, expression of adhesion molecules, and invasion of immune cells (Lucas et al., 2006).

1.3.1 Initiation of an immune response

Toll-like receptors (TLRs) are widely distributed in immune cells and are often the first trigger of an immune response as they are responsible for binding Pathogen Associated Molecular Patterns (PAMPs). TLRs recognize a range of pathogen-derived products including bacteria, viruses, fungi, and parasites. For example, bacterial lipopolysaccharide (LPS) is the main activator for TLR4, viral double-stranded RNA activates TLR3, and protozoal products bind to TLR2. This means that TLRs will pick up almost all pathogens that infect humans (Liew et al., 2005). TLRs are part of the TIR (Toll/Interleukin-1 receptor) superfamily which contains a TIR domain in the cytosolic region. All TLRs, except for TLR3, use a TLR signalling adaptor protein called MyD88 which interacts with the IRAK (IL-1 receptor-associated kinase) family and TRAF6 (TNF-receptor-associated factor 6). This leads to the activation of the transcription factor nuclear factor of kappa binding (NFκB) and mitogen-activated protein kinases (MAPK) such as p42/p44 MAPK and Jun N-terminal kinase (JNK). These pathways then lead to the production of cytokines and chemokines, with different ones being induced depending on the stimulus (Liew et al., 2005).

1.3.2 Cytokines

Cytokines are a set of structurally diverse small proteins (8-80 kDa) including the interleukins (IL), interferons (IFN), and tumour necrosis factors (TNF) (Roitt et al., 2001, Janeway et al., 2005). They are primarily involved in the immune response and are classed as pro- or anti-inflammatory depending on whether they promote or attenuate the inflammatory process respectively. Cytokines are also extremely important under normal physiological conditions and have a number of non-inflammatory functions including roles in cell growth and differentiation, and cell-cell communication (Roitt et al., 2001). They are mainly produced by immune cells such as microglia, macrophages, monocytes, T-cells, and dendritic cells, but also by some non-immune cells such as astrocytes, neurons and endothelial cells, and they mediate their effects by activating specific receptors which initiate intracellular signalling pathways resulting in gene transcription (Roitt et al., 2001). The pro-inflammatory cytokines IL-1β and TNFα are among the first pro-inflammatory mediators to be released in response to microglial activation. They activate a "cytokine cascade" which induces the production of other pro-inflammatory cytokines and inflammatory products such as prostaglandins and nitric oxide (NO) (Zetterstrom et al., 1998). As such, they are considered to be the primary mediators of the inflammatory response within the CNS. Usually this inflammatory response is resolved by anti-inflammatory cytokines, such as IL-10 and TGFβ, which inhibit further production of IL-1β and TNFα, thus playing a suppressive role in the immune response (Bogdan et al., 1992, Yoshimura et al., 2003).

IL-1β

Interleukin-1β (IL-1β) is an important pro-inflammatory cytokine involved in the innate inflammatory response and was the first cytokine identified to act in the brain (Lucas et al., 2006). It binds to IL-1 receptors (IL-1Rs) that belong to the TIR superfamily. There are two distinct plasma membrane IL-1Rs, IL-1R1 and IL-1R2. IL-1R1 mediates the signal transduction effects of IL-1 by binding to IL-1R accessory protein (IL-1RAcP) to form a heterodimeric type 1 IL-1R complex. IL-1RAcP alone is unable to bind IL-1 but functions as an additional chain in the receptor complex. IL-1R1 has a 215 amino acid cytoplasmic TIR-domain-containing tail and undergoes a conformational change upon ligand binding (Fitzgerald and O'Neill, 2000). Similar to TLR signalling, the TIR domain of the IL-1R recruits MyD88 which interacts with the IRAK and TRAF6, leading to the activation of NFκB and members of the MAPK family, ultimately resulting in pro-inflammatory gene expression (Fitzgerald and O'Neill, 2000, Liew et al., 2005). IL-1R1 associates with the cytosolic adaptor protein MyD88 which leads to phosphorylation of the tyrosine kinase Src (Davis et al., 2006, Tsakiri et al., 2008b). In contrast to IL-1R1, IL-1R2 does not mediate signal transduction. It shows high sequence similarity to IL-1R1 in its extracellular domain structure but it only has a 29 amino acid cytoplasmic domain meaning it is incapable of sending further signals. IL-1R2 is shed from the cell surface and functions as a decoy receptor, competing with IL-1R1 for IL-1β, thus acting as a negative regulator of the IL-1 system and eliciting an anti-inflammatory action (Colotta et al., 1994, Fitzgerald and O'Neill, 2000).

All cell types in the brain, including microglia, astrocytes, neurons, and invading leukocytes, can express and release IL-1β, either under normal conditions or in response to neuronal injury or inflammatory molecules such as LPS and TNF. IL-1R1 is also expressed on these cells under basal conditions, however only activated microglia express IL-1R1, meaning that they can respond to IL-1β in an autocrine manner, as well as a paracrine manner (Friedman, 2001, Allan et al., 2005, Wang et al., 2006a, Wang et al., 2006b). Neural precursor cells (NPCs) also express IL-1R1 and IL-1R2 (Wang et al., 2007b), and IL-1R2 is commonly expressed on immune cells in the brain (Lucas et al., 2006).

IL-1β signalling mediates the acute-phase of the inflammatory response and induces the expression of inflammatory molecules such as cycloxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Laflamme et al., 1999, Akama and Van Eldik, 2000), the production of chemokines (Tsakiri et al., 2008a), and neutrophil recruitment (Blond et al., 2002). Blond and colleagues (2002) showed that IL-1β induces TNFα expression in peripheral tissue, while in the brain parenchyma it induces *de novo* synthesis of additional IL-1β, but not TNFα. IL-1β, via IL-1R1 activation, induces cardiovascular effects, neuroendocrine effects, fever, reduced glutamate release, and altered gene expression of iNOS, cytokines, prostaglandins, neuropeptides, and growth factors. These effects can be inhibited by IL-1 receptor antagonist (IL-1ra), a molecule which binds to IL-1R1 thus preventing IL-1β binding and subsequent signal transduction (Lucas et al., 2006).

IL-1β treatment can cause retinal neuronal death (Abcouwer et al., 2008), increase apoptosis of NPCs (Wang et al., 2007b), and increase N-methyl-D-aspartate (NMDA) and glutamate induced hippocampal neuron death (Ma et al., 2002). IL-1β is found in higher concentration in the aged brain when compared to levels found in younger brains and is correlated with an increase in p38 MAPK which led to an increase in apoptosis (Martin et al., 2002). However another study showed that IL-1β can protect against retinal ganglion cell death (Diem et al., 2003). As well as its inflammatory actions, it has been linked to a wide variety of activities including appetite suppression, weight loss, sleep modulation, endocrine alterations, nervous system functions, behavioural changes, inhibition of synaptic plasticity and neuronal transmission, and neuronal cell death (Rothwell, 2003). IL-1β is also implicated in a variety of neurological and neurodegenerative disorders including stroke, AD, PD, MS and epilepsy (Allan et al., 2005, Lucas et al., 2006), and IL-1R1 expression is up-regulated following injury (Friedman, 2001, Wang et al., 2006a, Wei et al., 2008), and hypoxia (Froyland et al., 2008, Lam et al., 2008).

TNFα

In 1984, molecules which were cytotoxic to tumour cells were isolated and designated tumour necrosis factors (TNFs). Since then research has identified the existence of a TNF superfamily of proteins, consisting of 19 ligands signalling through 29 receptors (Aggarwal, 2003). TNFα is one of the most studied members of this family. It is a potent pro-inflammatory cytokine and is a primary mediator of the inflammatory response. TNF receptors (TNFRs) are of two main types, those that contain a death domain and can mediate apoptosis, such as TNFR1, and those that do not, such as TNFR2. A pre-ligand binding assembly domain in the extracellular region of the receptor mediates assembly of receptor trimers complexes and is necessary for signalling. TNFR1 (also called TNFr1a or p55) is the receptor for the soluble ligand and binds TNFα with high affinity. It is expressed on all cell types including astrocytes and neurons (Yang et al., 2002, Aggarwal, 2003, Ohtori et al., 2004, Kuno et al., 2006, Lucas et al., 2006). TNFR1 activates TNFR associated death domain (TRADD) which then activates caspase pathways leading to apoptosis. TRADD also binds to TNFR associated factor 2 (TRAF2) which then activates NFκB, JNK, and MAPK pathways. The apoptotic signals induced by TNFR1 do not require new protein synthesis (Aggarwal, 2003). In contrast to this, TNFR2 (also called TNFr1b or p75) is the receptor for membrane bound ligand and binds TNFα with low affinity. It is predominantly expressed on immune cells and endothelial cells as well as astrocytes (Yang et al., 2002, Aggarwal, 2003, Kuno et al., 2006, Lucas et al., 2006). TNFR2 does not contain a death domain and is responsible for cell proliferation. It signals via TRAF2 activation leading to protein synthesis which is mediated by activation of NFκB (Aggarwal, 2003). Despite the common signalling pathways, TNFR1 and TNFR2 have markedly different effects as TNFα is toxic to neurons that over-express TNFR1, but it is trophic to neurons with TNFR2 over-expression (Yang et al., 2002), and numerous *in vivo* and *in vitro* studies using knockout (KO) mice have shown that TNFR1 mediates apoptosis while TNFR2 is anti-apoptotic (Fontaine et al., 2002, Raivich et al., 2002, Yang et al., 2002, Marchetti et al., 2004).

TNFα is produced by immune cells including macrophages, NK cells, T and B cells, and from microglia and astrocytes in the CNS, where it plays a fundamental role in the pathophysiology of injury (Aggarwal, 2003, Ohtori et al., 2004, Lucas et al., 2006). TNFR1 and TNFR2 are expressed on neurons and glia (Neumann et al., 1997a, McGuire et al., 2001, Bette et al., 2003, Dziewulska and Mossakowski, 2003, Yan et al., 2003), and NPCs (Sheng et al., 2005), although TNFR2 is more commonly expressed on immune cells including macrophages and microglia (Neumann et al., 1997a, Yang et al., 2002, Aggarwal, 2003, Bette et al., 2003, Dziewulska and Mossakowski, 2003, Kuno et al., 2006). TNFα has a multitude of actions: It influences genes that are involved in inflammation, such as IL-1β, IL-6, MHC-class-I, and genes involved in cell adhesion. It also induces genes that regulate apoptosis such as Fas, TNFR1, and caspase-8. As well as this it regulates genes involved in survival, cell metabolism, and cell signalling. However, the survival pathways seemed to be unable to compete with the death signal after 24 hours (Buntinx et al., 2004a). The release of TNFα from microglia induces expression of iNOS in astrocytes (Akama and Van Eldik, 2000). In peripheral tissue, TNFα has been shown to induce IL-1β expression, but injection of TNFα into the brain parenchyma does not induce either itself or IL-1β. TNFα also induces mononuclear cell recruitment (Blond et al., 2002). TNFR1 is pro-inflammatory and is required for the TNFα-induced production of the chemokines IP-10 and MCP-1 from NPCs (Sheng et al., 2005), the recruitment of microglial cells and induction of astrocytic vascular cell adhesion molecule (VCAM-1) in the spinal cord of animals with EAE (experimental autoimmune encephalomyelitis, a model for MS) (Archambault et al., 2006), and microglial expression of the co-stimulatory molecule B7-2 (Bohatschek et al., 2004). Other research indicates that during the acute phase of CNS inflammatory responses TNFα seems to be pro-inflammatory but during the chronic phase it is immunosuppressive (Lucas et al., 2006).

TNFα also induces cell death in neurons (Williams et al., 2005), oligodendroglial cell lines (Buntinx et al., 2004b), and differentiated PC12 cells (Koski et al., 2004), and it increases NMDA-induced cell death in cultures of cortical neurons by causing a transient increase in extracellular signal-regulated kinase (ERK) and JNK activity leading to NMDA receptor-dependent calcium influx and cell death (Jara et al., 2007). TNFα induces apoptosis by rapid down-regulation of c-Myc protein expression and this was enhanced by IFNα (Nakashima et al., 2005). However TNFα has been shown to be neuroprotective against excitotoxic damage in primary cortical neurons via a process dependent on TNFR2 and NFκB activation (Dolga et al., 2008), and another study showed that TNFα protects against retinal ganglion cell death (Diem et al., 2003). The effect of TNFα is dependent on the age of the cells. For example co-treatment of TNFα plus amyloid-β (Aβ) is toxic to old neurons (from 24 month old rats), but is protective to middle-age neurons (from 10 month old rats) (Patel and Brewer, 2008).

TNFα and both receptors also have a role in the regulation of neural development (Mehler and Kessler, 1997) and TNFα promotes nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) production in astrocytes (Kuno et al., 2006). In particular, TNFR2 has a role in modulating NGF signalling as inhibition of TNFR2 increases NGF-induced ERK activation thus inducing differentiation of neuroblastoma cell lines, and it decreases NGF-induced Akt activation which is essential for survival signalling (Takei and Laskey, 2008).

TNFα can have both positive and negative effects: It can play an anti-cancer role and help protect against microbial infection, but it can also contribute to tumorigenesis and autoimmunity and has been identified in other diseases such as stroke, AD, PD, MS, AIDS, and chronic heart failure (Aggarwal, 2003, Lucas et al., 2006). Neuronal injury leads to the up-regulation of glial TNFα and TNFR1, and is associated with the expression of the TNFR1 receptor on adjacent neurons (Ohtori et al., 2004). TNFR1 is induced following hypoxia (Froyland et al., 2008, Lam et al., 2008), a short ischemic event (Yin et al., 2004, Pradillo et al., 2005), peripheral nerve injury (Ohtori et al., 2004), and constriction injury (Wei et al., 2008), and it is increased in the substantia nigra of PD brains (Mogi et al., 2000, Gomez-Santos et al., 2007). In AD brains, TNFR1 levels are increased while TNFR2 levels are decreased (Cheng et al., 2009), and in spinal cord injury neuronal expression of TNFR1 and TNFR2 is increased (Yan et al., 2003, Harrington et al., 2005).

IFNγ

Interferons (IFNs) are a group of cytokines which were originally described as molecules capable of protecting cells from viral infection (Bach et al., 1997). One of the most prominent members of this family, IFNγ (Type-II-IFN), is a pro-inflammatory cytokine made up of two identical 17-kDa polypeptide chains which form a noncovalent homodimeric glycosylated protein of 45-50 kDa (Billiau, 1996, Bach et al., 1997). IFNγ acts via the IFNγ receptor (IFNgR). There are two subunits in the IFNgR: the α-chain (IFNgR1 or CDw119) has high ligand binding affinity, while the β-chain (IFNgR2 or accessory factor-1, AF-1) is necessary for signalling. IFNgR signals via Janus kinases (JAKs) and signal transducer and activator of transcription (STATs). JAKs are the enzymes responsible for phosphorylating and activating STATs, and IFNgR1 is constitutively associated with JAK2 while IFNgR2 is associated with JAK1. In unstimulated cells, IFNgR is associated with an inactive form of JAK1, but binding of

IFNγ results in receptor oligomerization, which brings the JAKs into contact with one another. The JAKs become activated through tyrosine phosphorylation of each other and the cytoplasmic domains of the receptors. This activation results in recruitment and phosphorylation of STAT-1α, forming a homodimer which translocates into the nucleus. It initiates transcription of genes containing a gamma activated sequence (GAS), or STAT binding element (SBE), in their promoter region, particularly the interferon regulatory factor (IRF) family (Bach et al., 1997, Chen et al., 2006, Gough et al., 2008). Emerging evidence shows that IFNgR can also signal via other pathways that either co-operate with or act in parallel to JAK-STAT signalling, including MAPK, NFκB, phosphatidylinositide-3-kinase (PI_3K), and calcium (Ca^{2+}) and calmodulin (CaM) dependent protein kinases (CaMKII) (Gough et al., 2008).

IFNγ is induced by immune and inflammatory stimuli and is only released under pathologic conditions such as infection, trauma, cancer, or autoimmunity. It is primarily produced by Th1 cells and NK cells following their activation by cytokines or interaction with virus-infected or tumour cells, but can also be produced by B cells and professional antigen presenting cells (APCs) (Billiau, 1996, Bach et al., 1997, Gough et al., 2008). Cytokines are regulators of IFNγ production, for example IL-2 increases while IL-10 decreases production (Billiau, 1996). Practically all cells of the body express IFNgRs, giving it wide ranging actions (Billiau, 1996). Specific studies have shown that IFNgRs are found on astrocytes and microglia (Vikman et al., 1998, Hashioka et al., 2009, Tsuda et al., 2009), and in motoneurons (Linda et al., 1998), and that neurons express IFNγ and both IFNgR1 and IFNgR2 (Neumann et al., 1997a, Neumann et al., 1997b). IFNgRs are constitutively expressed at extremely low levels, but can be regulated by external stimuli in certain cell types (Bach et al., 1997).

De Veer and colleagues (2001) combined data from many IFNγ experiments to identify over 300 IFN-stimulated genes (ISGs), including adhesion molecules, kinases, antigen presentation and processing molecules, apoptotic and protective molecules, chemokines, signalling molecules, transcription factors, activators and repressors, and immune modulation and inflammation molecules. Other studies have also shown that IFNγ can enhance and induce expression of iNOS, the antigen presenting molecule MHC-class-II, the co-stimulatory molecule CD40, cytokines such as IL-12 and TNFα, adhesion molecules such as ICAM-l, and chemokines such as IP-10 and RANTES (Billiau, 1996, Bach et al., 1997, Neumann et al., 1997a, Nguyen et al., 1998, Chen et al., 2006, Gough et al., 2008). Buntinx and colleagues (2004b) showed that IFNγ induced apoptotic cell death in oligodendroglial cell lines in a time (24-72 hours) and dose (1-250 ng/ml) dependent fashion, and that this effect was amplified when IFNγ was given in conjunction with TNFα. Further study by this group revealed that IFNγ up-regulated transcription of the death receptors Fas and TNFR1, and caspase-8 (Butovsky et al., 2006). IFNγ resulted in dopaminergic cell loss, but only in the presence of microglia (Mount et al., 2007), and IFNγ also increases apoptosis of taste bud cells *in vivo* (Wang et al., 2007a). IFNgRs are important in mediating IFNγ induced toxicity (Mount et al., 2007), and media from astrocytes treated with IFNγ kills neuroblastoma cells (Hashioka et al., 2009). Interestingly, IFNγ can also occasionally have a beneficial effect as IFNγ activated microglia can induce neurogenesis (Butovsky et al., 2006). Also pre-treatment of hippocampal cell cultures with IFNγ protects neurons against glutamate-induced death by increasing recovery of intracellular Ca^{2+} concentrations (Lee et al., 2006) and treatment of neuronal progenitor cells with IFNγ enhanced neuronal differentiation and increased expression of the neuronal markers such as βIII-tubulin (Arien-Zakay et al., 2009). The effects of IFNγ are cell specific, for example it stimulates proliferation of mitogen-triggered T cells, but acts as a mild inhibitor of proliferation for most other cell types, and it promotes apoptosis of lymphocytes (Billiau, 1996). IFNγ can also protect cells from viral infection and plays an important role in the clearance of virus from spinal cord and brain stem neurons, but not from cortical neurons (Bach et al., 1997, Binder and Griffin, 2001).

In cases where inflammation is not due to infection, IFNγ has a disease-promoting effect, however, in some models of infectious disease it can have a protective role as it promotes non-specific cellular defence mechanisms and favours the generation of specific immunity (Billiau, 1996). IFNγ production is a key feature in MS; it kills oligodendrocytes (Popko and Baerwald, 1999) and a number of transgenic mouse studies have shown that it is involved in hypomyelination (Corbin et al., 1996, LaFerla et al., 2000). It is also critical in the initiation and amplification of the local immune response during EAE (Stoll et al., 2000). In humans, the inability to secrete IFNγ or complete IFNgR deficiency is associated with frequent infection and ultimately death from mycobacterium infection (Gough et al., 2008). Astrocytes express sharply up-regulated levels of IFNgR in a number of neurodegenerative diseases such as AD, PD, MS, and ALS (Hashioka et al., 2009).

IL-10

IL-10 is a potent anti-inflammatory cytokine with roles in inflammation, infection, anti-tumour activity, and the promotion of self-tolerance in autoimmune diseases (Commins et al., 2008, Mosser and Zhang, 2008). It binds to the IL-10 receptor (IL-10R), which is made up of a ligand binding α-chain (IL-10R1) and an accessory β-chain (IL-10R2) which is necessary for signal transduction (Ledeboer et al., 2002, Mosser and Zhang, 2008). IL-10 signals via the JAK-STAT pathway. IL-10R1 and IL-10R2 are associated with JAK1 and tyrosine kinase 2 respectively. Ligand binding activates these kinases causing phosphorylation of tyrosine residues in the cytoplasmic tails of the receptors and subsequent STAT3 recruitment. STAT3 forms a homodimer which is released from the receptor and translocates to the nucleus. Here it binds to STAT-binding elements in the promoters of several immune response genes including suppressor of cytokine signalling 3 (SOCS3) which is a negative regulator of various cytokine genes, and IL-10 itself (Mosser and Zhang, 2008). IL-10 also results in reduced NFκB activation (Mosser and Zhang, 2008).

IL-10 is primarily produced by type 2 helper T (Th2) cells and regulatory T cells, but can also be produced by many other immune cells such as monocytes, activated macrophages, and dendritic cells, and by non-immune cells such as epithelial cells and tumour cells (Mosser and Zhang, 2008). Astrocytes, and to a lesser extent microglia, also produce IL-10 (Ledeboer et al., 2002). IL-10R2 is expressed on most cell types, and microglia and astrocytes also express IL-10R1 (Ledeboer et al., 2002, Mosser and Zhang, 2008). IL-10R1, but not IL-10R2, is up-regulated in immune cells following activation (Ledeboer et al., 2002). IL-10 is induced by inflammatory stimuli such as LPS and the pro-inflammatory cytokines TNFα and IL-1β, in a time dependent manner (Sheng et al., 1995, Ledeboer et al., 2002, Souza et al., 2003). IL-10 can inhibit its own production from astrocytes, meaning that it is capable of auto-regulation (Ledeboer et al., 2002). Following LPS stimulation, IL-10 release is much later than that of pro-inflammatory cytokines and it inhibits further TNFα production, thus negatively regulating inflammation (Sheng et al., 1995). IL-10 inhibits LPS-induced production of TNFα, IL-1β, and IL-18 by degrading the cytokine mRNA, indicating a post-transcriptional mechanism (Bogdan et al., 1992, Denys et al., 2002, Ledeboer et al., 2002). However, IL-10 can also inhibit the promoter region of the TNFα gene, suggesting a transcriptional mechanism. This mechanism of inhibition is independent of p38 MAPK or NFκB activation (Denys et al., 2002). IL-10 also inhibits MHC-class-II expression and chemokine production, prevents the production of the Th1-associated cytokines IL-2 and IFNγ, increases the release of IL-1ra from macrophages (Mosser and Zhang, 2008), and suppresses LPS-induced expression of ICAM-1 on microglia (Wirjatijasa et al., 2002). It induces a morphological transformation in microglia from an amoeboid to a ramified phenotype, indicating microglial de-activation (Wirjatijasa et al., 2002), and can act as a growth factor by stimulating the proliferation of some $CD8^+$ T cells (Mosser and Zhang, 2008). IL-10 is extremely important in limiting autoimmune pathologies (Mosser and Zhang, 2008) and plays a role in Burkitt lymphoma and malignant B-cell lymphomas (Commins et al., 2008). It also prevents LPS-induced hypothermia and mortality (Gerard et al., 1993), and is protective during reperfusion injury, preventing the increase in TNFα, tissue injury, and lethality (Souza et al., 2003). Importantly, IL-10 also has direct trophic actions on neurons (Zhou et al., 2009).

TGFβ1

Another group of anti-inflammatory cytokines are the transforming growth factors-β (TGFβ), a large family of ligands, receptors, and binding proteins which have numerous roles in the regulation of cell growth, apoptosis, proliferation, adhesion, differentiation, motility, migration, and extracellular matrix production (Rahimi and Leof, 2007, Glasgow and Mishra, 2008). The active form of TGFβ1 consists of two polypeptides which interact via a disulfide bond and hydrophobic interactions to form a 25 kDa dimer (Rahimi and Leof, 2007). It signals by binding to the TGFβ receptor (TβR) which forms a heterotetrameric complex of types I and II serine/threonine kinase receptors. Binding results in the phosphorylation of type I receptor by the type II receptor, and subsequent activation of intracellular mediators known as SMAD proteins which translocate to the nucleus where they activate gene expression and regulate transcription (Rahimi and Leof, 2007, Glasgow and Mishra, 2008). The ERK, JNK, and p38 MAPK pathways have also been shown to play a role in SMAD regulation (Glasgow and Mishra, 2008).

TGFβ1 is produced by many cell types, including astrocytes and microglia (Vincent et al., 1997), and has varying actions depending on the type of stimulation and cell involved (Rahimi and Leof, 2007). TGFβ1 inhibits CD40-induced IFNγ expression (Nguyen et al., 1998), and LPS-induced production of TNFα from macrophages by suppressing translation of the TNFα mRNA. It was effective even when added 6 hours after LPS, but it required 12-16 hours to achieve suppression (Bogdan et al., 1992). It can also inhibit production of NO, but not IL-1β, from glia treated with endotoxin (Vincent et al., 1997). However another study showed that it increased LPS-induced IL-1β, but not TNFα, production (Wirjatijasa et al., 2002). TGFβ does not alter microglial morphology or suppress LPS-induced ICAM-1 expression (Wirjatijasa et al., 2002). TGFβ promotes neuronal survival and the expression of the cell survival gene, bcl2, in rat hippocampal neurons. It has been shown to modulate Ca^{2+} homeostasis and buffering and mitochondrial function which can help protect cells against oxidative and cytotoxic injury. It may also regulate the levels of neurotrophins produced by target tissues (Mehler and Kessler, 1997). In tumours, TGFβ promotes the accumulation of regulatory T cells but inhibits the actions of tumour-specific cytotoxic T cells (Wu, 2007). Expression of TGFβ was induced in microglia following *in vitro* ischemia (deprivation of glucose, oxygen, and serum), and this protected against ischemia-induced damage of microglia and neurons (Lu et al., 2005). It also has a role in the regulation of neural development (Mehler and Kessler, 1997), and in the maintenance of normal blood vessel wall structure and is key factor in a number of cardiovascular syndromes (Grainger, 2007) including angiogenesis (Bertolino et al., 2005). TGFβs are most likely not neurotrophic by themselves, however they are required to promote the survival of neuron populations that is induced by select neurotrophins and GDNF (Krieglstein et al., 2002).

1.3.3 Chemokines

Chemokines are a set of very small cytokines (8-14 kDa) that function as chemotactic proteins. They regulate leukocyte migration under normal and pathological conditions and are responsible for inflammatory cell recruitment in host defence by attracting immune cells to an area of inflammation (Mennicken et al., 1999, Roitt et al., 2001, Ubogu et al., 2006). Chemokines create a concentration gradient surrounding the inflammatory stimulus by binding to cell-surface heparan sulfate proteoglycans. Here they come into contact with leukocytes rolling on the endothelium, resulting in leukocyte migration to the site of inflammation (Luster, 1998).

There are four families of chemokines defined by their cysteine residue position. The two largest families of chemokines have four conserved cysteines linked by disulfide bonds and are divided according to the position of the first two cysteines. The α-chemokines (CXC chemokines) have cysteines separated by one amino acid, for example IFN-inducible protein-10 (IP-10), and attract neutrophils. The β-chemokines

(CC chemokines) have adjacent cysteines, for example macrophage inflammatory protein (MIP) and monocyte chemoattractant peptide (MCP), and attract monocytes, eosinophils, and basophils (del Pozo et al., 1995, Baggiolini et al., 1997, Luster, 1998, Ubogu et al., 2006). In humans, the genes of the CC and CXC chemokines are clustered on chromosome 17 and chromosome 4 respectively (Baggiolini et al., 1997). The two smaller families of chemokines are the γ-chemokines (C chemokines) which have only one cysteine, and the δ-chemokines (CX_3C chemokines) in which the cysteines are separated by three amino acids (Bazan et al., 1997, Mackay, 1997, Charo and Ransohoff, 2006, Ubogu et al., 2006).

Chemokines act by binding to specific G-protein coupled seven-transmembrane-domain receptors on the surface of target cells. These receptors have two conserved cysteines, one in the NH_2-terminal domain and the other in the third extracellular loop. These cysteines form a disulfide bond which is critical for the conformation of the ligand-binding pocket (Baggiolini et al., 1997). Receptor binding results in the activation of small guanosine triphosphate (GTP)-binding proteins of the Ras and Rho families. These proteins are responsible for leukocyte recruitment as they play a role in cell motility by regulating actin-dependent processes such as membrane ruffling, pseudopod formation, and assembly of focal adhesion complexes. Receptor binding also leads to the activation of protein kinase C (PKC), the formation of inositol 1,4,5-triphosphate (IP_3), and the release of intracellular Ca^{2+} (Luster, 1998). Chemokine receptors are divided into subgroups depending on the type of chemokine that binds to them: CXC receptors bind only CXC chemokines and CC receptors bind only CC chemokines. So far researchers have identified six human CXC chemokine receptors (CXCR1 through CXCR6), ten human CC chemokine receptors (CCR1 through CCR10), one human C chemokine receptor (XCR1), and one human CX_3C chemokine receptor (CX_3CR1) (Charo and Ransohoff, 2006, Ubogu et al., 2006). The effect of a chemokine is determined by the type of leukocyte on which its receptor is expressed. Interestingly, some chemokine receptors are also expressed on non-leukocyte cell such as neurons, astrocytes, and endothelial cells, indicating that they may have other functions. Some receptors are expressed on numerous cell types while others are restricted to specific cells. Chemokine receptors can also be constitutively expressed on some cells while on others they are induced by cytokine stimulation (Luster, 1998).

Chemokines are released by a number of cell types including endothelial cells, macrophages, and monocytes. During brain inflammation they are up-regulated in astrocytes and microglia, and neurons have been shown to express chemokine receptors (Mennicken et al., 1999). As well as their role in leukocyte attraction, they also have direct actions on target cells and have a number of functions including enzyme secretion, cellular adhesion, cytotoxicity, tumour cell growth, and T-cell activation (Mennicken et al., 1999, Roitt et al., 2001, Ubogu et al., 2006). Chemokine production is induced by cytokines such as IL-1, TNFα and IFNγ, viral infection, and LPS (Luster, 1998). They play a role in a number of disorders including MS, trauma, stroke, AD, asthma, acute respiratory distress, inflammatory bowel disease, rheumatoid arthritis, type 2 diabetes, HIV, atherosclerosis, and psoriasis (Luster, 1998, Mennicken et al., 1999, Charo and Ransohoff, 2006).

IP-10

IFN-inducible protein-10 (IP-10) is a CXC chemokine also known as CXCL10. It was first identified as the product of a gene induced by IFNγ, and has since been shown to be involved in the regulation of lymphocyte recruitment as it attracts human monocytes, T lymphocytes, and NK cells (Baggiolini et al., 1997). It also has a role in the inhibition of neovascularization, tumour growth, and metastasis, the spread of a disease from one organ or part to another non-adjacent organ or part (Farber, 1997, Luster, 1998). IP-10 binds to a selective CXC receptor, CXCR3, which consists of 368 amino acids. CXCR3 is highly expressed in IL-2-activated T lymphocytes but is not detectable in resting T lymphocytes, B lymphocytes, monocytes, or granulocytes (Loetscher et al., 1996). So far CXCR3 is the only chemokine receptor that is selectively expressed in activated CD4+ and CD8+ T lymphocytes (Baggiolini et al., 1997).

IP-10 is produced by immune cells such as microglia in response to cytokines and infectious stimuli such as IFNγ, LPS, autoimmune inflammatory lesions, and some viral infections (Kawai et al., 2001, Shen et al., 2006, Ubogu et al., 2006). Infiltration of peripheral leukocytes is a key factor in the pathogenesis of MS, and IP-10 and CXCR3 play an important role in this. IP-10 is produced by astrocytes around the MS lesions and IP-10 levels are increased in the cerebrospinal fluid (CSF) of MS patients. CXCR3 is also present on lymphocytes in the CSF and it plays an important role in recruiting microglia and the subsequent 'pruning' of denervated dendrites of interneurons in a mouse lesion model (Ubogu et al., 2006). Production of IP-10 in the CNS is also correlated with the development of EAE (Fife et al., 2001). Reports also indicate that

IP-10 expression is increased in AD brains and in the brains of aged mice in a transgenic mouse model of AD (Xia et al., 2000, Duan et al., 2008), and is implicated in the development of HIV-associated dementia (van Marle et al., 2004, Cinque et al., 2005).

1.3.4 Cell Surface Molecules of glial cell activation

VCAM-1

Leukocyte trans-endothelial migration (the migration of leukocytes from the blood, across the endothelium, into inflamed tissue) is an important aspect of the body's immune response. Chemokines create a concentration gradient surrounding the inflammatory stimulus to attract leukocytes to the site of inflammation where they are first tethered to the blood vessel wall, then rolled over the endothelial lining. Firm adhesion of the leukocytes to the endothelium is mediated by integrins and cell adhesion molecules (CAMs), and is followed by extravasation; the movement of leukocytes out of the circulatory system, towards the site of tissue damage or infection (Luster, 1998, van Buul and Hordijk, 2008). Integrins are a family of 24 cell-surface receptors that form αβ heterodimers from 18α and 8β subunits. They mediate contact between cells, the extracellular matrix, and pathogens, and they participate in leukocyte co-stimulation, migration, and phagocytosis (Galkina and Ley, 2007). CAMs are transmembrane glycoproteins made up of an extracellular region containing immunoglobin-like repeats and a short cytoplasmic tail (Preiss and Sattar, 2007). Members of the CAM superfamily include VCAMs (vascular CAMs), ICAMs (intercellular CAMs), and MAdCAMs (mucosal addressin CAMs). They bind to integrins that contain an 'inserted' (I) domain in their chain, which is thought to favour the formation of a heterophilic adhesive zipper (Aricescu and Jones, 2007). CAMs are normally expressed on vascular endothelial cells at low levels, allowing very little leukocyte adhesion or recruitment. However, following an inflammatory stimulus, expression is dramatically increased and the endothelium creates "open gates" for the passage of leukocytes. Although the primary role of CAMs is in cell adhesion, they also perform a variety of other functions at cell contacts including roles in mechanical support, target recognition, and the formation of membrane microdomains and signalling platforms (Aricescu and Jones, 2007, Galkina and Ley, 2007, Preiss and Sattar, 2007, van Buul and Hordijk, 2008).

VCAM-1, or CD106, is expressed by many different cell types, including activated endothelial cells, bone marrow and spleen stromal cells, epithelial cells, lymph node cells, dendritic cells (Wu, 2007), microglia and astrocytes (Lee and Benveniste, 1999, Wu et al., 2000, Peterson et al., 2002). It contains seven immunoglobulin domains and the intracellular domains interact with moesin and ezrin to form endothelial docking structures. It binds to heterodimeric α_4 integrins with non-covalent linkage to a β_1 or β_7 chain, in particular very late antigen (VLA-4 or $\alpha_4\beta_1$ integrin) and $\alpha_4\beta_7$ integrin, which are primarily expressed on monocytes and lymphocytes (Lee and Benveniste, 1999, Galkina and Ley, 2007, Wu, 2007, van Buul and Hordijk, 2008). Binding of VCAM-1 to α_4 integrins promotes cell migration, including $CD8^+$ T cells (Wu, 2007), and activates the small GTPase, Rac1, resulting in production of reactive oxygen species (ROS), which negatively regulates endothelial cell-cell contact, increasing trans-endothelial migration (van Buul and Hordijk, 2008). VCAM-1 binding also leads to the association of the α_4 integrin cytoplasmic tail to paxillin, a signalling adaptor protein, which leads to activation of focal adhesion kinase then Src kinases, ultimately regulating integrin-dependent cell migration (Wu, 2007). Interaction between VCAM-1 and $\alpha_4\beta_1$ is critical for foetal development (Preiss and Sattar, 2007). Cytokines, such as TNFα, IL-1β, IL-4, and IFNγ, increase VCAM-1 expression (Galkina and Ley, 2007, Wu, 2007), while NO and TGFβ decrease cytokine-stimulated endothelial VCAM-1 expression (Lee and Benveniste, 1999, Preiss and Sattar, 2007). Due to the central role played by CAMs in leukocyte trafficking they are of extreme importance during disorders such as MS. For example, numerous macrophages and microglia expressing VCAM-1 were detected at the edges of MS lesions (Peterson et al., 2002), and a soluble form of VCAM-1 has been found in the serum and CSF of MS patients (Lee and Benveniste, 1999). VCAM-1 also plays a vital role in atherosclerosis (Galkina and Ley, 2007, Preiss and Sattar, 2007), cerebral ischemia (Justicia et al., 2006), traumatic brain injury (Clausen et al., 2007), and tumour immune evasion (Wu, 2007).

CD40

Another important element of the immune response is the ability of APCs, such as microglia and macrophages, to present an antigen associated with an MHC-class-II molecule to helper T (Th) cells. Binding of the antigen to the Th cells results in T cell activation followed by the secretion of cytokines such as IFN and IL-2 from Th1 cells, IL-4, IL-5 and IL-6 from Th2 cells, and IL-10 and TGFβ from regulatory T cells (Mills and McGuirk, 2004). This process requires a number of co-stimulatory molecules such as CD40.

CD40 is a 50 kDa cell surface receptor and a type I phosphoprotein member of the TNF receptor superfamily (van Kooten and Banchereau, 1997). CD40 is activated by the binding of CD40 ligand (CD40L), which is presented by T cells, resulting in a number of intracellular signalling events including the activation of various protein tyrosine kinases and transcription factors, and the phosphorylation and activation of p44/42 MAPK. The formation of a CD40 trimer is necessary in order for it to be able to bind CD40L, which is also in a trimeric form. The cytoplasmic C-terminus of the CD40 molecule does not have intrinsic enzymatic activity, so CD40 signalling is mediated by interaction with TRAFs (Chen et al., 2006). CD40 was identified on B cells, and it is now known to be expressed on a variety of cells including macrophages, microglia, dendritic cells, endothelial cells, smooth muscle cells, fibroblasts, and tumour cells (van Kooten and Banchereau, 1997, Chen et al., 2006). Although some studies have shown that CD40 is expressed in astrocytes (Abdel-Haq et al., 1999, Chen et al., 2006), others claim it is only CD40L which is expressed (Calingasan et al., 2002).

In the normal brain the microglial expression of CD40 is down-regulated but it becomes up-regulated upon microglial activation (O'Keefe et al., 2002). LPS, TNFα and IFNγ induce CD40 expression via STAT-1 and NFκB signalling mechanisms (Nguyen et al., 1998, Chen et al., 2006). This leads to the production of NO, cytokines (including IL-1, TNFα, IL-6, and IL-10), chemokines (including IP-10 and MCP-1), cell adhesion molecules (including ICAM-1 and VCAM-1), co-stimulatory molecules (including B7-1, B7-2, MHC-class-II, and CD40 itself), neurotoxins (Chen et al., 2006), and microglial activation (Town et al., 2001). During antigen presentation to Th1 cells, MHC-class-II stimulates expression of CD40 which in turn induces expression of B7 molecules. This means that CD40 can be used as a phenotypic marker for microglial activation (Wei and Jonakait, 1999, Twig et al., 2005). CD40 plays an important role in a number of neurodegenerative diseases including AD, MS, and cerebral ischemia (Town et al., 2001, Chen et al., 2006).

1.3.5 Soluble molecules

NO and iNOS

Nitric oxide (NO) is one atom of nitrogen combined with one atom of oxygen, leaving an unpaired electron making it a radical species and therefore highly reactive

(Butler et al., 1995). It is synthesised from L-arginine by an enzyme called nitric oxide synthase (NOS). NO synthesis involves sequential oxidation, requiring NADPH and oxygen (O_2) as co-substrates, and generates reactive oxygen at two steps during the process. NO synthesis usually occurs in response to increased Ca^{2+}, but it can also be induced in response to stimuli like shear stress (Stuehr, 1999). Each NOS polypeptide consists of an N-terminal oxygenase domain, and a C-terminal reductase domain, with a recognition sequence for the Ca^{2+} binding protein CaM in between. NOS activity is regulated by a number of post-translational mechanisms including inhibition by NO, levels of H_4B (a critical cofactor), covalent modifications of NOS, and interactions between NOS and other proteins (Stuehr, 1999). NOS exists in three forms with each gene being located on a different chromosome. Two of these forms are constantly expressed, and are designated based on the cell types in which they were originally discovered; neuronal NOS (nNOS or NOS-I) and endothelial NOS (eNOS or NOS-III). The third form, inducible NOS (iNOS or NOS-II), is constitutively expressed only in certain tissues like lung epithelium, but is more typically produced in response to inflammation and pro-inflammatory mediators (Stuehr, 1999). iNOS was originally identified in macrophages: Resting macrophages do not produce NO, but when they are activated by an inflammatory stimulus there is an induction of iNOS that results in the production of large quantities NO that continues until substrate depletion (Moncada, 1999).

NO has many different physiological functions, both positive and negative. It can be extremely damaging as it is involved in oxidative stress, cytotoxicity, apoptosis, necrosis, DNA damage, and blockade of mitochondrial respiration (Pannu and Singh, 2006). The cytotoxic actions of NO are likely due to the substances it produces: NO reacts with superoxide to give peroxynitrite, a powerful oxidant that further decomposes to hydroxyl radicals which are highly reactive and biologically destructive molecules (Butler et al., 1995). NO provokes apoptosis, but it also antagonizes cell death pathways (Weigert and Brune, 2008). NO can be beneficial by its roles in vasodilation, increased vascular permeability, inhibition of platelet activation (Pannu and Singh, 2006), and antimicrobial effects (Fang, 1997). The amount of NO produced, and the environment and context in which this occurs, largely determines whether it will have positive or negative actions (Pannu and Singh, 2006), and there is a rough correspondence between small and large quantities of NO and its homeostatic and toxic functions respectively (Nathan, 1997). In the brain, NO is involved in memory, the regulation of cerebral blood flow and the formation of CSF. It can also act as a retrograde messenger with a potential role in long-term potentiation (LTP), the process underlying the formation of memory (Moncada, 1999).

NO released by iNOS from macrophages is cytostatic (it inhibits cell growth and proliferation) and cytotoxic for protozoan parasites, fungal cells and bacteria (Moncada, 1999). This NO inhibits enzymes in the nucleus responsible for the synthesis of DNA, as well as enzymes in the mitochondria, particularly complex I and complex IV (Moncada, 1999, Pannu and Singh, 2006). Tumour cells express iNOS which increases during tumour development. NO generated by iNOS is involved in tumour cell killing, however it can also be associated with tumour promoting effects due to growth and angiogenesis (Weigert and Brune, 2008). Inflammation in the brain often causes astrocytes and microglia to induce iNOS, leading to excessive levels of NO that are potently toxic to neurons and oligodendrocytes (Pannu and Singh, 2006). IL-1β and TNFα production from microglia results in iNOS activity and NO production from astrocytes (Akama and Van Eldik, 2000). NO can affect gene transcription directly, either by inhibiting or activating transcription factors; for example it down-regulates expression of VCAM and ICAM-1, and can modulate the balance of Th cells by favouring Th2 responses due to up-regulation of IL-4 production (Kolb and Kolb-Bachofen, 1998).

The expression of iNOS may be beneficial in defence against pathogens, but it is also linked to a number of inflammatory diseases (Nathan, 1997). NO is a key feature of sepsis, the body's systemic response to infection, usually caused by the contamination of the blood with bacteria. Patients with septic shock display increased levels of pro-inflammatory cytokines TNFα, IL-1β, IFNγ and IL-6, leading to cytokine-dependent induction of iNOS and excessive production of NO (Titheradge, 1999). Activation of iNOS and NO generation is also an accepted marker of inflammation in many neurodegenerative conditions including stroke, AD, spinal cord injury, and MS (Pannu and Singh, 2006) as well as inflammatory conditions including rheumatoid arthritis, asthma, and Crohn's disease (Moncada, 1999).

1.3.6 Neurotrophic factors

NGF

Nerve growth factor (NGF) belongs to a family of neurotrophic factors called the neurotrophins. These are the typical neurotrophic factors and are polypeptide growth factors consisting of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5). Neurotrophins are essential for the maintenance of a healthy nervous system as they play a role in proliferation, differentiation, survival and death of neuronal and non-neuronal cells. One of the primary functions of neurotrophins is to prevent neuronal death, but they also mediate activities, such as behaviour, learning, and memory (Dechant and Neumann, 2002, Chao et al., 2006). Neurotrophins also act on neurons affected by neurodegenerative, neurological and psychiatric pathologies including AD, PD, ischemia, Huntington's disease, epilepsy, and depression (Dechant and Neumann, 2002, Chao et al., 2006).

Neurotrophins are unique in that they act through two different receptors located on the surface of axons and dendrites (Moises et al., 2007), the receptor tyrosine kinase (Trk) and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the TNF receptor superfamily (Chao et al., 2006). NGF is selective for TrkA and $p75^{NTR}$ which do not bind to each other directly but can form complexes leading to increased ligand selectivity. Signal transduction through $p75^{NTR}$ increases JNK, NFκB, and ceramide, while TrkA signalling results in long lasting MAPK and ERK activity, and activation of the PI3K/Akt pathway. TrkA activation modulates neuronal survival and differentiation and is responsible for the hyperalgesic effects of NGF (Chao et al., 2006, Nguyen et al., 2009). However, $p75^{NTR}$ activation triggers apoptosis during developmental cell death and during conditions of stress, injury, or inflammation. Interestingly, pro-NGF is more effective than mature NGF in inducing this $p75^{NTR}$-dependent apoptosis indicating pro-NGF preferentially activates $p75^{NTR}$ to mediate apoptosis and mature NGF selectively activates TrkA to promote survival. Also an imbalance in the ratio of TrkA and $p75^{NTR}$ expression may result in increased NGF apoptotic signalling through $p75^{NTR}$ (Chao et al., 2006, Saez et al., 2006).

Cytokine-stimulated astrocytes can produce NGF and promote neurogenesis (Liberto et al., 2004). For example, TNFα induces expression of NGF in astrocytes (Kuno et al., 2006), and riluzole, an anti-excitotoxic agent known to have neuroprotective effects in animal models of brain ischemia, PD, and Huntington's disease, increased NGF and GDNF in cultured mouse astrocytes (Mizuta et al., 2001). Meanwhile, corticosterone down-regulated NGF production in astrocytes and to a lesser extent in mixed cultures (Gubba et al., 2004). NGF is hugely important in neuronal differentiation, growth and survival. NGF preferentially favours the differentiation of human embryonic stem cells toward ectodermal and mesodermal lineages (Inanc et al., 2008), and is necessary for axonal growth and branching of embryonic dorsal root ganglion sensory neurons (Wickramasinghe et al., 2008). NGF, through Trk activation, provides neuroprotection against neurotoxin-exerted apoptotic death in neural stem cells (Nguyen et al., 2009), and reverses the age-dependent loss of cortical cholinergic synapses in aged rats (Cuello et al., 2007). It suppress retrograde apoptotic signals in axons and inhibits activation of the pro-apoptotic transcription factor, c-jun, in the cell bodies (Mok et al., 2009). It promotes the survival and functioning of cholinergic neurons in the basal forebrain which are important for memory processes, and has roles in pain and temperature sensation (Chao et al., 2006). A shortage of NGF leads to defects in axonal regeneration (Tosaki et al., 2008), an accumulation of ROS and eventual cell death (Lomb et al., 2009). NGF is also found to be reduced in the brains of PD patients (Olanow and Tatton, 1999). NGF gene delivery protects against the atrophy of basal forebrain cholinergic neurons that occurs with aging in the non-human primate brain. Short-term administration reverses the atrophy while long-term delivery reverses age-related cholinergic decline and restores cholinergic neuronal markers to levels of young monkeys (Nagahara et al., 2009). However other studies show that NGF can be detrimental. NGF signalling in hippocampal neurons rapidly causes neuronal apoptotic death via activation of the amyloidogenic pathway which is important in AD (Matrone et al., 2008), and there is an up-regulation of the pro-apoptotic pro-NGF in AD patients which could help explain the vulnerability of cholinergic neurons in the AD pathology (Cuello et al., 2007).

GDNF

Glial cell line-derived neurotrophic factor (GDNF) is one of the most potent neurotrophic factors. It is a glycosylated homodimer of approximately 39 kDa and is a distantly related member of the TGFβ superfamily (Lin et al., 1993, Verity et al., 1999). There are four major GDNF family receptor alpha proteins (GFRα1, 2, 3, and 4) which require the presence of TGFβ1 to activate the transport of GFRα to the cell membrane. GDNF binds specifically to GFRα1 forming homodimers which bring two molecules of Trk together, triggering trans-phosphorylation of specific tyrosine residues. This activates the MAPK and PI3K pathways which are crucial for neuronal survival and neurite outgrowth. In cells without Trk, GDNF can bind with high affinity to the neural cell adhesion molecule (NCAM). NCAM and GFRα1 form a complex which activates

Fyn and FAK (Sariola and Saarma, 2003, Lin et al., 2006, Parkash et al., 2008). Expression of Trk and GFRα1 is dependent on NGF (Luo et al., 2007b).

GDNF is released from many cell types including neurons, astrocytes, microglia, and macrophages. Its release can be modulated by many molecules including LPS, IL-1β, TNFα, and PGE-2, and following brain or nerve injury (Verity et al., 1999, Lin et al., 2006). For example, TNFα induces expression of GDNF in astrocytes (Kuno et al., 2006), and riluzole, an anti-excitotoxic agent known to have neuroprotective effects in animal models of brain ischemia, PD, and Huntington's disease, increased GDNF in cultured mouse astrocytes (Mizuta et al., 2001). GDNF is a survival factor for midbrain dopaminergic neurons and motoneurons in the CNS, and sympathetic, parasympathetic, sensory and enteric neurons in the periphery (Sariola and Saarma, 2003). *In vitro* studies showed that GDNF did not increase total neuron or astrocyte numbers, but it did promote the survival and differentiation of dopaminergic neurons. It also increased high-affinity dopamine (DA) uptake by dopaminergic neurons, but not transmitter uptake by GABAergic and serotonergic neurons (Lin et al., 1993). GDNF, plus BDNF or db-cAMP, significantly protected DA neurons suggesting that GDNF, BDNF, and cAMP produce convergent signals to activate protein kinase A (PKA) and MAPK pathways which are involved in the survival of postnatal mesencephalic DA neurons *in vitro* (Lara et al., 2003), and GDNF can help restore motor function in a rat model of PD (Yoo et al., 2003). It significantly increased motoneuron survival and co-treatment of GDNF with thrombin significantly prevented the thrombin-induced death of motoneurons (Turgeon and Houenou, 1999). GDNF is also important during ischemia. Expression of GDNF was induced in microglia following *in vitro* ischemia and this protected against ischemia-induced damage of astrocytes and neurons (Lee et al., 2004, Lu et al., 2005). *In vivo* studies have also shown that following ischemia, GDNF production is increased and is neuroprotective (Verity et al., 1999, Lin et al., 2006). Following brain ischemia, GDNF is increased at 2 hours due to neuronal up-regulation, and at 72 hours due to increased astrocyte-derived GDNF synthesis (Tokumine et al., 2003). GDNF is reduced in the brains of PD patients (Olanow and Tatton, 1999), and may be useful in the treatment of neurodegenerative diseases, such as PD, ALS, motor neuron disease (MND), and cholinergic deficit-related dementia (Lapchak, 1996).

1.4 Role of glia in inflammation

1.4.1 Microglial Activation

Microglia are the key cellular mediators of a neuroinflammatory response. They respond rapidly and in a territorially highly confined way to subtle, acute and chronic pathological stimuli. They display a complicated range of inflammatory responses, far surpassing the original view that the only function of "reactive gliosis" was structural support and phagocytosis (Streit et al., 2004). During brain development and remodelling microglia assist in the removal of unneeded cells through apoptosis and phagocytosis. In the mature brain they normally exist in their resting ramified state; however they are particularly sensitive to changes in their microenvironment and are readily activated in three ways, indirect, mixed mode and direct neurotoxins (Figure 1.2) (Liu and Hong, 2003).

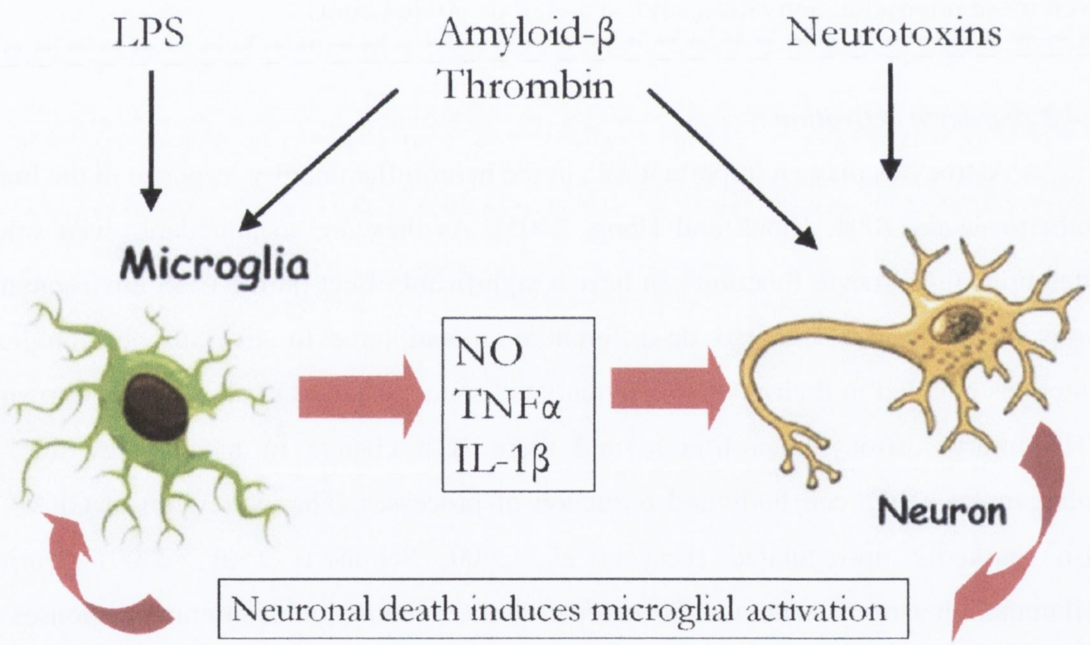


Figure 1.2: Methods of microglial activation (Liu and Hong, 2003)

Activation of microglia by indirect neurotoxins such as LPS, or mixed mode neurotoxins such as amyloid-β or thrombin, results in the release of inflammatory molecules such as NO, TNFα, and IL-1β. These inflammatory molecules can cause neuronal death which then induces microglial activation resulting in a positive feedback loop of microglial activation and neuronal death. The loop can also be activated by direct neurotoxins which induce neuronal death.

The term "activated microglia" is used to describe cells that are reproducing and showing immunophenotypic changes, but that are not yet phagocytic. Microglia only become phagocytic in response to neuronal death (Streit et al., 1988). Indirect neurotoxins have no known effect on neurons but they directly activate microglia: For example, the gram negative bacterial cell wall endotoxin LPS is widely used to induce a microglial immune response under experimental conditions. Mixed mode neurotoxins such as Aβ, an important factor in AD, directly activate microglia, but also have a direct effect on neurons. Direct neurotoxins attack and kill the neurons directly leading to neuronal death and neuronal death-induced microglial activation. Activated microglia then release a variety of potentially neurotoxic factors that further exacerbate the neuronal degeneration (Liu and Hong, 2003). Neurons have a suppressant effect on microglial reactivity and so a reduction in the ratio of neurons to microglia, due to neuronal death, reduces this modulatory effect (Zietlow et al., 1999) thus leading to even more microglial activation, and so a vicious circle begins.

1.4.2 Astrocyte activation

Astrocytes play an important role in the neuroinflammatory response in the brain (Liberto et al., 2004, Block and Hong, 2005). As they are so abundant, even small alterations in astrocyte function can have a significant effect on the CNS environment. Activated astrocytes undergo de-differentiation and have to give up physiological functions coupled to their mature differentiated state (Schubert et al., 2000). Following CNS injury, astrocytes proliferate and there is a change in morphology due to enlargement of the cell body and retraction of processes. The expression of GFAP is also markedly up-regulated (Eng et al., 2000, Schubert et al., 2000). During inflammation astrocytes help in tissue repair and the regulation of immune responses in the CNS (Farina et al., 2007). They express extracellular matrix proteins, neurotrophic factors, and membrane bound CAMs (Block and Hong, 2005). Astrocytes can form scars around the site of injury, creating a barrier which can impair microglial access to the site. This affects the function of glia-glia and glia-neuron gap-junction and intercellular signalling, halting apoptotic signals and the ability to respond to insults such as oxidative stress or infection (Kielian and Esen, 2004). Activated astrocytes have an increased capacity to clear glutamate via up-regulation of the glutamate transporter and glutamine synthase (Liberto et al., 2004). This is neuroprotective as it can prevent excitotoxicity resulting from excessive glutamate release. Cytokine-activated astrocytes generate an adaptive response post-injury to promote CNS repair as they can break down stored glycogen for glucose or lactate release, increase glutamate transporter levels to prevent excitotoxicity, increase cytosolic antioxidant proteins, promote revascularization, restore the BBB, promote re-myelination, enhance neuronal survival through the release of neurotrophic factors, and promote the formation of new synapses and neurons (Liberto et al., 2004).

1.4.3 Expression of inflammatory molecules

Giulian and colleagues (1993) did many studies investigating the factors released by glia in response to injury. They showed that astrocytes release proteins (greater than 10 kDa) that promote neuronal growth and diminish microglial toxicity. Activated microglia release small neurotoxic factors (less than 500 Da) that diminish neuronal survival but have no effect on other glial cells in culture. These neurotoxins are distinguishable from other microglial secretions such as cytokines. They are heat-stable, protease-resistant molecules with biologic activities blocked by NMDA receptor antagonists. Glia-neuron co-cultures (Giulian et al., 1994) showed the effect of soluble products from secretory microglia following stimulation by a phagocytic signal. There was an astrocyte proliferating effect, which was blocked by an IL-1 receptor antagonist, and a neurotoxic effect which was inhibited by an NMDA receptor antagonist. This implies that IL-1 promotes astrocyte growth.

Upon activation microglia undergo rapid transcriptional responses which are initiated by intracellular signalling cascades (Pawate et al., 2004). This process includes MAPK, NFκB, and JAK/STAT pathways leading to gene expression and production of pro-inflammatory cytokines. Activated microglia release many soluble factors. Some of these factors are beneficial to the survival of neurons, such as the GDNF. However, most of these factors are neurotoxic and pro-inflammatory. They include the cytokines TNFα and IL-1β, the free radicals NO and superoxide, fatty acid metabolites such as eicosanoids, and quiolinic acid (Liu and Hong, 2003). Many studies have shown that glial activation is implicated with the release of ROS which mediate many downstream events and can eventually lead to apoptosis. A study on the microglia BV-2 cells by Kang and colleagues (2001) showed that ROS functioned as signalling molecules for the activation of NFκB and the induction of IL-1β mRNA expression in response to treatment with Aβ. ROS scavengers and blockers of the ROS generating enzymes (such as NADPH oxidase, COX, and lipoxygenase) dramatically reduced the activation of

NFκB and IL-1β. Another study (Min et al., 2004) showed that plasminogen, a normal component of blood, activates microglia via stimulation of ROS production. This then induced expression of IL-1β, TNFα and iNOS mRNA. They found that ROS were generated within fifteen minutes of stimulation and that antioxidants significantly reduced IL-1β and TNFα production.

Activated astrocytes release cytokines such as IL-1β, TNF, IL-6, IFNβ, TGFβ, and granulocyte-macrophage colony-stimulating factor (GM-CSF), numerous chemokines including IP-10, and neurotrophic factors such as NGF and BDNF (Farina et al., 2007). The release of inflammatory molecules results in many pro-inflammatory effects including the activation of astrocytes, and the activation, differentiation and proliferation of microglia, as well as increased BBB permeability, and the recruitment of peripheral immune cells such as monocytes, macrophages, dendritic cells, neutrophils, and T and B lymphocytes. However the release of neurotrophic factors results in some pro-survival effects including the proliferation and survival of astrocytes, the regulation of microglial activity, the release of neuroprotective mediators, and the survival, differentiation, and regeneration of neurons (Farina et al., 2007). This balance between pro- and anti-inflammatory effects is fundamental to the control of inflammation.

Active microglia, like other macrophages, are able to present antigens to T cells using MHC-class-II. In their resting state there is a very low level of expression of B7-2 and CD40 and no surface expression of B7-1. Microglia become professional APCs only after activation through cytokines and signalling from a relevant T cell by B7-CD28 and CD40-CD40L interactions (Matyszak et al., 1999). The expression of MHC-class-II, along with the associated CD40 and B7 molecules, is up-regulated during an inflammatory response (O'Keefe et al., 2002). Treatment of microglia with LPS and IFN-γ causes up-regulation of B7-1 molecules on the cell surface (Becher and Antel, 1996). The expression of these cell surface receptors is under the control of the anti-inflammatory factors IL-10, IL-4, TGF, and NGF. IL-10 inhibits B7-2 expression while IL-4, TGFβ1, and NGF repress the induced expression of B7-2 and CD40 (Wei and Jonakait, 1999). Astrocytes also express MHC-class-II, CD40, and B7 and are able to activate T cells. Astrocyte CD40 is up-regulated by inflammatory cytokines such as TNFα, IL-1β, and IFNγ, and by inflammatory stimuli such as LPS. Moreover, activation of astrocyte CD40 by CD40L has been demonstrated to result in pro-inflammatory cytokine release by these cells *in vitro* (Abdel-Haq et al., 1999, Dong and Benveniste, 2001).

1.4.4 Role of glia in disease

Many studies have shown that there is a role for activated glia in a number of neurodegenerative diseases. It is likely that the activation of microglia, as a consequence of neuronal injury, represents a risk factor that may be the start of a cascade of events leading to chronic inflammation and the progression of neurological degeneration. AD and PD are both age-related neurodegenerative disorders in which microglial activation plays an important role in the pathogenic, and possibly even the initiation, stages (Liu and Hong, 2003). Reactive microglia were found to exist with the plaques in the cortical region of AD brains, and in large numbers in the substantia nigra of PD brains, the region where most of the degeneration of dopaminergic neurons occurs. Banati and colleagues (1994) recognised that the study of microglial cell biology was fundamental to understanding the brain's basic tissue reactions and cellular mechanisms in response to CNS disease. They demonstrated that the prime function of microglia is to protect the integrity of the CNS but they admitted that microglial cytotoxicity had the potential to cause neuronal damage, due to the release of proteinases, cytokines, reactive oxygen intermediates, and reactive nitrogen intermediates. They proposed that interference with microglial activation or production of cytokines by microglia might offer "new therapeutic opportunities for the prevention of neuronal cell death in CNS disease" (Banati et al., 1993). Detection of microglial activation provides diagnostically useful parameters of disease, such as accurate spatial localisation, disease progression, and any secondary neurodegenerative or adaptive changes away from the primary site of disease (Banati, 2003).

Two particularly important activators of glial cells in relation to disease are Aβ, a key factor in AD, and thrombin, which is important in stroke and PD. Both these molecules directly activate microglia, but also have a direct effect on neurons (Figure 1.2) (Liu and Hong, 2003, Stuchbury and Munch, 2005, Luo et al., 2007a, Sokolova and Reiser, 2008).

1.5 Amyloid β (Aβ)

Aβ is formed by the cleavage of amyloid precursor protein (APP), a transmembrane protein with a large extracellular N-terminal domain and a smaller intracellular tail (Blennow, 2004). The Aβ domain is partly embedded in the plasma membrane (Figure 1.3a). Normally APP is cleaved by α-secretase releasing α-secretase-cleaved soluble APP (α-sAPP) and an 83 amino acid C-terminal fragment (CTF) of APP (C83). Next C83 is cleaved by the γ-secretase complex releasing a shorter peptide called p3. APP cleavage within the Aβ domain prevents formation of free Aβ (Figure 1.3b). In the amyloidogenic pathway, APP is cleaved by β-secretase, releasing β-secretase-cleaved soluble APP (β-sAPP) and the 99 amino acid CTF of APP (C99). The C99 is cleaved by the γ-secretase complex releasing free Aβ (Figure 1.3c). Aβ is important as it forms the senile plaques in the brains of patients with AD (Blennow, 2004). Hence a vast quantity of the study on AD has focused on the role of Aβ.

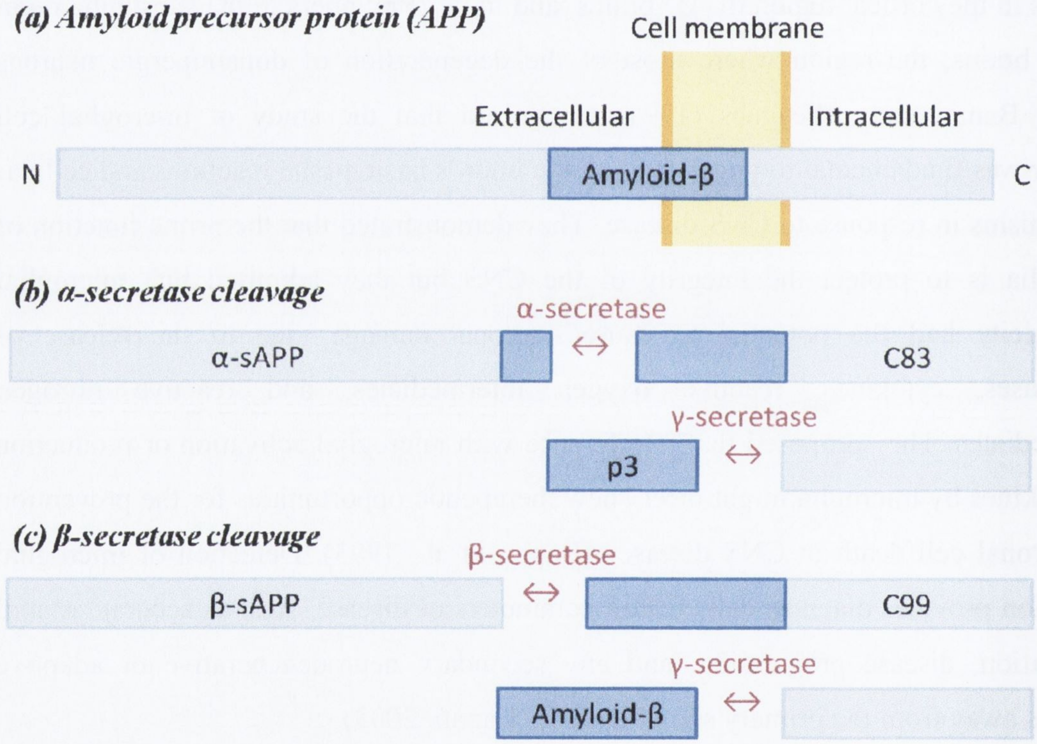


Figure 1.3: Schematic drawing of the generation of Aβ from APP (Blennow, 2004)
Red writing denotes enzymes. (a) APP is a transmembrane protein that contains a large extracellular N-terminal domain, a smaller intracellular C-terminal domain, and an Aβ domain that is partly embedded in the cell membrane. (b) Cleavage of APP by α-secretase releases α-sAPP and an 83 amino acid CTF called C83. When C83 is cleaved by the γ-secretase complex it releases a shorter peptide called p3. (c) Cleavage of APP by β-secretase releases β-sAPP and a 99 amino acid CTF called C99. When C99 is cleaved by the γ-secretase complex it releases free Aβ.

1.5.1 Aβ and Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease and the most common cause of dementia (Ott et al., 1995). Dementia is an acquired cognitive impairment sufficient to cause functional impairment and interfere with activities of daily living. AD is characterised clinically by progressive intellectual deterioration together with declining activities of daily living and eventually neuropsychiatric symptoms or behavioural changes. The most striking early symptom is memory loss which becomes steadily worse as the illness progresses. Other cognitive areas are also impaired such as language (aphasia), coordinated movement (apraxia), recognition (agnosia), and those functions closely related to the frontal lobe of the brain such as decision-making and planning. As the disease progresses behavioural changes also occur and patients can become aggressive or paranoid, suffer from hallucinations or delusions, and engage in inappropriate behaviours such as shouting and wandering.

The pathophysiology of AD is characterized by degeneration of the neurons and their synapses, and the presence of extensive amounts of senile plaques and neurofibrillary tangles (NFTs) (Burns et al., 1997). NFTs are caused by tau proteins, which belong to the microtubule-associated proteins (MAP) family. They are mainly expressed in neurons where their most important role is in axonal transport via the microtubules network. However during AD, abnormal phosphorylation on some of the 79 putative serine/threonine phosphorylation sites leads to tau aggregation and the formation of NFTs, resulting in loss of axonal transport and subsequent loss of proper neuronal function (Braak and Braak, 1991, Buee et al., 2000, Blennow, 2004). Another important factor is apolipoprotein E (ApoE), a heterogeneous protein which plays a critical role in lipid metabolism. ApoE4 is a risk factor for AD and has been shown to bind to senile plaques and NFTs (Buee et al., 1996). It is thought that ApoE may play a secondary role in NFT formation or that it accumulates within the neurons in response to neuronal damage (Benzing and Mufson, 1995). ApoE4 is also toxic to neurons (Jordan et al., 1998), and can contribute to the formation of lesions (Poirier et al., 1995). ApoE is capable of modulating glial activation both *in vitro* and *in vivo*, as measured by an increase in TNFα and IL-1β expression, following LPS stimulation (Lynch et al., 2001).

The predominant feature of AD is the formation of senile plaques, also known as neuritic plaques, which are formed from Aβ (Blennow, 2004). Aβ occurs in two forms, $A\beta_{1-40}$ and $A\beta_{1-42}$, containing 40 and 42 amino acids respectively. $A\beta_{1-42}$ aggregates more rapidly than $A\beta_{1-40}$, is the initial form of Aβ deposited in diffuse plaques, and is the prominent form of Aβ in senile plaques (Blennow, 2004). Aβ can exist in a fibrillar form which initiates a more potent inflammatory response than that from non-fibrillar forms of Aβ. Both *in vitro* and *in vivo* studies have shown that $A\beta_{1-42}$ exists in a β-sheet conformation, which makes it more fibrillogenic and therefore more cytotoxic than other forms of Aβ (Stuchbury and Munch, 2005). One study however found that $A\beta_{1-42}$, unlike $A\beta_{1-40}$, did not become fibrillar *in vivo*, but required incubation *in vitro* prior to injection in order to maintain a fibrillar structure (Shin et al., 1997). For these reasons most of the research into Aβ has focused on the $A\beta_{1-42}$ form. In particular it is proving to be very useful as a CSF biomarker. Blennow and colleagues (2004) found that in AD there was a decrease in CSF-$A\beta_{1-42}$ to about 40-50% of control levels. The hypothesis for this is that deposition of $A\beta_{1-42}$ in plaques led to lower levels diffusing to the CSF. They also showed a decrease in the ratio of $A\beta_{1-42}/A\beta_{1-40}$ in CSF. Preliminary data suggests that this ratio may be of particular importance and use in the early diagnosis of AD.

Inflammation has long been recognised as being pivotal to the progression of AD, and many papers and reviews have been published on neuroinflammation and how it contributes to neurodegeneration (Moore and O'Banion, 2002, Stuchbury and Munch, 2005). Studies of AD brains have identified the presence of activated glial cells, and many inflammatory proteins, including pro-inflammatory cytokines, complement factors, and acute-phase proteins, which all play a role in Aβ-mediated neurotoxicity (Stuchbury and Munch, 2005).

1.5.2 Aβ and Inflammation

Activated glial cells play an important role in AD and Aβ-mediated neurotoxicity. Microglia are found to localise in clusters around senile plaques in the cortical region of AD brains (Liu and Hong, 2003, McGeer and McGeer, 2003, Stuchbury and Munch, 2005), and are thought to mediate plaque formation as they can assist in the conversion of non-fibrillar Aβ to fibrillar Aβ (Moore and O'Banion, 2002). Many studies have shown that Aβ is capable of activating microglia in a dose and time-dependent manner. Glial activation results in a morphological response and the expression of pro-inflammatory cytokines, which in turn enhance Aβ-induced glial activation, resulting in a continuous inflammatory cycle (Moore and O'Banion, 2002). Microglia in AD are in a chronic state of activation, which involves the transformation of the microglia into phagocytic cells that are capable of not only ingesting particles, but releasing ROS, proteases, and several cytokines (Stuchbury and Munch, 2005). Microglia localised around plaques have a substantially increased expression of many inflammatory markers, including MHC-class-I and -II, the pro-inflammatory cytokines IL-1 and TNFα, and receptors for cytokines and other inflammatory agents (Halliday et al., 2000).

Astrocytes also play an important role in the process of neurodegeneration. Astrocytes are found to localise around senile plaques (Stuchbury and Munch, 2005), and reactive astrogliosis is frequently observed in the lesioned areas of AD brains (Liu and Hong, 2003, McGeer and McGeer, 2003). Isolated astrocytes from the brains of AD patients show increased expression of nNOS, indicating the production of NO (Stuchbury and Munch, 2005). *In vitro* studies have shown that astrocytes can create a barrier around plaques by deposition of proteoglycans, and this is backed up by investigation of post-mortem AD brains which showed localisation of proteoglycans to senile plaques (Moore and O'Banion, 2002).

Some studies showed that Aβ alone is able to activate microglia while others state that co-stimulatory molecules have to combine with Aβ in order for it to have a pro-inflammatory effect (Stuchbury and Munch, 2005). For example it was reported that when the THP-1 human monocyte cell line was treated with Aβ alone it resulted in a small increase in IL-1β and IL-6, but when treated with Aβ and the chemokine-like mediator C5a this increase in IL-1β and IL-6 was dramatically augmented (O'Barr and Cooper, 2000). Aβ acts in combination with CD40L to promote pro-inflammatory responses in microglia and endothelial cells, including secretion of IL-1β and TNFα (Town et al., 2001). Aβ also disrupts Muller glial cell function in the retina (Walsh et al., 2002). Importantly, human microglial cell lines and microglia extracted from post-mortem AD patients are sensitive to Aβ, and can be activated in the absence of co-stimulatory molecules (Stuchbury and Munch, 2005). An interesting study (von Bernhardi and Eugenin, 2004) examined the different responses of mixed glial cultures, microglia cultures, and astrocyte cultures to Aβ stimulation. Aβ (0.1, 1 and 5 μM) decreased cell viability (as measured using an MTT assay) in microglia and mixed glial cultures in a dose dependent manner, while only the highest dose decreased cell viability in astrocyte cultures.

Aβ-induced activation of microglia results in an increase in several markers of inflammation and oxidative stress. Aβ increased iNOS in microglia and in mixed glial cultures, and increased NO (over 1 to 4 days) in microglia but not in mixed glial cultures (von Bernhardi and Eugenin, 2004). However another study stated that Aβ did not increase NO in microglia, but did increase superoxide anion production (Moore and O'Banion, 2002). Exposure of microglia cultures and mixed glial-neuronal cultures to Aβ results in a dose-dependent increase in the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα (Rogers et al., 2002, McGeer and McGeer, 2003). IL-1β, IL-6, and TNFα are also elevated in AD brains (McGeer and McGeer, 2003). IL-1β is expressed in microglia associated with plaques. It influences synthesis and processing of APP leading to greater Aβ production and is thought to be a mediator of plaque and NFT formation (Moore and O'Banion, 2002). Other studies showed that Aβ induced expression of IL-1β from a THP-1 human monocyte cell line (O'Barr and Cooper, 2000), and from a microglial cell line, and this was modulated by ROS (Liu and Hong, 2003). IL-1β from microglia treated with Aβ mediates iNOS expression in reactive astrocytes (Moore and O'Banion, 2002). TNFα is also expressed in microglia associated with plaques and is essential for Aβ-induced neurotoxicity (Moore and O'Banion, 2002). Aβ-stimulated primary microglia produced the inflammatory cytokines IL-1α, IL-1β, TNFα, and IL-6, and the chemokine MCP-1 (Szczepanik et al., 2001). Another study showed that in astrocyte cultures treated with Aβ, the production of IL-1β and TNFα occurred before the production of iNOS (Akama and Van Eldik, 2000). Aβ induced IFNγ production from co-cultures of astrocytes and microglia (Yamamoto et al., 2007). Exposure of cultured microglia or monocytes to Aβ results in a dose-dependent increase in their secretion of the chemokines IL-8, MIP-1α, and MCP-1, and the growth factor macrophage-colony stimulating factor (M-CSF) (Rogers et al., 2002, McGeer and McGeer, 2003). The chemokine receptors CXCR2, CCR3 and CCR5, and the cell adhesion molecule ICAM-1 are also up-regulated in AD (McGeer and McGeer, 2003). Anti-inflammatory cytokines may also play role as TGFβ1 has been detected in microglia, plaques, serum, and CSF of AD patients (Moore and O'Banion, 2002, Rogers et al., 2002, McGeer and McGeer, 2003).

Another function of microglia is their ability to phagocytose and internally degrade Aβ deposits (Moore and O'Banion, 2002, Rogers et al., 2002). Astrocytes and microglia in the brains of non-demented subjects with cerebral Aβ deposition contained either Aβ-positive granules or Aβ aggregate in the cytoplasm (Yamaguchi, 2005). This may indicate the uptake of extracellular Aβ deposits. These findings mean that there is an active balance between plaque formation and clearance, and plaques may disappear when clearance is greater than formation. However there is also contradictory evidence which suggests that microglia not only decrease Aβ deposition, but in some cases may actually increase it (Stuchbury and Munch, 2005). Another study showed that microglial phagocytosis of Aβ is increased by intravenous immunoglobulin, a purified immunoglobulin fraction manufactured from the blood of healthy humans (Istrin et al., 2006). It has also been shown that astrocytes can degrade Aβ deposits *in situ* (Farina et al., 2007). This dynamic balance of cerebral Aβ deposition makes the temporal profile of the disease more complicated.

1.5.3 Aβ and Neurotoxicity

Aβ has been shown to be directly toxic to neurons. A single injection of Aβ into the retina was sufficient to induce chronic DNA fragmentation and neuronal loss, without direct contact being needed between the neurons and Aβ aggregates (Walsh et al., 2002). Neuronal apoptosis has been shown to result in Aβ accumulation through a caspase-3 mediated mechanism, which is involved in apoptotic cell death, thus perpetuating the cycle of neurodegeneration (Moore and O'Banion, 2002). There is also an 'amyloid cascade hypothesis' which suggests that an $A\beta_{1\text{-}42}$ seed is deposited and initiates the formation of diffuse plaques. These are initially fluid and non-fibrillar, but over time they compact, and become fibrillar and therefore neurotoxic (Halliday et al., 2000, Stuchbury and Munch, 2005). Neurotoxicity is dependent on glial activation and the resulting induction of iNOS and inflammatory cytokines (Akama and Van Eldik, 2000). The potency of Aβ-induced neurotoxicity on cultured neurons was increased dramatically in the presence of microglia, with this increase being primarily attributed to microglial activation (Liu and Hong, 2003). Conditioned media (CM) from microglial cultures which had been treated with Aβ caused apoptosis in cortical neurons (Combs et al., 1999, Combs et al., 2001). This neuronal cell death was prevented by pre-incubating the microglia with inhibitors of iNOS or antibodies against TNFα, but not IL-1β (Combs et al., 2001). Another study showed that CM from microglia cultures which had been treated with Aβ caused apoptosis in hippocampal neurons, while CM from mixed glial cultures which had also been treated with Aβ did not cause apoptosis, indicating that astrocytes could play a role in preventing apoptosis. Interestingly, when CM from astrocyte cultures which had been stimulated with LPS and IFNγ, was added to microglia cultures which were then treated with Aβ, and the resulting microglia CM was added to hippocampal cultures, it did not induce apoptosis, showing that astrocytes can mediate their effect even when cell contact is not present (von Bernhardi and Eugenin, 2004). This shows that astrocytes can modulate the inflammatory response, possibly having a protective role in the prevention of microglial-induced neuronal apoptosis.

The immune response in AD can be both beneficial and damaging (Moore and O'Banion, 2002, Rogers et al., 2002). Initially it is beneficial: reactive microglia degrade and phagocytose Aβ and eliminate cell debris, while reactive astrocytes isolate neurons from senile plaques and release cytokines and growth factors that may aid neuronal survival and recovery. However the immune response in AD is chronic, leading to prolonged glial activation, release of neurotoxins, and thus further neurodegeneration (Halliday et al., 2000).

1.5.4 Aβ signalling

Aβ activates tyrosine kinase based intracellular signalling cascade in microglia (Combs et al., 1999, Combs et al., 2001). It is likely that Aβ-induced inflammation is mediated by the activation of NFκB, a transcription factor which induces numerous genes including cytokines, immune-receptors and acute phase proteins. Consistent with this hypothesis, anti-sense NFκB oligonucleotides inhibited the Aβ-induced increase in IL-1β and IL-6 mRNA (Moore and O'Banion, 2002). THP-1 human monocyte cell line treated with Aβ and the chemokine-like mediator C5a activates NFκB, resulting in an increase in IL-1β and IL-6 (O'Barr and Cooper, 2000). Aβ also has chemokine like activity at formyl chemotactic receptors, which again results in NFκB activation (Moore and O'Banion, 2002). COX-2, an enzyme which produces prostaglandins such as prostaglandin-E2 (PGE-2, a potent pro-inflammatory agent), is also important in AD (Halliday et al., 2000, Moore and O'Banion, 2002, Stuchbury and Munch, 2005), and microglia treated with Aβ stimulate COX-2 pathways (Moore and O'Banion, 2002).

Aβ can also directly activate both the classical and alternative complement pathway (Rogers et al., 2002). There is evidence of activation of the complement cascade in AD where complement protein C1q is associated with plaques, and neurons have been shown to express the final membrane-attack complex (MAC) (Moore and O'Banion, 2002). Aβ has a high affinity for C1q protein, and aggregated complexes containing this segment bind strongly to the microglial C1q receptor making it a mechanism for activation of microglia (Figure 1.4) (Halliday et al., 2000).

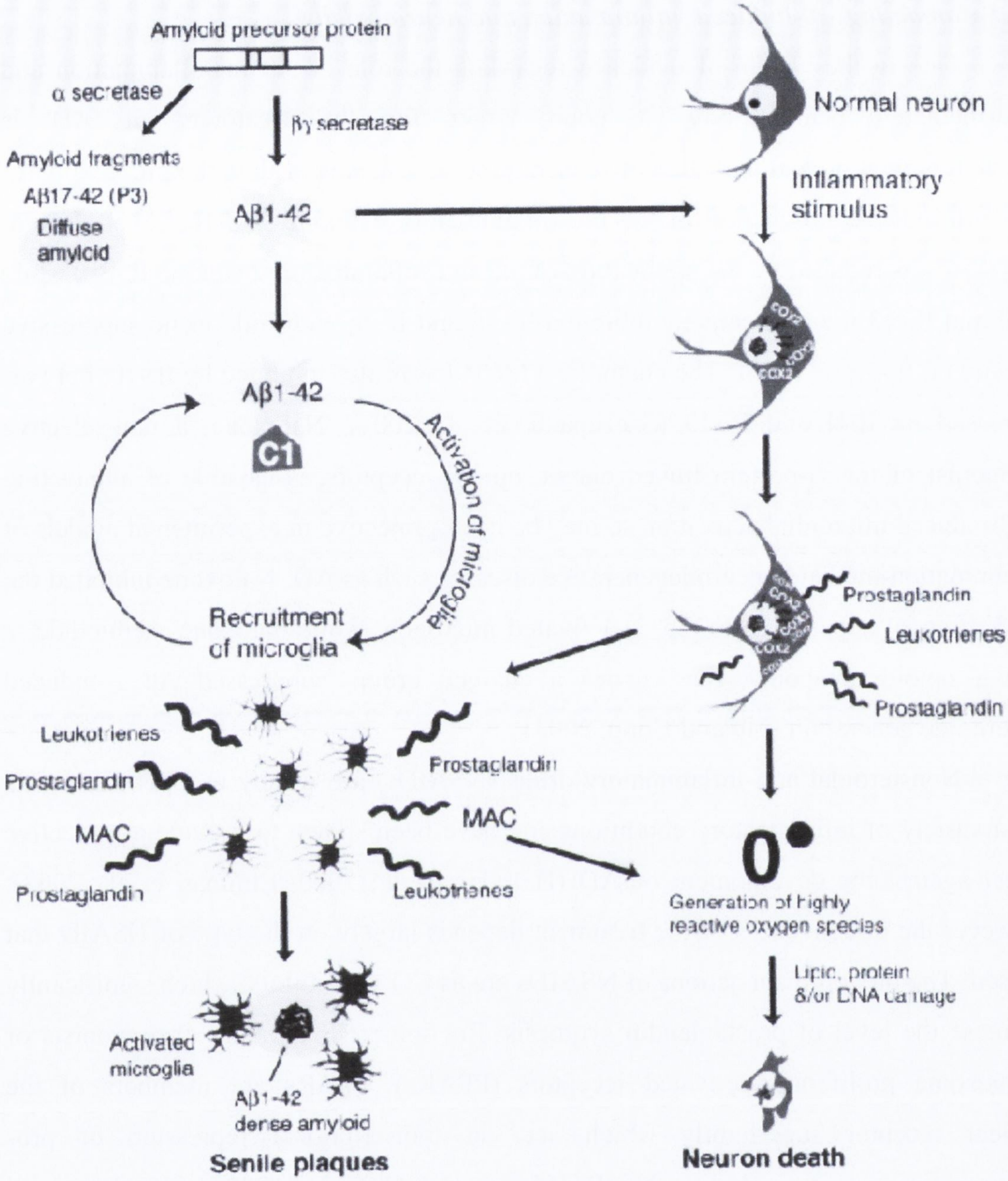


Figure 1.4: Aβ in inflammation and neurodegeneration (Halliday et al., 2000)

Aβ fragments are released into the extracellular space of the brain via the cleavage of APP by secretases. The fibrillogenic form $A\beta_{1-42}$ has a high affinity for the C1q protein and this complex activates microglia through their C1q receptor. Activated microglia produce inflammatory mediators to recruit more microglia and they release agents such as membrane attack complexes (MAC), prostaglandins, and leukotrienes, leading to the generation of free radicals that contribute to the neurodegeneration of neurons and to Aβ deposition and plaque formation.

1.5.5 Combating Aβ-induced inflammation and neurotoxicity

Due to the wealth of evidence regarding the role of Aβ in inflammation and glial-mediated neurotoxicity, a good target for the treatment of AD is neuroinflammation and the value of reducing this as a means to slow the progression of AD. Aβ-induced production of the pro-inflammatory cytokines, IL-1α, IL-1β, TNFα, and IL-6 was reduced by the application of the anti-inflammatory cytokine IL-10, while IL-4 and IL-13 more selectively inhibited IL-1β and IL-6, with little or no suppressive activity on IL-1α or TNFα. The chemokine MCP-1 was also inhibited by IL-10, but was increased by IL-4 and IL-13 (Szczepanik et al., 2001). Naloxone, a non-selective antagonist of the G-protein linked classic opioid receptors, is capable of attenuating LPS-induced microglial activation so may be neuroprotective in experimental models of inflammation-mediated neurodegenerative diseases such as AD. Naloxone inhibited the production of superoxide in $A\beta_{1-42}$-activated microglia, while naloxone methiodide, a partial opioid antagonist that carries a charged group, suppressed $A\beta_{1-42}$-induced superoxide generation (Liu and Hong, 2003).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as treatments for a variety of inflammatory conditions and have been shown to provide a protective effect against the development of AD (Halliday et al., 2000, Etminan et al., 2003). However the effectiveness of the treatment depends largely on the type of NSAID that is used. The predominant actions of NSAIDs are as COX inhibitors, which significantly decrease the level of prostaglandin synthesis. But some NSAIDs are also agonists of peroxisome proliferator-activated receptors (PPARs). PPARs are members of the nuclear receptor superfamily which act via transcriptional repression of pro-inflammatory genes, including NFκB (Moore and O'Banion, 2002, Stuchbury and Munch, 2005). COX-2 selective NSAIDs such as celecoxib and rofecoxib do not have a significant therapeutic benefit for AD patients, however, some NSAIDs such as ibuprofen, indomethacin and sulindac sulfide, which bind to PPARs, have demonstrated efficacy in AD. This indicates that the neuroprotective effects of NSAIDs may be the result of mechanisms other than the inhibition of COX (Stuchbury and Munch, 2005).

While these drugs are making progress, there is still a long way to go before a fully effective treatment for combating Aβ-induced inflammation and neurotoxicity is available.

1.6 Thrombin

Thrombin is a member of the serine protease family, generated from the proenzyme prothrombin, which is synthesised in the liver and then secreted into the bloodstream (Fenton, 1986). It plays important multifunctional roles in wound healing and blood coagulation. In particular it is crucial for the final stages of the coagulation cascade during haemostasis when it is responsible for the conversion of plasma fibrinogen into fibrin and the development of an insoluble fibrin clot. It plays a central role in determining the stability of the fibrin clot by maintaining a balance between coagulation and fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI) which protects the fibrin clot against lysis (Bouma and Mosnier, 2006). Thrombin is not solely restricted to the periphery: Prothrombin mRNA has been detected in the brain, indicating that thrombin can be produced there (Dihanich et al., 1991). Thrombin and its receptors (protease activated receptors, PARs) have also recently been identified as having an important role in a number neurodegenerative disorders including stroke, PD, AD, and MS, and can either promote neurodegeneration or neuroprotection making them attractive targets for therapeutic intervention (Luo et al., 2007a, Sokolova and Reiser, 2008).

1.6.1 Thrombin and Stroke

Stroke is a heterogeneous disease with more than 150 known causes. There are a number of different types of stroke, the main ones being ischemic and haemorrhagic stroke, but also subarachnoid haemorrhage, cerebral venous thrombosis, and spinal cord stroke (Amarenco et al., 2009). Ischemic stroke results from a shortage of the blood supply to the brain, while haemorrhagic stroke (intracerebral haemorrhage (ICH)), results from the leakage or rupture of a blood vessel in the brain. In the United States, ischemic stroke accounts for 87% of all strokes, with the remaining 13% of strokes being hemorrhagic in intracerebral or subarachnoid locations. Neuroimaging is required to reliably differentiate between ischemic stroke and ICH, as well as to diagnose entities other than stroke (Yew and Cheng, 2009). The characteristic symptom of stroke is experiencing or awakening with the abrupt onset of important neurologic deficits, with the most common presenting symptoms being difficulty with speech or weakness on one half of the body (Yew and Cheng, 2009). ICH often causes immediate death, but even if the patient survives the initial bleed, the resulting blood in the brain can lead to secondary brain injury and sometimes delayed fatality (Hua et al., 2007).

Inflammation is hugely important in aggravating brain injury following ICH. The BBB is disrupted, although it remains intact to large molecules for the first few hours, and the brain undergoes an inflammatory response which is immediately initiated but does not peak until several days later (Xi et al., 2006). Cytokines play an important role, in particular TNFα and IL-1β which usually exert neurotoxicity. However in the absence of iNOS, these cytokines may contribute to neuroprotection and plasticity (Stoll et al., 2000). Activation of the complement cascade is also seen (Xi et al., 2006). Apoptosis and necrosis both occur in ICH, as demonstrated by an increase in concentrations of NFκB protein and DNA fragmentation (Xi et al., 2006). It is thought that some of the injury in ICH may be due to products in the blood, in particular thrombin. Following ICH, a substantial amount of thrombin is released into the brain parenchyma due to the high concentration of prothrombin in the plasma. This thrombin is responsible for early cerebral oedema formation and acute brain injury (Hua et al., 2007, Hua et al., 2009).

1.6.2 Thrombin and Parkinson's disease (PD)

Parkinson's disease (PD) is a progressive neurological disorder characterised by a large number of motor and non-motor impairments. The four key features of PD can be described under the acronym TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability. Tremors are unilateral and usually involve the hands, lips, chin, jaw and legs. Rigidity is identified by increased resistance which is usually accompanied by the "cogwheel" phenomenon. Bradykinesia is a hallmark of basal ganglia disorders and refers to slowness of movement including difficulties with planning, initiating and executing movement, and with performing sequential and simultaneous tasks. Postural instability is due to loss of postural reflexes. Other motor symptoms include flexed posture, freezing, and shuffling gait, while non-motor symptoms include autonomic or cognitive dysfunction, sleep disorders, sensory abnormalities, and neuropsychiatric symptoms such as depression, apathy, anxiety, and hallucinations (Jankovic, 2008). The pathophysiology of PD is characterised by a loss of DA due to degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). This disrupts the dopaminergic control of movement coordination in the basal ganglia and gives rise to the movement problems. Another key feature of PD is the presence of Lewy bodies. These are aggregates of the α-synuclein protein which form inside neurons and disrupt cell function. Neurodegeneration and Lewy bodies can also be found in the locus coeruleus, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the autonomic nervous system. Other key features contributing to cell death in PD are oxidative stress and the release of hydrogen peroxide (H_2O_2) and other ROS, defects in complex I activity of the mitochondrial respiratory chain leading to decreased adenosine triphosphate (ATP) synthesis, and excitotoxicity (Olanow and Tatton, 1999). Inflammation, glial activation, and release of cytokines are also very important in the progression of PD (Olanow and Tatton, 1999, Liu and Hong, 2003, Tansey et al., 2007).

Thrombin has often been associated with PD. Expression of PAR-1 was shown to be increased in astrocytes in the SNpc, and immunoreactivity for thrombin and prothrombin was stronger in astrocytes and the vessel walls in SNpc of PD brains (Ishida et al., 2006). A mouse model of PD showed that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration increased levels of two activators of PAR-1 (Hamill et al., 2007). *In vivo* studies have shown thrombin and PAR-1 activation to be both beneficial (Cannon et al., 2005, Cannon et al., 2006) and detrimental (Hamill et al., 2007).

1.6.3 Thrombin and inflammation

Inflammation and glial activation are hugely important in aggravating brain injury following ICH (Xi et al., 2006) and in PD (Liu and Hong, 2003). Thrombin results in activation of microglia both *in vivo* (Choi et al., 2003, Choi et al., 2005, Lee da et al., 2006, Fujimoto et al., 2007, Huang et al., 2008a, Huang et al., 2008b, Ohnishi et al., 2009) and *in vitro* (Lee et al., 2005, Katsuki et al., 2006), and microglial proliferation (Moller et al., 2000). Microglial activation appears to occur in a PAR-1 independent manner as it is not blocked by hirudin, a thrombin specific inhibitor (Ryu et al., 2000, Lee et al., 2005, Lee da et al., 2006). Thrombin also activates human astrocytes, induces morphologic changes and increases proliferation (Ishida et al., 2006). Astrocytes treated with thrombin showed cell proliferation and reversal of stellation, with cell proliferation but not reversal of stellation being dependent on thrombin receptor-activated tyrosine kinase activity (Grabham and Cunningham, 1995, Suidan et al., 1996). Conversely, in an MPTP model of PD, the MPTP-induced microglial activation was reduced in mice treated with a PAR-1 antagonist and in PAR-1 knockout mice (Hamill et al., 2007).

Thrombin promotes an inflammatory environment by increasing expression of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 (Moller et al., 2000, Choi et al., 2003, Choi et al., 2005, Lee et al., 2005, Huang et al., 2008a) and inflammatory activators such as COX-2 (Lee da et al., 2006) which instigate and contribute to cell death. TNFα levels in the brain are increased after ICH and after intracerebral infusion of thrombin, and TNFα KO mice experienced less ICH-induced brain oedema than wild-type mice (Hua et al., 2007). It also significantly increases the secretion of the chemokines cytokine-induced neutrophil chemoattractant-1 (CINC-1) and CINC-3, by PAR activation (Wang et al., 2007c). Thrombin causes oxidative stress, as evident by an increase in ROS production and protein oxidation, which is mediated by activation of NADPH oxidase, and blocked by an NADPH oxidase inhibitor (Choi et al., 2005, Park and Jin, 2008). It also induces NO or iNOS expression both *in vivo* (Choi et al., 2003, Choi et al., 2005, Huang et al., 2008a, Huang et al., 2008b), and *in vitro* (Moller et al., 2000, Ryu et al., 2000, Lee et al., 2005). Nitrite levels in co-cultures of microglia and neurons treated with thrombin are approximately 10-fold higher than in untreated co-cultures and 20-fold higher than in microglia-deficient, neuron-enriched cultures treated with thrombin (Lee da et al., 2006). Thrombin induced transient intracellular Ca^{2+} signals in neurons, mediated via the endoplasmic reticulum, and improved the recovery of neuronal Ca^{2+} homeostasis after glutamate treatment (Kiseleva et al., 2004, Gorbacheva et al., 2006). It also induced transient intracellular Ca^{2+} signals in cultured microglia, which were blocked by hirudin, an inhibitor of the proteolytic activity of thrombin, demonstrating that these signals depend on enzymatic activity and not on other properties of thrombin (Moller et al., 2000).

Thrombin can also effect the functioning of the BBB. It activates matrix metalloproteinase-2 (MMP-2) in endothelial cells. MMPs are zinc-dependent proteases that can degrade extracellular matrix and cause BBB disruption (Hua et al., 2007). One study showed that thrombin induced endothelial cell contraction, opened intercellular tight junctions, increased MMP-2 expression, and triggered basal membrane degradation. These effects occurred due to the activation of PAR-1, possibly leading to the opening of the BBB, and increased BBB permeability may be one of the mechanisms behind thrombin-induced cerebral oedema (Guan et al., 2004).

1.6.4 Thrombin and neurotoxicity

Thrombin treatment causes whole tissue injury in mid-brain slice cultures (Katsuki et al., 2006), and results in loss of neurons in the cortex and the striatum (Lee da et al., 2006, Fujimoto et al., 2007, Herrera et al., 2008, Ohnishi et al., 2009), loss of dopaminergic neurons in the substantia nigra (Choi et al., 2003, Herrera et al., 2008, Huang et al., 2008b), and loss of hippocampal neurons (Choi et al., 2005). Thrombin also mediates neuronal injury in human ICH as treatment with the thrombin inhibitor, argatroban, results in a smaller perihaematomal brain oedema. This is caused by PAR-1 activation as injection of the PAR-1 antagonist, BMS-200261, or PAR-1 KO mice reduces infarct volume in transient focal cerebral ischemia in mice (Hua et al., 2007). PAR-1 KO mice in an MPTP model of PD showed greatly reduced loss of DA, DA transporter, and tyrosine hydroxylase in the striatum, leading to less striatal damage (Hamill et al., 2007).

The neurodegeneration caused by thrombin is a result of apoptosis which is mediated by its receptors, mainly PAR-1 (Luo et al., 2007a). A wide range of *in vitro* studies have shown that thrombin induces neuronal apoptosis and results in neuronal cell death (Rao et al., 2007, Park and Jin, 2008, Sanchez et al., 2009, Thirumangalakudi et al., 2009), especially when given in conjunction with MMP-3 and MMP-9 (Xue et al., 2009). Treatment of co-cultures of neurons and microglia with thrombin also results in the cell death of neurons (Lee et al., 2005, Lee da et al., 2006, Huang et al., 2008a). This direct toxicity of thrombin on neurons is mediated in a PAR dependent manner as it is blocked by hirudin (Lee da et al., 2006). Mixed neuron and astrocyte cultures were treated with different doses of thrombin (1-100 U/ml) for 24 hours, and cell viability was determined using media lactate dehydrogenase (LDH) concentration. While the low doses of thrombin did not induce cell death, doses greater than 5 U/ml resulted in a dose-dependent release of LDH, indicating rising levels of cell death (Hua et al., 2007). Where both microglial activation and neuronal loss were measured, the microglial activation preceded the neuronal loss (Huang et al., 2008b), or the neuronal loss was reduced by inhibition of microglial activation (Fujimoto et al., 2007). Reducing the number of microglia present, using a macrophage depletion method, protected the neurons from cytotoxicity (Katsuki et al., 2006). This indicates that microglia play an important role in thrombin-induced neuronal cell death. CM from microglia cultures treated with thrombin induced degeneration of dopaminergic neurons, with this cell death being prevented by inhibiting microglial activation using MAPK pathway inhibitors (Lee et al., 2005). Thrombin-induced neuronal cell death appears to be mediated by NO as it is prevented by a NOS inhibitor (Lee da et al., 2006). Also antioxidative drugs prevented thrombin-induced dopaminergic cell death, but did not prevent whole tissue injury (Katsuki et al., 2006).

1.6.5 Thrombin as beneficial agent

Despite its neurotoxic and glial activating ability, thrombin, at low doses, can be beneficial. Following ICH, thrombin has been found to stimulate differentiation of bone marrow-derived endothelial progenitor cells and astrocyte proliferation, and enhance synthesis and secretion of NGF in glial cells (Hua et al., 2009). At low doses thrombin was shown to play a role in neurogenesis following induced ICH by increasing levels of doublecortin (DCX, a microtubule-associated protein used as a marker for neurogenesis) in the basal ganglia (Yang et al., 2008) and the ipsilateral caudate (Hua et al., 2009). Hirudin blocked this ICH-induced up-regulation of DCX (Hua et al., 2009). Thrombin, via PAR-1 activation, promotes angiogenesis, the growth of new blood vessels from pre-existing vessels, by stimulating cells to secrete vascular endothelial growth factor (VEGF), a specific mitogen of endothelial cells, and up-regulating VEGF receptors in endothelial cells (Hua et al., 2009). Low dose thrombin activation of PAR-1 protects rat hippocampal neuron and astrocyte cultures from cell death in response to oxidative stress, Aβ aggregation, or hypoglycemia (Luo et al., 2007a). Thrombin also protects neurons from glutamate-induced excitotoxicity via activation of PAR-1 (Gorbacheva et al., 2005, Gorbacheva et al., 2006). Some CM studies have shown that thrombin treatment can increase cell viability. CM from astrocyte cultures treated with thrombin promoted the survival of human hybrid neurons. This protective effect was completely reversed with an inhibitor of glutathione peroxidase (an enzyme which protects against oxidative damage), indicating that glutathione peroxidase released from thrombin-treated astrocytes has a key role in the neuroprotection (Ishida et al., 2006). CM obtained from PAR-activated astrocytes similarly protected cortical neurons (Wang et al., 2007c). Thrombin preconditioning (the treatment of neurons with a low dose of thrombin several days before a brain insult) provides protection in a 6-hydroxydopamine (6-OHDA) model of PD. It reduces dopaminergic terminal loss and ventricular enlargement, but does not prevent DA depletion (Cannon et al., 2005). This protection is mediated through PAR-1, but not PAR-4, activation (Cannon et al., 2006). Interestingly, co-administration of thrombin or a PAR-1 agonist and 6-OHDA results in considerably greater behavioural deficits while thrombin treatment 1 or 7 days after 6-OHDA again results in protection. This shows that the effects of thrombin can be dependent on the time of administration and that while pre- or post-injury treatment is beneficial, co-treatment is in fact detrimental (Cannon et al., 2007).

1.6.6 Thrombin signalling

Thrombin signals mainly via PARs which are seven transmembrane G protein coupled receptors that are activated by proteolytic cleavage instead of ligand binding. The main target for thrombin is PAR-1, but it can also activate PAR-3 and PAR-4. PAR-1 can couple to members of the G-protein-coupled receptor families, linking it to a wide variety of intracellular signalling cascades such as IP_3, 1,2-diacylglycerol (DAG), and adenylate cyclase (Luo et al., 2007a, Hua et al., 2009). Most studies agree that all PARs are widely expressed in the brain, including neurons, microglia, astrocytes, and oligodendrocytes (Luo et al., 2007a), but one study showed that PAR-1 was expressed in human astrocytes and neurons, but not in oligodendrocytes or microglia (Ishida et al., 2006). However another study, using PCR and flow cytometry, showed that PARs are expressed on primary mouse microglial cells, as well as the murine microglial cell lines BV-2 and N9 (Balcaitis et al., 2003). PARs are necessary in brain development and function and can mediate cell death or survival, depending on the strength and duration of activation (Luo et al., 2007a).

As well as PAR activation, the effects of thrombin can be mediated in a number of different ways including the complement cascade (Hua et al., 2007), an increase in COX-2 and PGE-2 (Thirumangalakudi et al., 2009), a decrease in Bcl2 with corresponding increase in caspase-3 activity (Sanchez et al., 2009), an increase in cyclin-dependent kinase 4 and the pro-apoptotic protein Bim (Bcl2-interacting mediator of cell death) (Rao et al., 2007), and most commonly by activation of ERK and MAPK pathways (Choi et al., 2003, Fujimoto et al., 2007). One particular study (Ryu et al., 2000) showed that thrombin-induced microglial activation was mediated via PKC, MAPK and NFκB activation. Another study showed that thrombin-induced microglial activation was mediated via activation of JAK2 and phosphorylation of STAT3, with inhibition of this JAK-STAT pathway protecting the neurons in co-cultures of neurons and microglia (Huang et al., 2008a). Two studies by the same research group claimed that the microglia-activating potential of thrombin was not in fact due to the molecule itself, but rather due to an issue with its preparation or quality (Hanisch et al., 2004, Weinstein et al., 2005).

1.7 Noradrenaline

Noradrenaline (NA, norepinephrine) is a monoamine neurotransmitter and a member of the catecholamine family of neurotransmitters which also contains DA and adrenaline (epinephrine).

1.7.1 NA synthesis and metabolism

NA is synthesised from the dietary amino acid tyrosine, and contains a benzene ring and an amine-containing side chain (Leonard, 1997, Rang et al., 1999). Tyrosine, an aromatic amino acid, is converted to dihydroxyphenylanlanine (DOPA) by tyrosine hydroxylase (TH). DOPA is converted to DA by DOPA decarboxylase, and dopamine-β-hydroxylase (DβH) converts DA to NA. NA is then converted to adrenaline by phenylethanolamine N-methyl transferase (PNMT) (Figure 1.4).

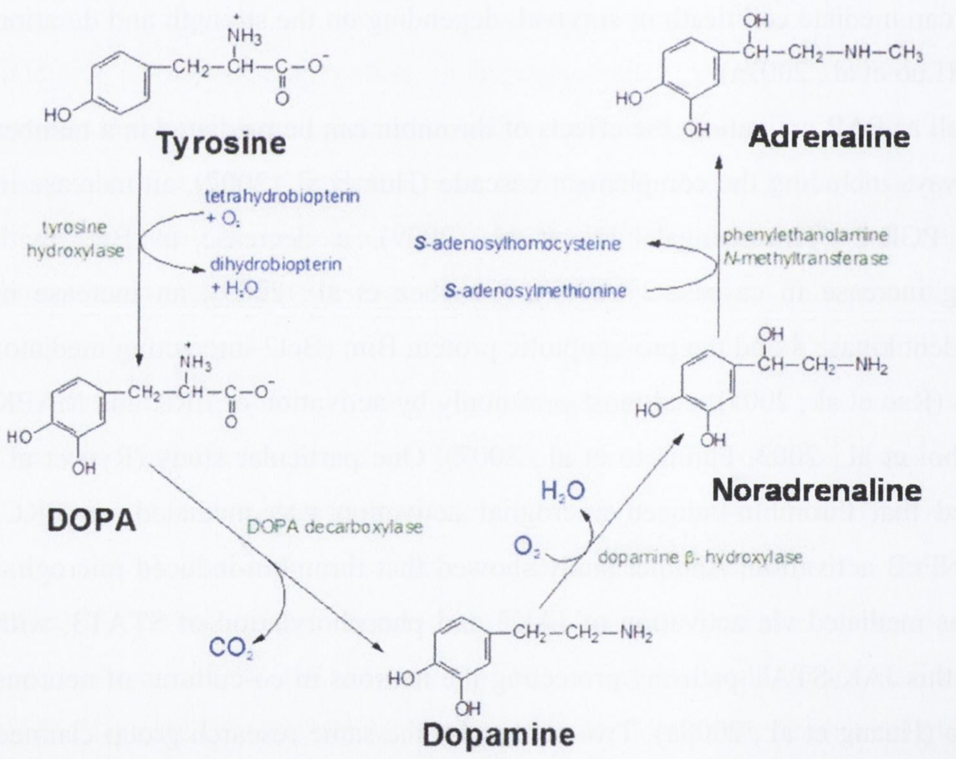


Figure 1.5: Catecholamine synthesis

Adapted from: http://themedicalbiochemistrypage.org/nerves.html#catecholamines

Green writing denotes enzymes. Blue writing denotes other molecules involved in the reactions. TH converts tyrosine to DOPA. DOPA decarboxylase converts DOPA to dopamine and CO_2 is released. DβH converts DA to NA and O_2 is converted to H_2O. PNMT converts NA to adrenaline.

The synthesis of DOPA by TH is the only rate-limiting step in this process, while DβH is only found in cells capable of catecholamine synthesis, such as adrenergic neurons in the brain and chromaffin neurons in the adrenal medulla (Rang et al., 1999). NA can up-regulate its own production as high activity of the noradrenergic neurons leads to increased expression of the TH gene and synthesis of the enzyme so that demand for NA synthesis can be met. After synthesis, NA is moved to synaptic vesicles by the vesicular monoamine transporter (VMAT) where it is stored in high concentrations with ATP and the protein chromogranin A. Both of these molecules, as well as a small quantity of DβH, are co-released with NA (Rang et al., 1999).

After NA has been released, it is not confined solely to vicinity of the synaptic cleft, but can reach surrounding glial cells (Aoki, 1992). NA is taken back up into the pre-synaptic neuron by the noradrenergic transporter (NET). The NET is a sodium (Na^+) and Cl^- dependent, high-affinity catecholamine transporter, which clears both DA and NA from the synaptic cleft and extracellular fluids (Eisenhofer et al., 2001). It is also found on non-neuronal cells, including astrocytes (Inazu et al., 2003). After re-uptake, approximately 70% of NA is recycled by being sequestered into the vesicles by the intracellular VMAT and stored for re-release (Eisenhofer et al., 2001). The remaining NA is metabolised by the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), both of which are located in neurons and glia.

1.7.2 NA location and function

NA has numerous roles both peripherally and centrally. In the brain it is involved in memory formation, attention, anxiety, arousal, and REM sleep. In the periphery NA controls smooth muscle contraction in the sympathetic nervous system (the “fight or flight” response) where it induces changes in a range of functions including heart rate, blood pressure and gastrointestinal activity. Apart from its actions as a classical neurotransmitter, NA also has a role in influencing the survival, maintenance and plasticity of CNS neurons, including regulation of glial function, energy consumption, extracellular homeostasis, endogenous neurotrophin systems, and anti-inflammatory effects (Marien et al., 2004). Importantly in this regard, electron microscopy studies of the visual cortex suggest that NA released from nerve terminals is not confined to the synaptic cleft, meaning NA may be able to diffuse to neighbouring glia (Aoki, 1992).

NA is released from noradrenergic neurons which are located mainly in the pons and the medulla, with the largest group of neurons being the locus coeruleus (LC). There are two main sources of noradrenergic neurons that innervate the brain: The dorsal bundle arises from cell bodies in the LC that project to the spinal cord and cerebellum and via the median forebrain bundle to the entire cortex, hippocampus, amygdala, and olfactory bulbs. The ventrally located cell bodies (lateral tegmental nuclei) are more diffusely localised and project fibres, termed the ventral bundle, to innervate the brainstem and hypothalamus. The total number of estimated noradrenergic neurons in the LC of a normal young adult human brain ranges is from 45,000 to 60,000 (German et al., 1988). There are two different types of LC axonal terminals; conventional synaptic structures and varicosities (varicose enlargements or swellings) that are believed to result in the extra-synaptic release of NA, which may then diffuse over some distance before affecting noradrenergic receptors on neurons, glia, and blood vessels (Parnavelas and Papadopoulos, 1989, Marien et al., 2004). NA effects appear to be cell specific. In some cells NA stimulation increases the probability of neuronal spiking in response to white matter stimulation, whereas in other cells NA stimulation decreases the probability of neuronal spiking in response to afferent pathway stimulation (Waterhouse et al., 2000). For example, in the somatosensory cortex, NA facilitates the transfer of information by altering the responsiveness of neurons to other powerful excitatory (glutamatergic) and inhibitory (GABAergic) inputs. With low level activation, facilitation of excitatory and inhibitory responses occurs, however at higher stimulation somatosensory cortical neuron activity was suppressed (Waterhouse et al., 1998).

1.7.3 NA Receptors

NA signals via a number of different cell surface adrenergic receptors (ARs or adrenoceptors), all of which are seven trans-membrane metabotrophic G-protein coupled. There are two basic types of ARs, the alpha (α) and beta (β)-ARs, defined on the basis of their affinities and actions.

α-ARs

The main α-ARs are α_1 and α_2. The α_1-ARs are typically postsynaptic and coupled to the G-protein Gq, which stimulates phospholipase C (PLC), activating the IP_3/DAG second messenger system (Rang et al., 1999). This results in an increase in

PKC, phospholipase A_2 (PLA_2), phospholipase D (PLD), arachidonic acid release, cAMP (3'-5'-cyclic adenosine monophosphate) formation, and activation of Ca^{2+} channels (Figure 1.6) (Robinson and Hudson, 2000). The α_2-ARs are located on peripheral sympathetic nerve terminals, are pre-synaptic, and act as auto-regulatory inhibitory receptors. They are coupled to the inhibitory G-protein (Gi) that inhibits adenylate cyclase thereby preventing the formation of the second messenger cAMP and inhibiting Ca^{2+} channels (Rang et al., 1999). There is also evidence of activation of K^+ channels, PLA and Na^+ / hydrogen (H^+) exchange (Robinson and Hudson, 2000).

In the peripheral nervous system, α_1-ARs and α_2-ARs are predominantly located on vascular and non-vascular smooth muscle where activation of the receptor results in contraction, and helps mediate the response to endogenous neurotransmitter release. The α_1-ARs are also found on the heart, where they mediate a positive inotropic effect, and on the liver where they activate glycogen phosphorylation. The α_2-ARs are also located on fat cells where they inhibit lypolysis, and on sympathetic ganglia where they contribute to hyperpolarisation (Robinson and Hudson, 2000), and in the cerebellum (Russo-Neustadt and Cotman, 1997). In the CNS, α_2-ARs play an important role in regulating NA release, helping to mediate effects such as regulation of central cardiovascular function, blood pressure, hypothermia, pupil diameter and a role in cognitive function. Studies on mRNA have shown α_1-AR mRNA to be present in the hippocampus and the cortex (Robinson and Hudson, 2000).

β-ARs

The main β-ARs are β_1 (located in cardiac tissue) and β_2 (located in airway smooth muscle), initially subdivided on the basis of the relative potencies of a series of catecholamines. There are also two atypical subtypes; β_3 (located in adipose tissue) which is insensitive to typical β-AR antagonists, and β_4 (located in cardiac tissue) which has a low affinity for adrenaline and NA but is blocked by some β-AR antagonists (Robinson and Hudson, 2000). The human β-AR gene is situated on the long arm of chromosome 5 and there is a 65-70% homology between β_1/β_3- and β_2-receptors (Johnson, 1998 2001). β-ARs are positively coupled to the stimulatory G-protein (Gs) which contains three intracellular and three extracellular loops, one extracellular N-terminal domain, and one intracellular C-terminal tail (Wallukat, 2002). Binding of NA results in a β-AR → adenylate cyclase → PKA cascade. There are two forms of β-AR, activated and inactivated, which the receptor oscillates between, with the inactivated state being predominant under resting conditions. The activated form occurs when the receptor is associated with the α-subunit of the Gs protein and a molecule of GTP. When GTP is replaced by guanosine diphosphate (GDP) it dramatically reduces the affinity of the α-subunit for the receptor, causing dissociation and inducing the receptor to return to its low energy inactivated form (Johnson, 2006). Stimulation of the β-AR activates membrane bound adenylate cyclase and cytosolic ATP to produce increased levels of intracellular cAMP. This leads to a subsequent increase in cAMP dependent PKA (Figure 1.6). PKA is a ubiquitous serine/threonine kinase that exists in an inactive state; its catalytic domains are bound to regulatory domains. cAMP binds to the regulatory subunits of PKA, releasing the catalytic subunits in an enzymatically active form. The PKA catalytic domains can further phosphorylate serine and threonine residues on a broad spectrum of protein substrates, thereby increasing or decreasing the target enzyme activity (Lodish et al., 2003). PKA can also induce expression of genes that encode a cAMP-response element (CRE). This occurs when the catalytic subunit of the PKA translocates to the nucleus and phosphorylates the transcription factor CRE-binding protein (CREB), which interacts with a co-activator CBP/300 to initiate gene transcription of CRE-encoding genes (Lodish et al., 2003). The action of cAMP can be mediated through more than one PKA and this can differ between cell types so that elevation of cAMP can exert different effects. CREB is the most common transcription factor activated by PKA, however it can also activate NFκB, CREB-binding protein and CCAAT enhancer binding protein depending on cell and gene type (Lodish et al., 2003).

Activation of cAMP can also alter Ca^{2+} activity, resulting in reduction of membrane Ca^{2+} entry, and sequestration of and inhibition of release from intracellular stores (Johnson, 2006). It has been shown that β_2-AR activation can lead to cAMP activation of the guanine nucleotide exchange factor EPAC (exchange protein directly activated by cAMP) (Figure 1.6) (Schmidt et al., 2001). EPAC proteins are small GTPases of the Rap family which facilitate guanine nucleotide exchange and GTP loading of the Rap GTPase Rap2B. This leads to PLC activation which then leads to IP3 formation and Ca^{2+} release. This process is dependent on cAMP but independent of PKA (Schmidt et al., 2001). As well as Ca^{2+} release, EPAC has a role in the control of cell adhesion and cell-cell junction formation (Bos, 2006). EPAC binds cAMP at a much higher concentration than PKA binds cAMP, meaning that higher concentrations of cAMP are required to ensure EPAC activation (Bos, 2003). Occasionally, β_2-ARs can also couple to Gi proteins, resulting in stimulation of the extracellular signal-regulation kinase and p38 MAPK pathways, although this occurs much less frequently than the Gs proteins and the cAMP-dependent PKA system (Johnson, 2006).

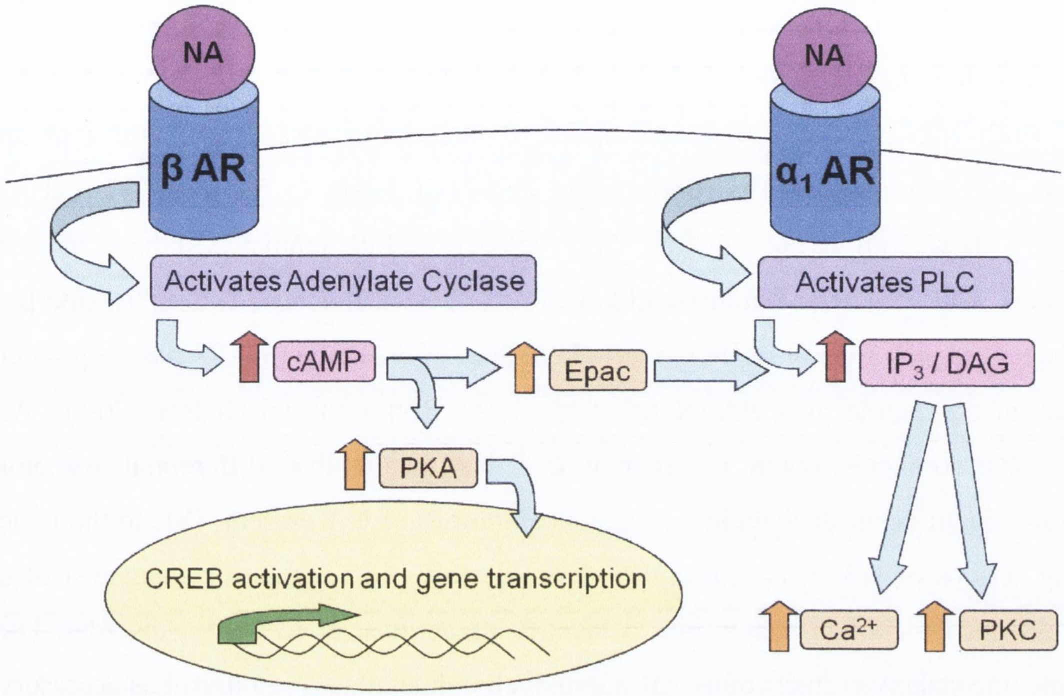


Figure 1.6: NA receptor signalling

Activation of the β-AR leads to activation of adenylate cyclase, which increases cAMP concentrations, leading to activation of PKA or EPAC. PKA activates CREB, resulting in gene transcription, while EPAC activates IP_3 and DAG, leading to increased Ca^{2+} release. Activation of the α_1-AR leads to activation of PLC, leading to activation of IP_3 and DAG, resulting in an increase in Ca^{2+} release and PKC activation.

In the periphery, β_1-ARs are located primarily in the heart and on adipose tissue, while β_2-ARs are located primarily on vascular, uterine and airway smooth muscle (Robinson and Hudson, 2000). In the CNS, the distinct patterns of β_1- and β_2-AR distribution have been determined using hybridisation studies. Immunostaining showed β_1-AR expression throughout the entire rat brain, particularly in the cerebellar cortex and basal ganglia, with smaller amounts being found in the brainstem areas (ventrolateral medulla, nucleus ambiguus and the nucleus of the solitary tract), the hypothalamus (paraventricular nucleus and the median eminence), and the hippocampus (Paschalis et al., 2009), as well as the frontal cortex and cerebellum (Russo-Neustadt and Cotman, 1997). β_2-AR expression has been shown in the hippocampus (Cox et al., 2008), the cerebellum and the frontal cortex (Russo-Neustadt and Cotman, 1997). Immune cells have also been found to express β-ARs. Macrophages express β_2-ARs only, while microglia express functional β_1- and β_2-ARs, but not β_3-ARs (Tanaka et al., 2002). Astrocytes also express functional β_2-ARs (De Keyser et al., 2004b).

AR agonists and antagonists

Both NA and adrenaline are capable of activating ARs. The potency of an agonist is determined by its affinity (how keenly it binds to its receptor) and its efficiency (how well it can activate the receptor). A full agonist will have a high efficiency, while a pure antagonist will have low or zero efficiency. The α-ARs and β_2-ARs have a higher affinity for NA over adrenaline, whereas the β_1-ARs have a greater affinity for adrenaline over NA (Rang et al., 1999, Robinson and Hudson, 2000). As many cells co-express both receptor types, this means that differential receptor activation could occur depending on the concentrations of NA present. Due to the wide ranging actions of NA, it is often more desirable to use an agonist or antagonist of a particular receptor when attempting to identify its actions, or to treat a disorder. For example, the sedative effects of α_2-AR agonist, clonidine, makes it a useful as accessory to general anaesthetics, while antagonists for the α_1-AR, such as indoramin and prazosin, can provide an effective therapy for hypertension (Robinson and Hudson, 2000).

All β-agonists include a β-OH group giving them an asymmetric centre. Site-directed mutagenesis identified the active site of the β_2-AR protein, with which β_2-AR agonists must interact for β_2-AR agonist binding and G protein coupling, as being located approximately one third of the way into the receptor core (Johnson, 2006). Two commonly used β-AR agonists are salbutamol and salmeterol which are used as therapy for the treatment of asthma and other bronchospastic conditions (Robinson and Hudson, 2000). Salbutamol is a non-selective β-AR agonist which binds with a relatively low affinity to both β_1- and β_2-ARs, and directly activates the receptor with moderate efficiency, while salmeterol is a selective β_2-AR agonist which has a three- to four-fold higher affinity than salbutamol, and interacts with a receptor-specific, auxiliary binding site (Johnson, 2001). Beta-blockers (β-AR antagonists) are primarily used for the treatment of angina pectoris and cardiac arrhythmias, but have also been used for management of alcohol withdrawal syndrome, anxiety disorders, migraine, hyperthyroidism and tremor. However, these treatments have to be managed carefully as too much β-AR blockade can result in heart failure, heart-block, and bronchospasm (Robinson and Hudson, 2000). One theory for the way in which β-AR agonists and antagonists exert their effects is that they bind to and temporarily stabilize the receptors in their activated or inactivated states respectively, thereby shifting the equilibrium in favour of that form and allowing greater or lesser activation to occur (Johnson, 2006).

1.7.4 NA and inflammation

The concept is emerging that endogenous mechanisms in the CNS could be an important factor for initiation, maintenance, and lack of resolution of brain inflammation. The activation of endogenous neuronal pathways, particularly those of NA, can provide immunomodulatory functions and protection in neuroinflammatory conditions, and their dysregulation can contribute to increased inflammation in disease (Feinstein et al., 2002, Galea et al., 2003).

NA and glial activation

There are numerous studies using NA and a variety of AR agonists and antagonists, as well as activators and inhibitors of the cAMP pathway, that show that the anti-inflammatory actions of NA are mediated via β_2-AR activation and increased cAMP: Studies using microglia, astrocytes, and brain endothelial cells have demonstrated that NA, by binding to β_2-ARs and activating cAMP signalling pathways, can inhibit the expression of a number of inflammatory genes including iNOS, IL-1β, TNFα, MHC-class-II, and ICAM-1 (Feinstein et al., 2002). Activation of β_1- and β_2-ARs on macrophages and microglia with NA and β-AR agonists resulted in an increase in the level of intracellular cAMP and the subsequent expression of IL-1β mRNA. These effects were prevented by the β-AR antagonist propranolol (Tanaka et al., 2002). Activation of astrocyte β_2-ARs increases intracellular levels of cAMP resulting in a wide range of downstream effects including suppression of iNOS, MHC-class-II, and CAM expression, suppression of IL-1β and TNFα secretion, production of NGF and BDNF, inhibition of astrocyte proliferation, and stimulation of glutamate uptake by astrocytes (De Keyser et al., 2004b). NA also inhibits IFNγ-induced MHC-class-II expression on astrocytes, and expression can be restored by a β-AR antagonist or the cAMP analogue dibutyryl-cAMP (db-cAMP), but not a selective β_1-AR or α-AR antagonist (Frohman et al., 1988). Induction of IFNγ, GM-CSF, and IL-3 mRNA is dose-dependently inhibited by a selective β_2-AR agonist in a process related to the accumulation of intracellular cAMP levels. The effect is blocked by a selective β_2-AR antagonist, but not a selective β_1-AR antagonist (Borger et al., 1998). The IL-1β-induced expression of iNOS and

ICAM-1 was decreased by stimulation of cAMP-dependent pathways with db-cAMP or forskolin, a cyclic AMP mimic (Balyasnikova et al., 2000). Another study showed that treatment of astrocytes with NA, db-cAMP, forskolin, and PGE-2 all increase cAMP-dependent protein kinase-mediated signalling pathways, resulting in suppression of IL-1β- and TNFα-induced expression of VCAM-1 and ICAM-1 (Ballestas and Benveniste, 1997). NA also reduces LPS-induced production of IL-1β mRNA and protein from microglia by over 80%, an effect that was mimicked by a β-AR agonist and reversed by a selective β_2-AR antagonist, but was not affected by an α-AR antagonist (Madrigal et al., 2005). NA significantly decreased MCP-1 mRNA levels in microglia, but increased levels in astrocytes. It acts at the transcriptional level as it also increased activation of an MCP-1 promoter, an effect which was replicated by db-cAMP and by a selective β_2-AR agonist, and reduced by the β-AR antagonist propranolol and by a selective β_2-AR antagonist (Madrigal et al., 2009). Zilpaterol, another β-AR agonist, inhibits LPS-induced TNFα and MIP-1β release in a dose-dependent manner both *in vitro* in macrophages and in rats *in vivo* (Verhoeckx et al., 2005a, Verhoeckx et al., 2005b). Research on another catecholaminergic neurotransmitter, DA, showed that it also inhibited LPS-induced release of TNFα and NO in astrocytes. This effect was blocked by selective antagonists of β_1-ARs and β_2-ARs, and mimicked by db-cAMP and forskolin, indicating that the anti-inflammatory actions of DA are mediated via noradrenergic receptors (Facchinetti et al., 2004).

The anti-inflammatory actions of NA via cAMP are mediated in a cell- and gene-specific manner through a number of transcription factors, in particular the reduction in the level of NFκB activation, as NA can increase the levels of inhibitor of kappa binding (IκB) proteins, which bind to and maintain NFκB in the cytosol (Feinstein et al., 2002). Another study showed that NA, through β_2-AR activation, reduced iNOS expression and NO generation via a reduction in the loss of inhibitory IκBα protein (Dello Russo et al., 2004). In primary astrocytes, NA dose-dependently increased the expression of IκBα mRNA and protein in both the cytosol and the nucleus. These effects were blocked by a β-AR antagonist and by PKA inhibitors but not by an NFκB inhibitor. In addition to this, lesions in the LC depleted NA in the frontal cortex of normal adult rats which led to a reduction of IκBα. This was paralleled by an increased inflammatory response to LPS, demonstrating that IκBα regulation could contribute to the observed anti-inflammatory properties of NA *in vitro* and *in vivo* (Gavrilyuk et al., 2002). NA also acts by modulating gene promoter activity: NA suppresses both cytokine- and LPS-induced iNOS expression due to modification of transcription factor activity in a particular 27 base pair region of the promoter of the iNOS gene, and may help explain findings that cAMP can potentiate, rather than suppress, iNOS expression in some cells (Feinstein, 1998, Gavrilyuk et al., 2001). The activation of β_2-ARs, but not β_1-ARs, in astrocytes led to a decrease in LPS-induced TNFα and IL-6 promoter activity, causing decreased mRNA and protein levels (Nakamura et al., 1998).

NA and prevention of neurotoxicity

Another action of NA is to protect against glial-induced neuronal cell death. Co-treatment of microglia with NA (0.1-10 μM) significantly reduced neuronal cell death in response to treatment with LPS CM (Madrigal et al., 2005). It did this by blocking IL-1β production from glia and dramatically decreasing the amount of NO induced in neurons by LPS CM. A follow up study by the same group (Madrigal et al., 2006) showed that NA reduced LPS-CM-induced neuronal cell death, whether it was added to the microglia or the neurons. However neuronal iNOS expression in response to LPS-CM was reduced by 70% when NA was added to the microglia, but it was only reduced by 35% when NA was added to the neurons (Madrigal et al., 2006). This indicates that NA has a better protective effect when used to inhibit microglial activation than when used solely on the neurons to prevent neuronal damage. Another study showed that when CM from astrocytes treated with NA was given to neurons it reduced NMDA-dependent glutamate release and the toxic consequences of oxygen and glucose depletion, an effect that was mediated by MCP-1 (Madrigal et al., 2009).

NA and anti-inflammatory agents

Another important observation is that NA and DA, and to a lesser extent adrenaline and forskolin, increased BDNF mRNA in astrocytes (Zafra et al., 1992). Adrenaline, also via ARs and cAMP, suppressed the LPS-induced production of TNFα, but increased synthesis of the anti-inflammatory cytokine IL-10. This IL-10 increase was reversed by a competitive inhibiter of the cAMP induced activation of PKA (Siegmund et al., 1998). Activation of β_2-ARs also increases expression of the anti-inflammatory cytokine TGFβ1 in non-ischemic rats and after transient forebrain ischemia (Zhu et al., 2001).

Overall it is clear that there is substantial evidence indicating that NA and β_2-AR signalling can exert anti-inflammatory effects in the CNS. Dysfunction of NA signalling could, therefore, allow the initiation of inflammatory processes within the CNS.

1.7.5 NA and neurodegenerative disease

NA has been shown to be implicated in a number of neurodegenerative diseases: Computer visualization techniques showed that there was approximately 60% loss of LC neurons in the brains of patients with AD, PD, and Down syndrome compared with normal age matched controls (German et al., 1992). Astrocytes in MS show a loss of β_2-ARs, which might account for the breakdown of tolerance to myelin antigens, facilitating the development of inflammatory lesions, and the axonal degeneration that leads to progressive disability (De Keyser et al., 2004a, De Keyser et al., 2004b). In patients with Huntington's chorea, a nearly complete loss in the number of β_1-ARs in the basal ganglia was observed, corresponding with an increase of β_2-ARs in the posterior putamen (Waeber et al., 1991).

In AD, the loss of LC neurons correlates with NA concentration, the number of Aβ plaques and NFTs, and the severity and estimated duration of dementia (Bondareff et al., 1987). NA has been shown to influence symptoms of dementia, such as agitation, depression, aggression, and psychosis (Kalinin et al., 2007), and there is a small but significant increase in total β-AR density, and to a lesser extent in α_2-AR density, in the cerebellums of aggressive demented patients versus both healthy controls and nonaggressive AD patients (Russo-Neustadt and Cotman, 1997). One recent epidemiological study reported that there was a decreased incidence of AD in patients who used β_2-AR agonists (Khachaturian et al., 2006). β_2-ARs also play an important role in the aetiology of Late Onset Alzheimer's disease (LOAD) as there is a significant interaction between the polymorphisms of β_2-AR and incidence of sporadic LOAD (Yu et al., 2008). Transgenic mice with mutations in the gene for APP are often used as models for AD as they exhibit symptoms of AD such as plaque formation and cognitive dysfunction. Studies by Kalinin and colleagues (2007) used transgenic APP mice treated with DSP4, a drug used to selectively lesion LC noradrenergic neurons, resulting in an exacerbation of the inflammatory response to Aβ and an increase in the number of amyloid plaques. Microglial activation was increased in the frontal cortex, while astrocyte activation was increased in the hippocampus, particularly in areas surrounding the plaques. The same study also showed that treating primary microglial cells with NA, as well as the β-AR agonist isoproterenol, increased microglial phagocytosis of Aβ (Kalinin et al., 2007). Another *in vivo* study showed that loss of LC neurons caused by treatment with the selective neurotoxin DSP4 led to increased Aβ-induced inflammation, including IL-1β and iNOS expression, and that these effects were attenuated by treatment with NA or a β-AR agonist (Heneka et al., 2002). Also a mouse model of AD showed that β_2-AR activation led to enhanced γ-secretase activity causing increased Aβ production, and that a β_2-AR-selective antagonist ameliorated Aβ plaque pathology in transgenic mice (Ni et al., 2006). These results show that NA, both through its anti-inflammatory properties and its ability to regulate Aβ clearance, has an important role in moderating AD progression.

The above neurodegenerative disorders are associated not only with deficiencies in noradrenergic signalling, but also contain an inflammatory component. As such, it is possible that these two factors are inter-related and may contribute to disease occurrence or progression.

1.8 Objectives

The objective of this project was to investigate the ability of thrombin and Aβ to induce a neurodegenerative phenotype in glial and neuronal cells *in vitro*, and the ability of NA interventions to prevent or reverse this by promoting an anti-inflammatory phenotype. The role of the β-AR in the anti-inflammatory actions of NA was also studied. Specific experiments focused on:

1. The ability of thrombin and Aβ to induce an inflammatory response in mixed glial and separated glial cultures, and the nature of this inflammatory response.

2. The ability of NA to reduce these inflammatory responses and to investigate the pathways by which NA mediates its effects, with particular emphasis on β-AR activation and subsequent downstream actions.

3. The ability of β-AR agonists to alter cytokine receptor expression on neurons and to protect against cytokine-induced neurotoxicity.

4. The ability of thrombin and Aβ to alter neuronal viability.

5. The ability of CM from glial cells treated with thrombin or Aβ to alter neuronal viability, and the ability of β-AR agonists to modulate this.

6. The ability of thrombin, Aβ, and β-AR agonists to induce production of anti-inflammatory cytokines and neurotrophic factors in mixed glial cultures.

Overall, this project has yielded significant insights into the inflammatory response induced by thrombin and Aβ, and how NA and subsequent β-AR activation exert an anti-inflammatory phenotype *in vitro*. It has also shown that thrombin can induce expression of anti-inflammatory cytokines and neurotrophic factors and that β-AR activation is capable of augmenting production of these molecules, resulting in increased neuronal viability.

2 Materials and Methods

2.1 Materials

Animals

Wistar rats (male and female, 0-3 days old)	Bioresources, TCD

Cell Culture Materials

Acrodisc syringe filter (0.2 µm)	Pall Corporation
Biocidal ZF ™	WAK-Chemie
B-27 growth factor	Invitrogen
Cell strainers (40 µM)	BD Falcon
Coverslips (plastic, 13 mm)	Sarstedt
Coverslips (glass, 13 mm)	VWR International
Cytosine β-D-arabino-furanoside (Ara-C)	Sigma
Cytotox 96-non-radioactive LDH assay kit	Promega
Disposable sterile scalpels	Swann-Morton
DNase	Sigma
Dulbecco's modified Eagle's medium: F-12 (DMEM)	Invitrogen
Dulbecco's phosphate buffered saline (PBS) (10X)	Sigma
Foetal Bovine Serum (FBS)	Invitrogen
Glutamax	Invitrogen
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	R&D Systems
Haemocytometer	VWR international
Horse serum	Invitrogen
Macrophage colony-stimulating factor (M-CSF)	R&D Systems
Neurobasal media (NBM)	Invitrogen
Penicillin-streptomycin	Invitrogen
Plastic syringe (50 ml, 20 ml and 1 ml)	Becton Dickenson
Poly-L-lysine	Sigma
Serological pipette (25 ml)	Sarstedt
Steri-Cycle CO_2 Incubator	Bio-Sciences
Sterile Petri dishes	Sarstedt
Sterile transfer pipettes	Sarstedt
Sterile Combitips plus (2.5 ml)	Eppendorf

Sterile falcon tubes (50 ml)	Sarstedt
Sterile falcon tubes (15 ml)	Sarstedt
Sterile microtubes (2 ml)	Sarstedt
Sterile 24 well plates	Sarstedt
Trypan Blue	Sigma
Trypsin	Sigma
Trypsin inhibitor	Sigma
Experimental Treatments	
β-amyloid (Aβ) [1-42] (Human)	Biosource
Butoxamine	Sigma
Dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP)	Sigma
Escherichia coli Lipopolysaccharide (LPS) serotype 0111:B4	Sigma
KT5720	Sigma
Metoprolol	Sigma
(-)-Norepinephrine (+) bitartrate salt hydrate (99%) (NA)	Sigma
N^6-Benzoyladenosine-3', 5' cyclic monophosphate (BNZ)	Biolog
8-(para-Chlorophenylthio)-2'-O-Methyladenosine-3', 5' cyclic monophosphate (pCPT)	Biolog
Propranolol hydrochloride	Sigma
Recombinant Rat IL-1β	R&D Systems
Recombinant Rat IFNg	R&D Systems
Recombinant Rat TNFα/TNFSF1A	R&D Systems
Salbutamol hemisulfate	Sigma
Salmeterol	Tocris
SQ22536	Tocris
Thrombin	Sigma
Xamoterol hemifumarate	Tocris
Molecular Reagents	
Absolute ethanol (EtOH)	Sigma
Biosphere filter tips (1000, 100 and 10 μl)	Sarstedt
High capacity cDNA archive kit	Applied Biosystems
Molecular grade water	Sigma

Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt
RNAlater™	Ambion
RNase away	Invitrogen
RNase-free 1.5 ml and 2 ml microfuge tubes	Ambion
RNase Zap wipes	Ambion
Total RNA isolation kit	Macherney-Nagel
TaqMan gene expression assays (see Table 2.2)	Applied Biosystems
TaqMan universal PCR master mix	Applied Biosystems
96-well optical reaction plates	Applied Biosystems

General Laboratory Plastics

Laboratory roll	Parafilm
Microtest 96-well flat bottomed plates	Sarstedt
Microtubes (1.5 ml)	Sarstedt
Microtubes (0.5 ml)	Sarstedt
Pipette tips	Sarstedt
Plastic transfer pipettes	Sarstedt
Plastic syringe (1 ml)	Becton Dickenson

General Laboratory Chemicals

Bovine serum albumin 96% (BSA)	Sigma
Bromophenol blue	Sigma
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	BDH
N-(1-naphthyl)-ethylenediamine dihydrochloride	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glycerol	Sigma
Glycine	Sigma
Hydrochloric acid (HCl)	BDH
Industrial methylated spirits	Lennox
Normal goat serum	Vector
Magnesium chloride ($MgCl_2$)	Sigma
Magnesium sulphate (Mg_2SO_4)	Sigma
β-Mercaptoethanol	Sigma

Methanol (MeOH)	BDH
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH
Potassium chloride (KCl)	Merck
Sodium carbonate (Na_2CO_3)	BDH
Sodium bicarbonate ($NaHCO_3$)	BDH
Sodium Chloride (NaCl)	BDH
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sulphuric acid (H_2SO_4) 98%	BDH
3,3',5,5',-Tetramethyl-benzidine (TMB)	Dako
Tris Base	Sigma
Tris-HCl	Sigma
Tween 20	Sigma

Protein Reagents

Maxisorp immunoplates for ELISA	Nunc
Rat Interleukin-1β (IL-1β) ELISA kit (Duoset)	R&D systems
Rat TNFα ELISA kit (Cytoset)	Biosource

Staining Reagents and Antibodies

Anti-β_1-AR IgG	Santa Cruz Biotech
Anti-β_2-AR IgG	Santa Cruz Biotech
Anti-βIII-tubulin IgG	Promega
Anti-CD11b IgG	Millipore
Anti-GFAP IgG	Dako
Anti-mouse IgG	Santa Cruz Biotech
Anti-Neu-N IgG	Millipore
Anti-rabbit IgG	Santa Cruz Biotech
Goat anti-rabbit Alexa 488	Invitrogen
Goat anti-mouse Alexa 488	Invitrogen
Goat anti-rabbit Alexa 546	Invitrogen
Hoescht	Invitrogen
Normal Goat Serum	Vector

2.2 *In vitro* cell culture studies

2.2.1 Preparatory work for cell culture

Aseptic Technique: Aseptic techniques were utilised during all cell culture work and also in the preparation of cell culture reagents. This was necessary in order to maintain a sterile environment which was free from fungal, bacterial and viral infections which could alter normal cellular functions. The aseptic techniques utilised included the sterilisation of glassware, plastics (pipette tips) and H_2O by autoclaving at 121 ^oC for 30-60 minutes, and the use of sterile disposable plastics. Dissection equipment was baked for a minimum of 2 hours at 200 ^oC to ensure sterility. All cell culture work was carried out in a laminar flow hood (Hera Safe, category 2). This allows only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The interior of the hood was sterilized with 70% ethanol (EtOH; 30% ddH_2O and 70% EtOH v/v) before and after use. The hood surface was also exposed to ultraviolet (UV) light for 15-30 minutes after use. Any items taken into the flow hood were lightly sprayed with 70% EtOH to prevent introduction of any pathogens to the hood work area. Disposable latex gloves were worn and sprayed with EtOH before use. Gloves were changed regularly during cell culture work. Cells were maintained in a sterile incubator (humidified 5% CO_2: 95% air environment at 37 ^oC) and any items put in the incubator were lightly sprayed with EtOH to prevent contamination with any pathogens. Both the incubator and laminar flow hood were regularly cleaned with Biocidal ZF™ to maintain a sterile environment.

Preparation of culture plates: 25 mg of poly-L-lysine was reconstituted with 3 ml of autoclaved distilled water (ddH_2O). The solution was mixed using a vortex, and when fully dissolved a further 22 ml of ddH_2O was added, to give a stock concentration of 1 mg/ml. This solution was then filter-sterilised using a 0.2 mm syringe filter and frozen at -20 ^oC in 1 ml aliquots for future use. To prepare 24 well plates for culture, 1 ml of poly-L-lysine was diluted 1:25 by the addition of 24 ml of ddH_2O and 100 μl (40 μg/ml) was used to coat each well. The plates were incubated for 30 minutes before the poly-L-lysine was removed and plates were left in the incubator overnight to ensure they were completely dry before use. When glass coverslips were required they were added to a 50 ml falcon tube containing 70% EtOH. The coverslips were incubated for 30 minutes, and then laid out on tissue paper in the laminar flow hood overnight under the UV light to ensure they were completely sterile. The coverslips were then added to another 50 ml falcon tube containing a 40 μg/ml solution of poly-L-lysine (1 ml of poly-L-lysine in 24 ml of ddH_2O). The coverslips were incubated for 30 minutes, and then laid out on tissue paper in the laminar flow hood overnight to ensure they were completely dry before use. They were then added to 24 well plates so that there was 1 coverslip per well.

2.2.2 Preparation of Culture Media and test compounds

Glial culture media: Complete glial culture media was made up using Dulbecco's modified Eagle's medium (DMEM:F12, Invitrogen, USA) containing 10% (v/v) heat inactivated Fetal Bovine Serum (FBS, Sigma, USA) and 1% (v/v) Penicillin-Streptomycin (100 units/ml, Gibco Brl, USA). To prepare it, 50 ml FBS and 5 ml Penicillin-Streptomycin was filter-sterilised using a 0.2 mm syringe filter and added to a 500 ml bottle of DMEM.

Neurobasal media: Complete neurobasal media (NBM, Invitrogen) was made up using 10% (v/v) heat inactivated horse serum (HS, Invitrogen), 1% (v/v) Penicillin-Streptomycin (100 units/ml, Gibco Brl, USA) and 1% (v/v) glutamax (Invitrogen). To prepare it, 50 ml HS, 5 ml Penicillin-Streptomycin, and 5 ml glutamax was filter-sterilised using a 0.2 mm syringe filter and added to a 500 ml bottle of NBM. A working solution was prepared by adding 500 μl of B-27 (Invitrogen) growth supplement to 50 ml of complete-NBM.

Phosphate buffered saline (PBS): A working 1x solution of PBS was prepared by adding 1 ml Dulbecco's sterile 10x PBS (Sigma, UK; 100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4) to 9 ml ddH_2O.

Granulocyte-macrophage colony-stimulating factor (GM-CSF): A 25 μg/ml stock solution of GM-CSF (R&D Systems) was prepared by dissolving 25 μg GM-CSF in 1 ml 0.1% BSA (Bovine Serum Albumin; 0.005 g BSA in 5 ml 1x PBS). Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

Macrophage colony-stimulating factor (M-CSF): A 50 μg/ml stock solution of M-CSF (R&D Systems) was prepared by dissolving 50 μg M-CSF in 1 ml 0.1% BSA (Bovine Serum Albumin; 0.005 g BSA in 5 ml 1x PBS). Stock solution was frozen at -20 °C in 20 μl aliquots for future use.

Lipopolysaccharide (LPS): A stock solution was prepared by reconstituting 25 mg Escherichia coli LPS (serotype 0111:B4) in 1 ml ddH_2O. The solution was vortexed and, once fully dissolved, 500 μl (25 mg/ml) was added to 62 ml complete DMEM (1:125 dilution) to give a 200 μg/ml stock solution which was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 1 ml aliquots for future use.

Thrombin: A 400 U/ml stock solution was prepared by dissolving 10 KU thrombin (Sigma, UK) in 25 ml ddH_2O. Stock solution was frozen at -20 °C in 1 ml aliquots for future use.

Amyloid beta (Aβ): A stock solution was prepared by dissolving 1 mg Aβ ($A\beta_{1-42}$, Biosource, USA, Formula weight 4515) in 166 μl ddH_2O. This was then diluted with 834 μl 1x PBS to form a 221.5 μM stock solution. This was incubated at 37 °C in a humidified 5% CO_2: 95% air environment for 48 hours in order to allow the protein to aggregate. This aggregated form of $A\beta_{1-42}$ initiates a more potent inflammatory response and is more cytotoxic than other forms of Aβ (Stuchbury and Munch, 2005), however it requires incubation *in vitro* prior to use in order for it to aggregate and maintain a fibrillar structure (Shin et al., 1997). Stock solution was frozen at -20 °C in 200 μl aliquots for future use.

Noradrenaline (NA): A 200 mM stock solution was prepared by dissolving 0.337 g NA (Sigma, UK, Formula weight 337) in 5 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 200 μl aliquots for future use.

Salbutamol: A 100 mM stock solution was prepared by dissolving 25 mg of salbutamol (β-AR agonist; Sigma, UK, Formula weight 228.35) in 1.71 ml ddH_2O. Stock solution was frozen at -20 °C in 15 μl aliquots. Before use it was filter-sterilised using a 0.2 mm syringe filter and diluted to a working concentration in pre-warmed media.

Salmeterol: A 100 mM stock solution was prepared by dissolving 10 mg salmeterol (selective β_2-AR agonist; Sigma, UK, Formula weight 415.57) in 240.6 μl DMSO. Stock solution was frozen at -20 °C in 12 μl aliquots. Before use it was filter-sterilised using a 0.2 mm syringe filter and diluted to a working concentration in pre-warmed media.

Xamoterol: A 50 mM stock solution was prepared by dissolving 10 mg xamoterol (selective β_1-AR agonist; Sigma, UK, Formula weight 397.43) in 503.2 μl ddH_2O. Stock solution was frozen at -20 °C in 12 μl aliquots. Before use it was filter-sterilised using a 0.2 mm syringe filter and diluted to a working concentration in pre-warmed media.

Di-butyryl adenosine 3', 5'-cyclic monophosphate (db-cAMP): A 50 mM stock solution was prepared by dissolving 25 mg of db-cAMP (cyclic AMP analogue; Sigma, UK, Formula weight 491.4) in 1 ml ddH_2O. Stock solution was frozen at -20 °C in 15 μl aliquots. Before use it was filter-sterilised using a 0.2 mm syringe filter and diluted to a working concentration in pre-warmed media.

N^6-Benzoyladenosine-3', 5' cyclic monophosphate (N^6-Benzoyl-cAMP or BNZ): A 10 mM stock solution was prepared by dissolving 10 μmol (4.6 mg) BNZ (PKA activator; Biolog, Germany, Formula weight 455.3) in 1 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 100 μl aliquots for future use.

8-(para-Chlorophenylthio)-2'-O-Methyladenosine-3', 5' cyclic monophosphate (8-pCPT-2'-O-Me-cAMP or pCPT): A 5 mM stock solution was prepared by dissolving 5 μmol (2.5 mg) pCPT (EPAC activator; Biolog, Germany, Formula weight 507.8) in 1 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 100 μl aliquots for future use.

SQ22536: A 100 mM stock solution was prepared by dissolving 10 mg SQ22536 (adenylate cyclase inhibitor; Sigma, UK, Formula weight 205.22) in 487.28 μl ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

KT5720: A 932 μM stock solution was prepared by dissolving 50 μg KT5720 (PKA inhibitor; Sigma, UK, Formula weight 537.61) in 100 μl DMSO. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

Propranolol: A 50 mM stock solution was prepared by dissolving 50 mg propranolol (non-selective β-AR antagonist; Sigma, UK, Formula weight g/mol) in 3.38 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

Metoprolol: A 100 mM stock solution was prepared by dissolving 137 mg metoprolol (selective β_1-AR antagonist; Sigma, UK, Formula weight 684.8) in 2 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

Butoxamine: A 50 mM stock solution was prepared by dissolving 30.38 mg butoxamine (selective β_2-AR antagonist; Sigma, UK, Formula weight 303.8) in 2 ml ddH_2O. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -20 °C in 50 μl aliquots for future use.

IL-1β: A 20 μg/ml stock solution was prepared by dissolving 20 μg IL-1β (R&D Systems) in 1 ml 1% BSA/PBS. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -80 °C in 20 μl aliquots for future use.

TNFα: A 10 μg/ml stock solution was prepared by dissolving 10 μg TNFα (R&D Systems) in 1 ml 1% BSA/PBS. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -80 °C in 20 μl aliquots for future use.

IFNγ: A 100 μg/ml stock solution was prepared by dissolving 100 μg IFNγ (R&D Systems) in 1 ml 1% BSA/PBS. This was then filter-sterilised using a 0.2 mm syringe filter. Stock solution was frozen at -80 °C in 20 μl aliquots for future use.

Alamar Blue (Resazurin): A 440 μM stock solution of alamar blue was prepared by dissolving 25 mg resazurin (Sigma, UK, $C_{12}H_6NNaO_4$, Formula weight 251.17) in 226.214 ml ddH_2O. Stock solution was stored in a dark bottle for future use.

Table 2.1: Agonists, antagonists, activators, and inhibitors of the NA signalling pathway

Agonists and Activators		**Antagonists and Inhibitors**	
Salbutamol	β-AR agonist	Propranolol	β-AR antagonist
Salmeterol	selective β_2-AR agonist	Butoxamine	selective β_2-AR antagonist
Xamoterol	selective β_1-AR agonist	Metoprolol	selective β_1-AR antagonist
db-cAMP	cAMP analogue	SQ22536	adenylate cyclase inhibitor
BNZ	PKA activator	KT5720	PKA inhibitor
pCPT	EPAC activator		

2.2.3 Preparation of Primary Mixed Glial cultures

The culturing of primary glial cells is an *in vitro* technique that involves the dissection of the brain, removal of the area of interest and dissociation of the tissue to obtain a population of glia. Cells are then allowed to mature over a 14-day period before being used in experimental procedures. Glial cultures obtained in this way typically contain astrocytes (70%) and microglia (30%), which simulates the approximate ratio of glia in the normal brain (unpublished observations). Primary cultures such as these also represent unaltered phenotypes that have not been transformed.

Mixed glial enriched cultures were prepared from brains of neonatal male and female Wistar rat pups (postnatal day 1-3; BioResources Unit, Trinity College, Dublin, Ireland). Tissue from the neonates was pooled and each plate represented an individual N number. Pups were taken into the laminar flow hood where they were decapitated using a large scissors. Using a smaller scissors the skin was cut down the midline to reveal the skull which was carefully cut on each side, at the level of the ears. The skull was gently pulled back to reveal the exposed brain. A curved forceps was then used to remove the meninges, which was discarded, and dissect out the cortex which was placed on a Petri dish containing 1x PBS. Any remaining meninges and adherent blood vessels were removed using a straight fine forceps. The cortical tissue was then cross-chopped using a scalpel and added to pre-warmed media. Complete media contained Dulbecco's modified Eagle's medium (DMEM:F12, Invitrogen, USA) with 10% heat inactivated Fetal Bovine Serum (FBS, Sigma, USA) and 1% Penicillin-Streptomycin (100 units/ml, Gibco Brl, USA). Tissue was incubated for 20 minutes, then triturated. The suspension was filtered through a sterile mesh filter (40 μm), centrifuged at 2000 x *g* for 3 minutes at 20 °C, and the resulting pellet was resuspended in warmed DMEM. Resuspended cells (50 µl per well) were seeded onto the centre of each of the poly-L-lysine-coated (40 µg/ml) coverslips in 24-well plates and allowed to adhere for 2 hours before addition of 300 µl warmed media. Cells were grown at 37 °C in a humidified 5% CO_2: 95% air environment and media was changed every 3 days. Data were obtained from a total of 6 cultures.

2.2.4 Preparation of Enriched Microglial and Astrocyte cultures

Separated glial enriched cultures were prepared from brains of neonatal male and female Wistar rat pups (postnatal day 1-3; BioResources Unit, Trinity College, Dublin, Ireland) according to the same protocol as the primary mixed glia cultures with the following adaptations. Resuspended cells (1 ml per flask) were pipetted onto T25 flasks and allowed to adhere for 2 hours before addition of 7 ml warmed media. Cells were grown at 37 °C in a humidified 5% CO_2: 95% air environment. On day 1 and day 7 the media was removed and new media containing the growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/ml; R&D Systems) and macrophage colony-stimulating factor (M-CSF; 5 ng/ml, R&D Systems) was added.

On day 10 the cells were checked for affluency and separated. Flasks were placed on an orbital shaker (2 hours at 110 rpm at room temp) then tapped (30 times) to dislodge microglia into the media. The media was then collected in a 50 ml falcon tube and centrifuged at 2000 x *g* for 3 minutes at 20 °C. The resulting pellet was resuspended in 1 ml warmed complete media and a cell count was performed using a Haemocytometer (10 µl cell suspension, 30 µl 1x PBS, 10 µl Trypan Blue). Media was added and the resuspended cells (50 µl per well) were seeded onto poly-L-lysine-coated (40 µg/ml) coverslips in 24-well plates at a density of $3x10^5$ cell/ml. The cells adhered for 2 hours prior to flooding with 300 µl complete media.

Astrocytes which remained adhered to the culture flasks were washed twice with 1x PBS before being incubated in 4 ml Trypsin-EDTA for 15 minutes. The flasks were tapped vigorously to dislodge the astrocytes and the suspension collected. The astrocytes were counted as before. Media was added and the resuspended cells (50 µl per well) were seeded onto poly-L-lysine-coated (40 µg/ml) coverslips in 24-well plates at a density of $3x10^5$ cell/ml. The cells adhered for 2 hours prior to flooding with 300 µl complete media.

Cells were allowed to grow separately for a few days before treatment occurred on day 13 or 14. Data were obtained from a total of 6 cultures.

2.2.5 Preparation of Primary Cortical Neuronal cell cultures

Cultures of primary rat cortical neurons were prepared from brains of neonatal male and female Wistar rat pups (postnatal day 0-1; BioResources Unit, Trinity College, Dublin, Ireland) according to the same protocol as the primary mixed glia cultures with the following adaptations.

3 solutions of trypsin or trypsin inhibitor were prepared as follows:
Solution 1: Trypsin (0.3 mg/ml) in sterile PBS.
Solution 2: Trypsin inhibitor (0.1 mg/ml), DNase (0.2 mg/ml) and Mg_2SO_4 (0.1 M) in sterile PBS.
Solution 3: 30% solution 2 in sterile PBS i.e. 3 ml solution 2 in 7 ml PBS.

When the meninges were removed from the cortices, they were placed in a Petri dish containing a few drops of a 3 mg/ml Trypsin/sterile PBS (w/v) solution and mechanically dissociated. There were then placed in a 15 ml falcon tube containing 2 ml of solution 1 and incubated for 25 minutes at 37 ^{o}C. Next, 4 ml of a solution 3 was added to inactivate the trypsin. The solution was flicked and the tissue allowed settle. The solution was aspirated off the cortical tissue using a disposable pipette. Then 2 ml of solution 2 was added to the tissue and it was dissociated by triturating with a sterile disposable pipette. The solution was passed through a 40 μm cell strainer into a sterile 50 ml falcon tube and centrifuged at 2000g for 3 minutes at 20 ^{o}C. The supernatant was aspirated off and the pellet re-suspended in 15 ml of pre-warmed NBM-B27. The pellet was gently triturated with a fresh sterile Pasteur pipette until a homogenous cellular suspension was obtained. At this stage, a cell count was performed by adding 10 μl of the cell suspension to 30 μl of 1x PBS and 10 μl of trypan blue in a 2 ml eppendorf microtube. This solution was vortexed and 50 μl was placed under a microscope coverslip on a haemocytometer. The cell number was counted and calculated using a light microscope at the 40x magnification.

Resuspended cells (50 μl per well) were seeded onto the centre of each of the poly-L-lysine-coated (40 μg/ml) coverslips in 24-well plates at a density of $2x10^5$ cell/ml and allowed to adhere for 2 hours before addition of 500 μl complete B27-NBM warmed media. Cells were grown at 37 °C in a humidified 5% CO_2: 95% air environment. The primary neuronal cultures were treated within 4-7 days of preparation. Data were obtained from a total of 6 cultures.

2.2.6 Quality of Primary Neuronal cultures

In order to assess the quality and purity of the primary neuronal cultures, cultures were grown on glass coverslips for up to 6 days. They were then fixed and stained using fluorescent immunocytochemistry, and imaged using a confocal microscope (methods described in Section 2.6). Neurons were identified in two ways by using either an antibody specific to βIII-tubulin to identify neuronal processes (Figure 2.1), or an antibody specific to Neu-N to identify neuronal nuclei (Figure 2.2). Tubulins are globular proteins and the principal structural components of microtubules, essential cell cytoskeleton structures that are vital for the formation and function of neuronal axons. Tubulin is a predominantly composed of α and β subunits, which each have a number of different subtypes. Of these, βIII-tubulin is expressed almost exclusively by neurons and is considered to be one of the earliest neuron-associated cytoskeletal marker proteins (Katsetos et al., 2003). Nue-N (*Neu*ronal *N*uclei) is a soluble neuron-specific nuclear protein that binds to DNA *in vitro* and serves as an excellent marker for neurons in the central and peripheral nervous systems (Mullen et al., 1992). Cultures were also stained green using an antibody specific to GFAP as a marker for astrocytes in order to investigate glial contamination of the cultures (Figure 2.2). Hoescht was used to identify cell nuclei.

2.2.7 Cell culture treatments

For each experiment, 24-well tissue culture plates were prepared with mixed glial, separated microglia/astrocytes or neuronal cultures as described previously. The cells were grown until the adherent monolayer was between 70-100% confluency. This occurred after 10-14 days for glia or 4-7 days for neurons. Each 24 well plate was divided into 6 or 8 treatment groups, of which either 3-4 wells were pooled to give 1 experimental sample, unless otherwise stated. Each plate was considered as an n=1. On the day of treatment culture media was removed from the wells and treatments administered in a final volume of 300 μl of pre-warmed complete DMEM:F12 for glia or complete NBM for neurons. All pre-treatments were administered 2 hours prior to the experimental treatment. If a further pre-treatment was required this was administered 30 minutes prior to the first pre-treatment. Cell cultures were incubated at 37 °C with 5% CO_2 with the various drug treatments for either 6 hours to assess gene expression, or 24 hours to assess protein production or cell viability, unless stated otherwise.

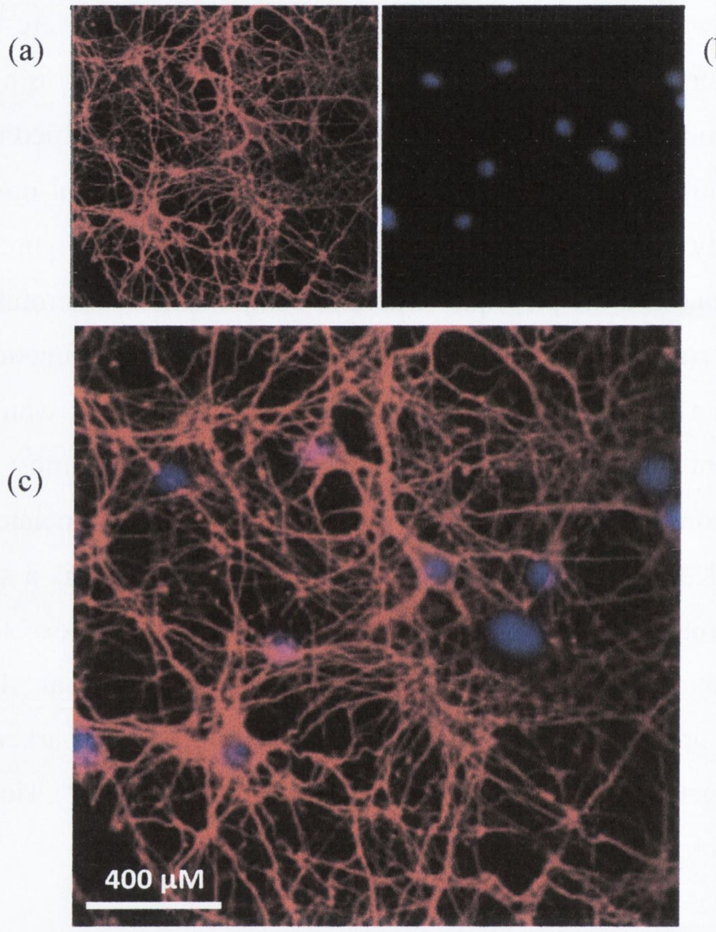


Figure 2.1: Neurons express βIII-tubulin

Fluorescent immunocytochemistry was carried out on primary neuronal cultures using antibodies against the marker of neuronal axons, βIII-tubulin. The cells were imaged at 20X magnification. (a) Red staining indicates βIII-tubulin positive neurons, (b) Blue staining indicates cell nuclei stained with Hoescht, (c) Enlarged image showing the colocalisation of (a) and (b). The axonal processes of the neurons are clearly visible. The white line represents a length of 400 µM.

(a) (b)

(c) (d)

(e)

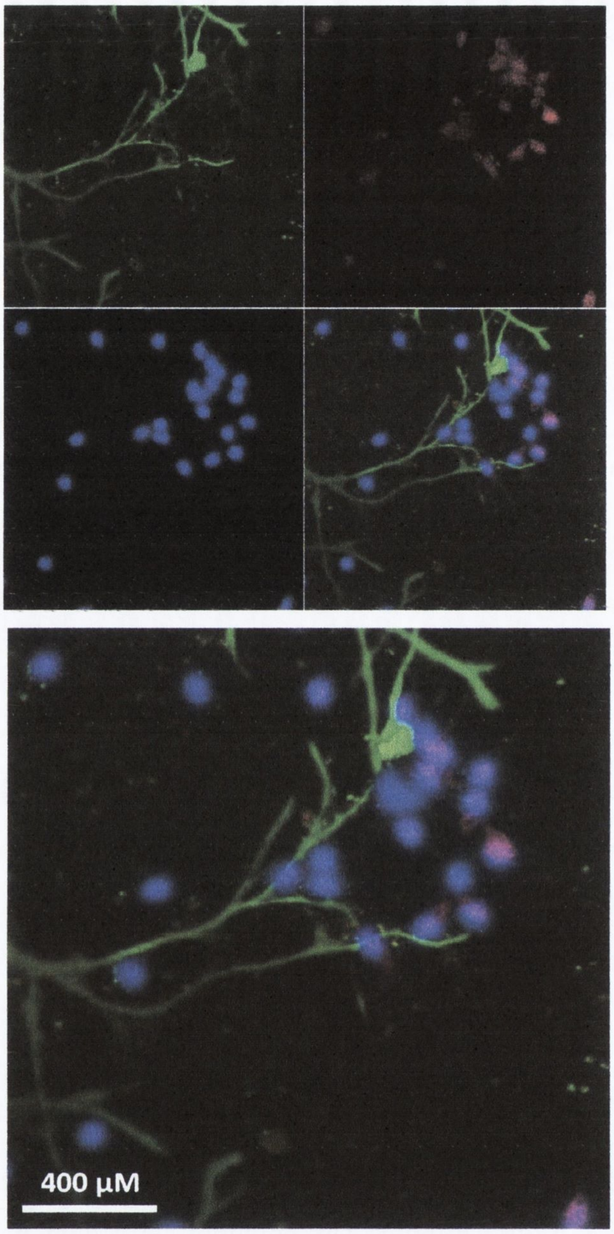


Figure 2.2: Neurons express Neu-N

Fluorescent immunocytochemistry was carried out on primary neuronal cultures using antibodies against the marker of neuronal nuclei, Neu-N, and the marker of astrocytes, GFAP. The cells were imaged at 20X magnification. (a) Green staining indicates GFAP positive astrocytes, (b) Red staining indicates Neu-N positive neurons, (c) Blue staining indicates cell nuclei stained with Hoescht, (d) Shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). While a few astrocytic processes are visible, the vast majority of the staining is for neurons. The white line represents a length of 400 μM.

2.3 RNA and RT-PCR procedures

Real time polymerase chain reaction (RT-PCR) is a technique used to provide a quantitative measure of messenger RNA (mRNA). It is able to detect the accumulation of product during the reaction which is then measured at the exponential phase of the PCR technique.

2.3.1 Harvesting cultured cells for mRNA analysis

The bench, equipment and gloves were wiped with RNase Away wipes and rinsed with DEPC water prior to use to prevent RNA contamination. Supernatant was removed, the cells were harvested using 100-125 μl Lysis buffer (RA1 (Macherey-Nagel, Germany), 1% β-mercaptoethanol) per well and 3-4 wells were pooled to create a sample. Samples were either immediately extracted or frozen at -20 °C.

2.3.2 RNA extraction

RNA was extracted from the cells using a NucleoSpin RNA II kit (Macherey-Nagel, Germany) according to the kit protocol. Samples were either used immediately following harvesting, or were defrosted before use. Samples were centrifuged at 11000 x g for 1 minute, the pink column was discarded and 350 μl of 70% ethanol was added to the bottom tube. This was mixed by pipetting and the solution transferred to the blue column, this column now contained the RNA. Samples were centrifuged at 11000 x g for 30 seconds and the blue column was moved to a new tube. The membrane was desalted by the addition of 350 μl of MDB (membrane drying buffer) and samples were centrifuged at 11000 x g for 1 minute. Any potential DNA contamination was digested by the addition of 95 μl reconstituted Dnase reaction mixture directly on to the membrane of the column and incubating for 15 minutes at RT. The membrane was then washed with 200 μl RA2 to inactivate the Dnase and centrifuged at 11000 x g for 30 seconds. The blue column was placed in a new tube and the membrane was dried by the addition of 600 μl RA3 and centrifuged at 11000 x g for 30 seconds, then 250 μl RA3 and centrifuged at 11000 x g for 2 minutes. Finally the blue column was placed in a micro-centrifuge tube and the RNA was eluted in 60 μl RNase free H_2O and centrifuged at 11000 x g for 1 minutes. The RNA was stored at -80 °C until RNA equalisation.

2.3.3 Measuring RNA concentration and Equalising RNA

The RNA concentration from each sample was measured using a NanoDrop®

ND1000 spectrophotometer. Purity was demonstrated by the $A_{260/280}$ ratio. RNA samples (30 µl) were equalised to the lowest concentration of RNA detected by addition of RNase free H_2O.

2.3.4 Reverse Transcription of equalised RNA

Complementary DNA (cDNA) is a doublestranded DNA version of an mRNA molecule. cDNA was made from equalised RNA using ABI High Capacity cDNA archive kit (Applied Biosystems). Equal volumes of Mastermix (containing RT Buffer (Reverse Transcriptase), dNTPs, random primers, multiscribe RT and RNase free H_2O) were added to an equivalent volume of equalised RNA (e.g. 30 µl Mastermix with 30 µl RNA giving 60 µl reaction volume) in PCR tubes. The samples were centrifuged and run on a PTC-200 Peltier Thermal Cycler DNA Engine on the ABI-RT program which runs for 10 minutes at 25 °C and then for 120 minutes at 37 °C. The cDNA was then removed and frozen at -80 °C until needed for RT-PCR.

2.3.5 RT-PCR procedure

A 1 in 4 dilution of cDNA was prepared with Sigma water (25 µl cDNA and 75 µl H_2O). Multiplex PCR was carried out, meaning that a probe for the endogenous marker β-actin (VIC-labelled MGB Taqman probe, Applied Biosystems) was included in each sample with the target probe, allowing each sample to be corrected for the amount of cDNA present. RT-PCR was performed using Taqman Gene Expression Assays (Applied Biosystems) which contain forward and reverse primers, and a FAM-labelled MGB Taqman probe to each gene of interest. The assay IDs for the genes examined as on the National centre for biotechnology information (NCBI) website are in Table 2.2.

A 10 µl volume of diluted cDNA was added to each well and 15 µl of reaction mix (12.5 µl Taqman Universal PCR Mastermix, 1.25 µl β-actin primer, 1.25 µl target primer) was added to give a final reaction volume of 25 µl. Electronic pipettes (EDP3 2-20 µl, 10-100 µl and 20-200 µl) were used to ensure pipetting accuracy. Samples were run on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Germany) for 40 or 45 cycles. The samples are first heated to 95 °C for 10 minutes to start the process before the cycles begin. The samples are heated to 95 °C for 15 seconds to allow denaturation of the double-stranded cDNA. The temperature then decreases to allow annealing and extension of the cDNA. The target probe is designed to anneal to the single-stranded cDNA as it has a higher melting point than that of the target primers (Applied Biosystems, Germany).

Table 2.2: List of genes from Applied Biosystems used in RT-PCR studies

Target	Gene Symbol	Gene Name	Taqman Gene Expression Assay ID*
β-actin	ACTB	actin, beta	4352340E
β_2-AR	Adrb2	adrenergic receptor, beta 2	Rn00560650_s1
CD40	Tnfrsf5	tumor necrosis factor receptor superfamily, member 5	Rn01423583_m1
GDNF	Gdnf	glial cell line derived neurotrophic factor	Rn00569510_m1
IFNgR1	Ifngr	interferon gamma receptor 1	Rn00587255_m1
IFNgR2	Ifngr2	interferon gamma receptor 2	Rn01458484_m1
IL-10	Il10	interleukin 10	Rn00563409_m1
IL-1R1	Il1r1	interleukin 1 receptor, type I	Rn00565482_m1
IL-1R2	Il1r2	interleukin 1 receptor, type 2	Rn00588589_m1
IL-1β	Il1b	interleukin 1 beta	Rn00580432_m1
iNOS	Nos2	nitric oxide synthase 2, inducible	Rn00561646_m1
IP-10	Cxcl10	chemokine (C-X-C motif) ligand 10	Rn00594648_m1
NGFβ	NGF	nerve growth factor, beta	Rn01533872_m1
TGFβ1	Tgfb1	transforming growth factor, beta 1	Rn00572010_m1
TNFR1	Tnfrsf1a	tumor necrosis factor receptor superfamily, member 1a	Rn01492348_m1
TNFR2	Tnfrsf1b	tumor necrosis factor receptor superfamily, member 1b	Rn00709830_m1
TNFα	Tnf	tumor necrosis factor (TNF superfamily, member 2)	Rn99999017_m1
VCAM-1	Vcam1	vascular cell adhesion molecule 1	Rn00563627_m1

*Gene reference as listed on the National centre for biotechnology information (NCBI) Entrez-Nucleotide website:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide

As well as the FAM/VIC dye, the probe contains a proprietary non-fluorescent quencher (NFQ) dye which blocks the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology. The temperature then falls and remains at 60 ºC for 1 minutes to allow transcription, the primers anneal and the cDNA strand is extended by the 5' nuclease activity of the Taq polymerase. This induces the release of the FAM/VIC labelled probe, which causes the FRET between the dye and the quencher to be broken. This generates a fluorescent signal. Due to the specificity of the probe and primers for the cDNA, one fluorescent signal is produced for each new cDNA copy and measured during the annealing phase of the PCR cycle.

2.3.6: RT-PCR analysis

The ΔΔCt method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression, as calculated relative to the endogenous control β-actin, and the control samples to give a Relative Quantification value (RQ) ($2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle). This method compares gene expression of treated samples to an untreated control sample instead of quantifying the exact copy number of the gene. This means that the fold-difference (increase or decrease) can be assessed between treated and untreated samples. The fold-difference is assessed using the cycle number (CT) difference between samples. A threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression, the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Samples with low CT readings demonstrate high fluorescence, indicating greater amplification and thus greater gene expression. To measure this fold-difference relative to control, the CT of the endogenous control (β-actin) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the CT difference (ΔCT). The ΔCT of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the ΔΔCT value (cycle difference corrected for β-actin). The ΔΔCT is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, the fold-difference in gene expression between the control and treated samples is given by 2 to the power of the -ΔΔCT. Thus when PCR is 100% efficient a five-cycle difference between samples means a 5-fold difference is a 32-fold difference (2^5). The control sample always has a ΔΔCT value of 0, thus 2^{-0} gives a $2^{-\Delta\Delta CT}$ of 1, against which all other samples are referenced, as outlined in Table 2.3 below.

Table 2.3: Example of the ΔΔCT method of analysis

	Target CT	β-actin CT	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$ Fold difference
Control	**20**	**16**	**20 -16 = 4**	**4 - 4 = 0**	$2^{-0} = 1$
Sample 1	**21**	**16**	**21 – 16 = 5**	**5 - 4 = 1**	$2^{-1} = 0.5$
Sample 2	**22**	**16**	**22 – 16 = 6**	**6 - 4 = 2**	$2^{-2} = 0.25$
Sample 3	**19**	**16**	**19 – 16 = 3**	**3 - 4 = -1**	$2^{-(-1)} = 2$
Sample 4	**18**	**16**	**18 – 16 = 2**	**2 – 4 = -2**	$2^{-(-2)} = 4$
Sample 5	**17**	**16**	**17 – 16 = 1**	**1 – 4 = -3**	$2^{-(-3)} = 8$

2.4 Protein analysis procedures

2.4.1 Harvesting cell-free supernatants for cytokine analysis

After the required incubation time, the 24 well plates were removed from the incubator and the supernatant was removed. Wells that received the same treatment were pooled into a 1.5 ml microtube. All samples were kept on ice before being centrifuged at 11000 x g for 3 minutes at 4 °C to remove any cell debris. Samples were then aliquoted (3 aliquots per sample) into new tubes and frozen at -80 °C until cytokine analysis. This procedure was repeated for each plate respectively.

2.4.2 Analysis of cytokine concentrations by ELISA

The sandwich enzyme-linked immunosorbent assay (ELISA) method was used to determine cytokine concentrations in supernatants taken from primary cell cultures.

TNFα: The concentration of TNFα was assessed in the supernatants from cultured cells. Analysis of TNFα was carried out by ELISA (R&D Systems, UK) as per instructions from manufacturers. The capture antibody (1.0 µg/ml goat anti-rat TNFα antibody) was diluted in coating buffer (1:250 dilution; 0.84 g $NaHCO_3$, 0.356 g Na_2CO_3, 100 ml dH_2O) and 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100 µl antibody and incubated overnight at 4 °C. They were then washed with wash buffer (three times with 300 µl Phosphate Buffered Saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) containing 0.05% Tween, then blotted on tissue paper to dry), blocked for 1 hour at room temperature (RT) with 200 μl blocking buffer (PBS, pH 7.3; 10% fetal calf serum (FCS)), washed with wash buffer, and incubated with 100 μl TNFα standards (0-2000 pg/ml) or samples in duplicate for 2 hours at RT. Wells were washed with wash buffer, incubated with 100 μl detection antibody (350 ng/ml biotinylated goat anti-rat antibody for TNFα, 1:250 dilution in blocking buffer) for 2 hours at RT, and washed again. They were then incubated in horseradish peroxidase (HRP)-conjugated streptavidin (1:250 dilution in blocking buffer) for 30 minutes at RT. Substrate solution (100 μl 3,3',5,5',-Tetramethyl-benzidine (TMB); R&D Systems, UK) was added. Incubation continued at RT in the dark for 30 minutes, or prior to the colorometric reaction reaching saturation, and the reaction was stopped using 50 μl 1 M H_2SO_4. Absorbance was read at 450 nm using a microtitre plate reader (Elx 800 Bio-Tek instruments Inc.). A standard curve was constructed by plotting the standards against the absorbance. The results obtained were expressed as TNFα concentration in pg/ml of supernatant or % control.

IL-1β: The concentration of IL-1β was assessed in the supernatants from cultured cells. Analysis of IL-1β was carried out by ELISA (DuoSet® ELISA Development System, R&D Systems, USA) as per instructions from manufacturers. The capture antibody (0.8 μg/ml goat anti-rat IL-1β antibody) was diluted in PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4; 1:180 dilution) and 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100 μl antibody and incubated overnight at RT. They were then washed with wash buffer (three times with 300 μl PBS containing 0.05% Tween, then blotted on tissue paper to dry), blocked for 1 hour at RT with 300 μl reagent diluent (1% BSA in PBS), washed with wash buffer, and incubated with 100 μl IL-1β standards (0-2000 pg/ml) or samples for 2 hours at RT. Wells were washed with wash buffer, incubated with 100 μl detection antibody (350 μg/ml biotinylated goat anti-rat antibody for IL-1β diluted in PBS containing 1% BSA and 2% normal goat serum (Vector, USA)) for 2 hours at RT and washed again. They were then incubated in HRP-conjugated streptavidin (1:250 dilution in blocking buffer) for 20 minutes at RT. Substrate solution (100 μl TMB; R&D Systems, UK) was added. Incubation continued at RT in the dark for 30 minutes, or prior to the colorometric reaction reaching saturation, and the reaction was stopped using 50 μl 1 M H_2SO_4. Absorbance was read at 450 nm using a microtitre plate reader (Elx 800 Bio-Tek instruments Inc.). A standard curve was constructed by plotting the standards against the absorbance. The results obtained were expressed as IL-1β concentration in pg/ml of supernatant or % control.

2.5 Analysis of cell viability and cell death

2.5.1 Alamar Blue cell viability assay

The Alamar Blue Assay was used in this study to assess cell viability. It is based on the ability of living cells to convert a redox dye (resazurin, $C_{12}H_6NNaO_4$) into a fluorescent end product (resorufin). Viable cells retain the ability to reduce resazurin into resorufin. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. A 440 μM solution of Alamar Blue dye was made by dissolving Resazurin (Sigma) in ddH_2O. Cells were treated as required (As), and one set of wells which contained media only (Am) and another set which had cells with no treatments (Ac) were also included. Plates were incubated at 37 °C and 5% CO_2 for 24 hours. At 1 to 4 hours prior to the endpoint the Alamar Blue dye was added to the wells at 10%v/v (e.g. 25 μl dye added to 250 μl media on cells). When colour change had occurred, 200 μl of supernatant was transferred to 96 well plates. Absorbance was read at 600 nm using a microtitre plate reader (Elx 800 Bio-Tek instruments Inc.). Results were calculated as follows:

Am = Average absorbance of wells containing Media only

Ac = Average absorbance of wells containing Cells without treatments

As = Absorbance of a particular sample

% Viability = ((Am – As)/(Am – Ac))*100

2.5.2 Lactate dehydrogenase (LDH) cytotoxicity assay

The lactate dehydrogenase (LDH) assay (CytoTox 96® kit, Promega) was used in this study, according to manufacturer's instructions, to measure cell toxicity. LDH is a stable cytosolic enzyme that is released from cells upon lysis. Measuring the levels of LDH in the supernatant inversely allows for an indirect measurement of LDH present in the cytoplasm of intact 'healthy' cells, and thus a measurement of cell toxicity. The supernatants of primary cell culture were removed and stored in 1.5 ml microtubes on ice, before centrifuging them at 15000 x rpm to pellet any cellular debris. The adherent primary cells were exposed to 15 μl of lysis solution (10x; 9% (v/v) Triton-x-100 in water) per 100 μl of culture medium, followed by incubation at 37 °C for 45-60 minutes. 50 μl of the cell lysate was measured in conjunction with 50 μl of supernatant for every experimental treatment (in duplicate) by incubating with 50 μl of reconstituted substrate mix in a 96-well plate, for 30 minutes at 37 °C. Following this, 50 μl of stop solution was added to arrest the reaction. Absorbance was measured at 490 nm on a microtitre plate reader (Elx 800 Bio-Tek instruments Inc.). The data was interpreted by expressing "% max LDH release" as LDH absorbance in the supernatant (released) / LDH absorbance in the cell lysate (total cellular LDH).

2.6 Fluorescence immunocytochemistry

The following describes the general protocol used to identify proteins of interest, which were carried out following *in vitro* cell treatments on cells grown on glass coverslips. The specific details for every protein assessed by fluorescent immunocytochemistry in this thesis are presented in Table 2.4.

2.6.1 General protocol for fluorescence immunocytochemistry

Cells were fixed with ice-cold methanol for 5 minutes at -20 °C, and washed twice with PBS. Non-reactive sites were blocked for 2 hours at RT in blocking buffer (PBS containing animal serum; Vector laboratories, Peterborough, UK). Cell manipulations were carried out in a light protected humidified environment from this point. The blocking buffer was removed and cells were incubated overnight at 4 °C with primary antibody raised against the protein of interest. The primary antibody was washed off with PBS and cells were incubated with an appropriate secondary antibody conjugated to an Alexa (Invitrogen). The cells were then washed in PBS. Nuclei were counterstained with Hoechst stain (Hoechst 33258, Invitrogen, Paisley, UK). The coverslips were mounted onto glass slides using Vectashield fluorescent mounting media (Vector Laboratories, Peterborough, UK). The edge of each coverslip was sealed with clear nail varnish and slides were stored in the dark at 4 °C until needed for analysis. The incorporated fluorophores were examined with a confocal microscope (Zeiss, LSM-510-META, Karl Zeiss, UK) using appropriate excitation wavelengths and filter sets (Table 2.5).

This procedure was carried out as outlined below using GFAP/β1-AR as an example, using the combinations of antibodies as described in Table 2.4. The blocking buffer for all antibodies was PBS containing 10% goat serum (v/v) and all primary and secondary antibodies were made up in PBS containing 5% goat serum (v/v).

Table 2.4: Combinations of antibodies used for fluorescence immunocytochemistry

1st Primary antibody	1st Secondary antibody	2nd Primary antibody	2nd Secondary antibody
Anti-GFAP (1:2000)	Goat anti-rabbit Alexa 488 (1:4000)	Anti-β_1-AR (1:200)	Goat anti-rabbit Alexa 546 (1:500)
Anti-GFAP (1:2000)	Goat anti-rabbit Alexa 488 (1:4000)	Anti-β_2-AR (1:200)	Goat anti-rabbit Alexa 546 (1:500)
Anti-CD11b (1:4000)	Goat anti-mouse Alexa 488 (1:4000)	Anti-β_1-AR (1:200)	Goat anti-rabbit Alexa 546 (1:500)
Anti-CD11b (1:4000)	Goat anti-mouse Alexa 488 (1:4000)	Anti-β_2-AR (1:200)	Goat anti-rabbit Alexa 546 (1:500)
Anti-Neu-N (1:300)	Goat anti-mouse Alexa 546 (1:500)	Anti-GFAP (1:2000)	Goat anti-rabbit Alexa 488 (1:4000)
Anti-Neu-N (1:300)	Goat anti-mouse Alexa 546 (1:500)	Anti-β_1-AR (1:200)	Goat anti-rabbit Alexa 488 (1:500)
Anti-Neu-N (1:300)	Goat anti-mouse Alexa 546 (1:500)	Anti-β_2-AR (1:200)	Goat anti-rabbit Alexa 488 (1:500)
Anti-βIII-tubulin (1:300)	Goat anti-mouse Alexa 546 (1:500)		

Table 2.5 List of confocal microscope configurations

Fluorophore	Peak excitation/emission wavelength (nm)	Beam splitters
Alexa Fluor 488TM	488/520	HFT 488; LP 505
Alexa Fluor 546TM	546/520	HFT 546; LP 505
Hoechst 33258	345/487	HFT UV/488/543/633 LP 456 - 499

GFAP/β1-AR: Following treatment, cells were fixed with ice-cold methanol for 5 minutes at -20 ºC, and washed twice with PBS. Non-reactive sites on cells were blocked in blocking buffer (PBS containing 10% goat serum (v/v); Vector Laboratories, Peterborough, UK) for 2 hours at RT. Cell manipulations were carried out in a light protected humidified environment from this point. The blocking buffer was removed and cells were incubated with the first primary antibody, rabbit anti-GFAP (1:2000 dilution in PBS containing 10% goat serum, Dako), overnight at 4 °C. Cells were washed 3 times for 5 minutes in PBS. Cells were incubated with the first secondary antibody, goat anti-rabbit IgG conjugated to Alexa 488 (1:4000 in PBS containing 5% goat serum (v/v); Invitrogen), for 1.5 hours at RT. Cells were washed 3 times for 5 minutes in PBS. Cells were once more blocked in blocking buffer (PBS containing 10% goat serum (v/v); Vector Laboratories, Peterborough, UK) for 2 hours at RT. The blocking buffer was removed and cells were incubated with the second primary antibody, rabbit anti-β_1-AR (1:200 dilution in PBS containing 10% goat serum (v/v); Santa Cruz Biotechnology Inc., California, USA) overnight at 4 °C. Coverslips were washed 3 times for 5 minutes in PBS. Cells were incubated with the second secondary antibody, goat anti-rabbit IgG conjugated to Alexa 546 (1:500 in PBS containing 5% goat serum (v/v); Invitrogen), for 1.5 hours at RT. Cells were washed 3 times for 5 minutes in PBS. Nuclei were counterstained with Hoechst stain (1:1000 in PBS, Hoechst 33258, Invitrogen, Paisley, UK). Cells were washed for 1 hour every 5 minutes in PBS before mounting on-to glass slides using fluorescent mounting medium (Vectashield®; Vector Laboratories, Peterborough, UK). The edge of each coverslip was sealed with nail varnish and slides were stored in the dark at 4 °C until needed for analysis. The incorporated fluorophores were examined with a confocal microscope (Zeiss, LSM-510-META, Karl Zeiss, UK) using appropriate excitation wavelengths and filter sets (Table 2.5).

Primary antibodies:

Mouse Anti-Neu-N (1:300; Millipore)
Mouse Anti-βIII-tubulin (1:300; Promega)
Mouse Anti-CD11b (1:4000; Millipore)
Rabbit Anti-GFAP (1:2000; Dako)
Rabbit Anti-β1-AR (1:200; Santa Cruz Biotechnology)
Rabbit Anti-β2-AR (1:200; Santa Cruz Biotechnology)

Secondary antibodies:

Goat anti-mouse Alexa 488 (Invitrogen)
Goat anti-mouse Alexa 546 (Invitrogen)
Goat anti-rabbit Alexa 488 (Invitrogen)
Goat anti-rabbit Alexa 546 (Invitrogen)

2.7 Statistical Analysis of Results

All data was analysed using Prism 4 (GraphPad Prism, California, USA) or a GB-STAT routine. Statistical comparisons were initially performed using a one- or two-way analysis of variance (ANOVA), without repeated measures, or a student t-test with pooled variance, as indicated in the experimental sections. If significant changes were observed, the data was further analysed using Newman-Keuls or Fisher's LSD *post hoc* test as appropriate. A p value of less than 0.05 was considered statistically significant. Results are expressed as means and standard error of the mean (SEM) or mean percentage control and SEM.

3. The anti-inflammatory actions of NA in glial cells – a role for β_2-ARs and cAMP?

3.1 Introduction

Glial cell activation and neuroinflammation play an important role in the pathogenic, and possibly even the initiation, stages of many neurodegenerative disorders. It has long been known that microglia are the innate immune cells of the CNS and that they become activated and produce an immune response when exposed to an inflammatory stimulus (Liu and Hong, 2003). However recent studies have also implicated astrocytes in the immune response (Liberto et al., 2004). The aim of this section was to investigate the ability of LPS, thrombin, and Aβ to induce an inflammatory response in primary mixed glial, microglial, and astrocyte cultures and to examine the ability of NA interventions to prevent this inflammatory response.

Many different molecules are capable of activating glia and inducing an inflammatory response. One such molecule is LPS, a gram negative bacterial cell wall endotoxin which is commonly used to induce an inflammatory response under experimental conditions (Liu and Hong, 2003). Primary microglia stimulated with LPS produced inflammatory cytokines including IL-1β and TNFα, and the chemokine MCP-1 (Szczepanik et al., 2001). Astrocytes also produce iNOS and pro-inflammatory cytokines such as IL-1β, TNFα, and IL-6 in response to LPS (Forloni et al., 1997, Nicholson et al., 2004, Iwai et al., 2006). LPS is extremely useful in inducing an inflammatory response but it is not representative of the inflammation that occurs during neurodegenerative disorders. For this reason it was decided to use two other molecules that are involved in the pathology of neurodegeneration; thrombin, which is involved in the acute neuroinflammation induced following a stroke or traumatic brain injury and during PD, and Aβ, which is involved in the chronic neuroinflammation induced during AD.

Thrombin is a blood protein that leaks into the brain parenchyma when the BBB is damaged, such as occurs in hemorrhagic stroke and traumatic brain injury, causing acute brain injury (Hua et al., 2007, Hua et al., 2009). *In vitro* studies on thrombin showed that it results in activation of microglia (Lee et al., 2005, Katsuki et al., 2006), and induces morphologic changes in and increases proliferation of astrocytes (Ishida et al., 2006). It also promotes an inflammatory environment by inducing iNOS and increasing expression of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6

(Moller et al., 2000, Ryu et al., 2000, Choi et al., 2003, Choi et al., 2005, Lee et al., 2005, Huang et al., 2008).

Aβ is a protein which aggregates to form plaques in the brains of patients with AD (Blennow, 2004). Activated astrocytes and microglia are found to localise around the plaques (Liu and Hong, 2003, Stuchbury and Munch, 2005). Aβ-stimulated primary microglia produced the inflammatory cytokines IL-1α, IL-1β, TNFα, and IL-6, and the chemokine MCP-1 (Szczepanik et al., 2001). Another study showed that in astrocyte cultures treated with Aβ, the production of IL-1β and TNFα occurred before the production of iNOS (Akama and Van Eldik, 2000). Exposure of cultured microglia or monocytes to Aβ results in a dose-dependent increase in their secretion of the chemokines IL-8, MIP-1α, and MCP-1, and the growth factor M-CSF (Rogers et al., 2002, McGeer and McGeer, 2003).

Upon activation, glia release pro-inflammatory molecules that can directly or indirectly cause damage to neural cells (Liberto et al., 2004). The nature of the inflammatory response in these studies was assessed by measuring a range of inflammatory molecules: iNOS, IL-1β, TNFα, CD40, IP-10, and VCAM-1. The enzyme iNOS is typically produced in response to inflammation and pro-inflammatory mediators (Stuehr, 1999), resulting in the production of large quantities of NO (Moncada, 1999) leading to effects such as oxidative stress and cytotoxicity (Pannu and Singh, 2006). IL-1β and TNFα are pro-inflammatory cytokines and among the first pro-inflammatory mediators to be released in response to glial activation (Liberto et al., 2004, Lucas et al., 2006). They are potent modulators of the immune response, causing the induction of cytokines, chemokines, adhesion molecules, and inflammatory enzymes such as iNOS (Laflamme et al., 1999, Akama and Van Eldik, 2000, Aggarwal, 2003). CD40 is a co-stimulatory molecule necessary for the activation of APCs and their ability to present antigen to T-cells (Mills and McGuirk, 2004). It can be induced by LPS and cytokines (Nguyen et al., 1998, Chen et al., 2006), and becomes up-regulated upon microglial activation making it a useful indicator of an inflammatory response (O'Keefe et al., 2002). The pro-inflammatory chemokine IP-10 is a potent chemoattractant for monocytes, T lymphocytes, and NK cells (Baggiolini et al., 1997), and it is produced in response to cytokines and LPS (Kawai et al., 2001, Shen et al., 2006, Ubogu et al., 2006). The cell adhesion molecule VCAM-1 is involved in the firm adhesion of leukocytes to the endothelium, a process that is essential for the movement of leukocytes out of the circulatory system, towards the site of tissue damage or infection (Luster, 1998, van Buul and Hordijk, 2008).VCAM-1 is also increased by cytokines (Galkina and Ley, 2007, Wu, 2007). These molecules are markers of glial activation, and are extremely useful in determining the magnitude and nature of an immune response. Microglia and astrocytes have been shown to express iNOS (Guo and Bhat, 2006), IL-1β (Allan et al., 2005), TNFα (Aggarwal, 2003, Ohtori et al., 2004, Lucas et al., 2006), CD40 (van Kooten and Banchereau, 1997, Abdel-Haq et al., 1999, Chen et al., 2006), IP-10 (Kawai et al., 2001, Shen et al., 2006, Ubogu et al., 2006), and VCAM-1 (Lee and Benveniste, 1999, Wu et al., 2000, Peterson et al., 2002).

A major focus of research in the treatment of neurodegeneration is the inhibition of neuroinflammation. With regard to this, this project has focused on NA, a monoamine neurotransmitter which has been reported to elicit anti-inflammatory actions in the CNS. NA signalling is also important in neurodegenerative disorders. For example, loss of LC noradrenergic neurons in AD increases neuronal damage, inflammatory responses, and iNOS, IL-1β, and IL-6 expression, and reduces Aβ clearance (Heneka et al., 2002, Heneka et al., 2003, Kalinin et al., 2006, Kalinin et al., 2007), and traumatic brain injury causes decreased cAMP levels and downstream activation of PKA (Atkins et al., 2007). Both astrocytes and microglia have been shown to express receptors for NA indicating that NA is capable of eliciting effects upon glial cells (Tanaka et al., 2002, De Keyser et al., 2004). Studies using microglia, astrocytes, and brain endothelial cells have demonstrated that NA, by binding to β_2-ARs and activating cAMP signalling pathways, can inhibit the expression of a number of inflammatory genes including iNOS, IL-1β, TNFα, MHC-class-II, and ICAM-1 (Feinstein et al., 2002, Tanaka et al., 2002, De Keyser et al., 2004). NA, β-AR agonists, and cAMP activators can also suppress the IL-1β- and TNFα-induced expression of iNOS, VCAM-1, and ICAM-1 (Ballestas and Benveniste, 1997, Balyasnikova et al., 2000), the LPS-induced production of IL-1β mRNA and protein from microglia (Madrigal et al., 2005), and the LPS-induced TNFα and MIP-1β release in a dose-dependent manner in macrophages *in vitro* and *in vivo* (Verhoeckx et al., 2005a, Verhoeckx et al., 2005b).

The objective of these experiments was to investigate the ability of thrombin and Aβ to induce an inflammatory phenotype in glial cells. LPS was used as a positive control to ensure that the cells were capable of producing an inflammatory response. Of particular interest was the ability of thrombin and Aβ to induce an inflammatory response in mixed glial, microglial and astrocyte cultures, and the nature of this inflammatory response. Once an inflammatory response had been achieved the objective was to investigate the ability of NA interventions to prevent this by promoting an anti-inflammatory phenotype, and to investigate the pathways by which NA mediates its effects, with particular emphasis on β-AR activation and subsequent downstream actions.

3.2 LPS, thrombin, and Aβ induce an inflammatory response in mixed glial cells

The following studies were carried out to assess the ability of LPS, thrombin, and Aβ to induce an inflammatory response in mixed glial cells. Vehicle, LPS (100 ng/ml), thrombin (10 or 20 U/ml), or Aβ (2.5, 5 or 10 μM) was administered to cortical mixed glial cultures for 6 hours prior to the analysis of gene expression by RT-PCR. Concentrations of the drugs were chosen based on the literature and on previous results achieved in the laboratory with similar concentrations. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Specifically, the mRNA expression of the pro-inflammatory enzyme iNOS, the pro-inflammatory cytokines, IL-1β and TNFα, the co-stimulatory molecule CD40, the inflammatory chemokine IP-10 which is predominantly chemotactic for T-cells and monocytes, and the VCAM-1 which promotes adhesion and infiltration of peripheral leucocytes, were examined as they are reliable indicators of an inflammatory response. Vehicle, LPS, thrombin, or Aβ was also administered to cortical mixed glial cultures for 24 hours and the cell-free supernatants were harvested for the assessment of cytokine production by ELISA. Specifically, the protein expression of the pro-inflammatory cytokines, IL-1β and TNFα, were examined, as they are good indicators of whether or not any changes in mRNA expression were being translated to the protein level.

LPS induces an inflammatory response: LPS was used as a positive control to show that the cultures were capable of producing an inflammatory response. LPS significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression (Figure 3.2.1). LPS also significantly increased TNFα and IL-1β protein expression (Figure 3.2.2). This shows that LPS is capable of inducing an inflammatory response in mixed glial cells.

Thrombin induces an inflammatory response: Both doses of thrombin (10 and 20 U/ml) significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression (Figure 3.2.3). Thrombin (10 and 20 U/ml) also significantly increased TNFα protein expression and thrombin (20 U/ml) increased IL-1β protein (Figure 3.2.4). This shows that thrombin is capable of inducing an inflammatory response in mixed glial cells.

Aβ induces an inflammatory response: All doses of Aβ (2.5, 5, and 10 μM) significantly increased IL-1β and TNFα mRNA expression. Only the highest dose (10 μM) significantly increased CD40. There was no effect on iNOS, VCAM-1, or IP-10 mRNA (Figure 3.2.5). Aβ also significantly increased IL-1β protein expression, but not TNFα protein (Figure 3.2.6). This shows that Aβ is capable of inducing an inflammatory response in mixed glial cells, although it is slightly less robust than the response induced by LPS or thrombin.

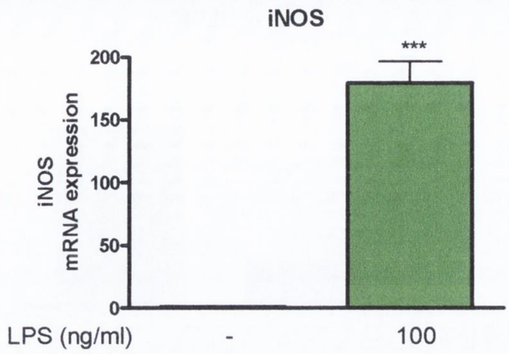
Figure 3.2.1: LPS induces an inflammatory response in mixed glial cells

LPS (100 ng/ml) was administered to mixed glial cells for 6 hours. A Student t-test demonstrated that LPS (100 ng/ml) significantly increased (a) iNOS ($P<0.0001$), (b) IL-1β ($P=0.0003$), (c) TNFα ($P<0.0001$), (d) CD40 ($P=0.0005$), (e) VCAM-1 ($P<0.0001$), and (f) IP-10 ($P<0.0001$) mRNA expression.

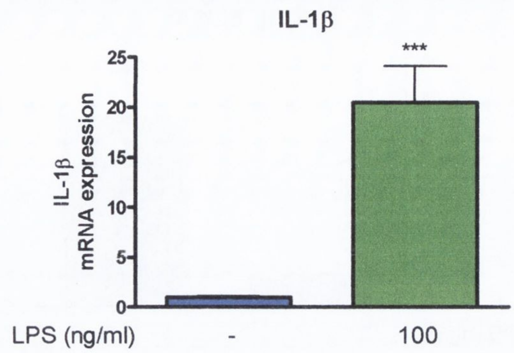
Data expressed as means ± SEM (n=5-6), ***$P<0.001$ vs. control (Student t-test).

Figure 3.2.1:

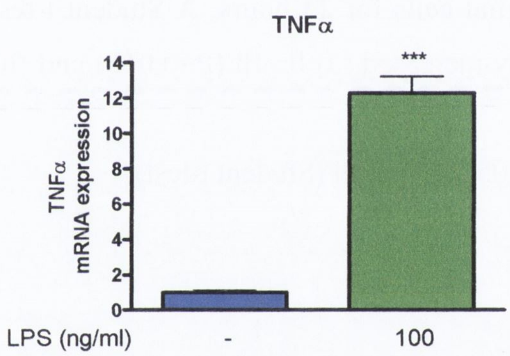
(a) iNOS mRNA



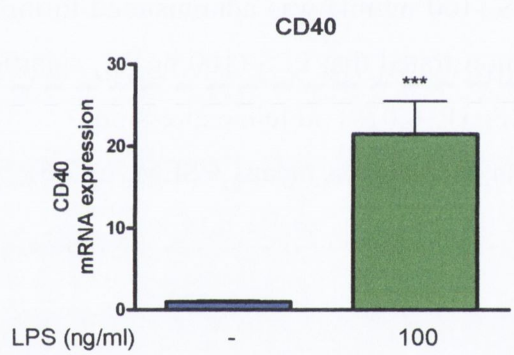
(b) IL-1β mRNA



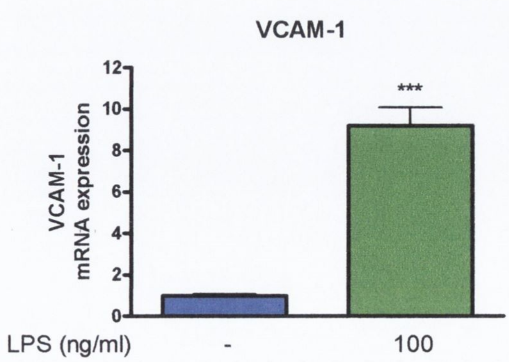
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

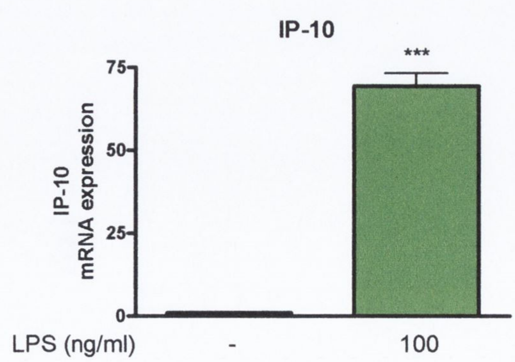


Figure 3.2.2:

(a) IL-1β protein (b) TNFα protein

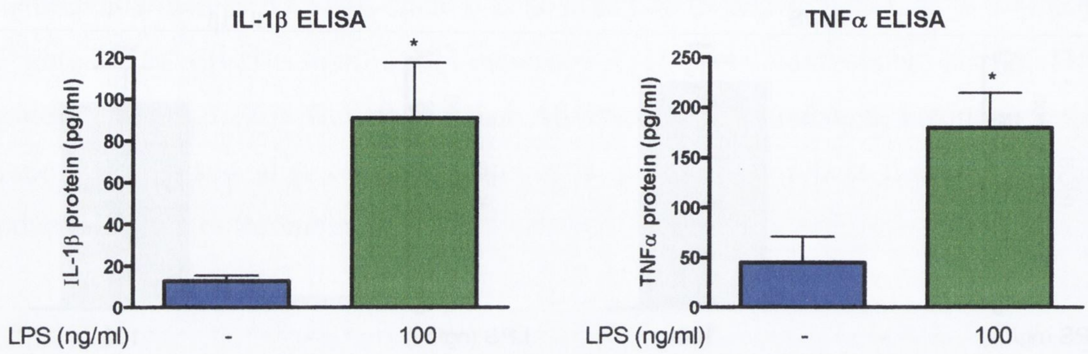


Figure 3.2.2: LPS induces an inflammatory response in mixed glial cells

LPS (100 ng/ml) was administered to mixed glial cells for 24 hours. A Student t-test demonstrated that LPS (100 ng/ml) significantly increased (a) IL-1β (P=0.015) and (b) TNFα (P=0.015) protein expression.

Data expressed as means ± SEM (n=5-6), *P<0.05 vs. control (Student t-test).

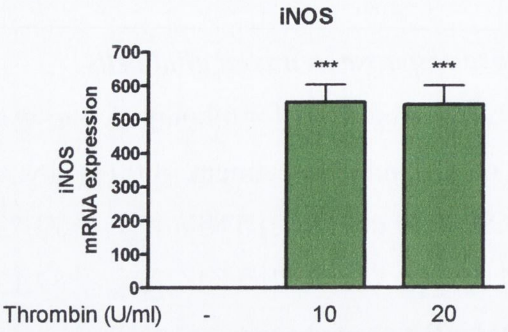
Figure 3.2.3: Thrombin induces an inflammatory response in mixed glial cells

Thrombin (10 or 20 U/ml) was administered to mixed glial cells for 6 hours. A one-way ANOVA demonstrated a significant effect of thrombin treatment on (a) iNOS [$F_{(2,15)}$=49.45, P<0.0001], (b) IL-1β [$F_{(2,15)}$=101.50, P<0.0001], (c) TNFα [$F_{(2,15)}$=72.73, P<0.0001], (d) CD40 [$F_{(2,14)}$=116.10, P<0.0001], (e) VCAM-1 [$F_{(2,15)}$=37.13, P<0.0001], and (f) IP-10 [$F_{(2,15)}$=97.56, P<0.0001] mRNA expression.

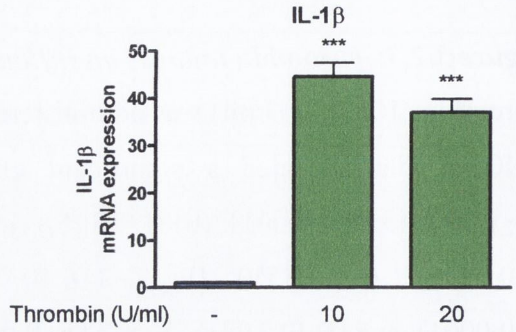
Data expressed as means ± SEM (n=5-6), ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 3.2.3:

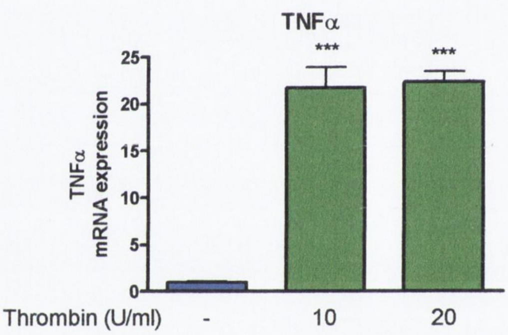
(a) iNOS mRNA



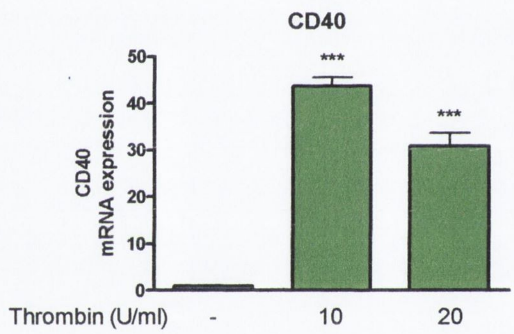
(b) IL-1β mRNA



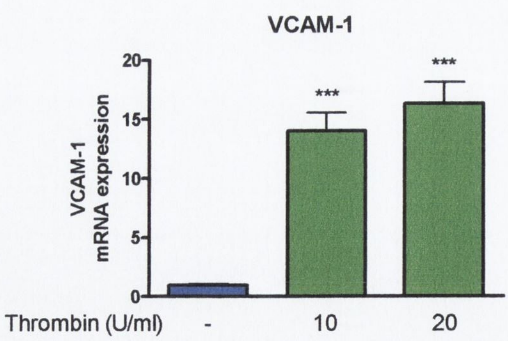
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

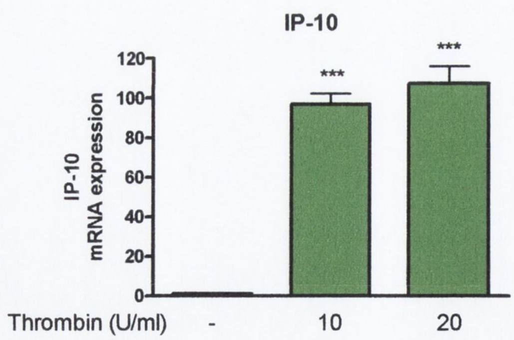
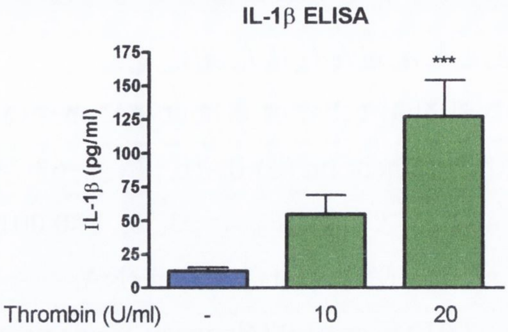


Figure 3.2.4:

(a) IL-1β protein



(b) TNFα protein

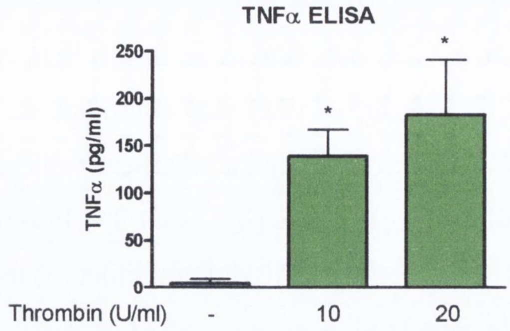


Figure 3.2.4: Thrombin induces an inflammatory response in mixed glial cells

Thrombin (10 or 20 U/ml) was administered to mixed glial cells for 24 hours. A one-way ANOVA demonstrated a significant effect of thrombin treatment on (a) IL-1β [$F_{(2,14)}$=12.13, P=0.0009] and (b) TNFα [$F_{(2,9)}$=5.58, P-=0.027] protein expression.

Data expressed as means ± SEM (n=5-6), *P<0.05, ***P<0.001 vs. control (Newman-Keuls post-hoc test).

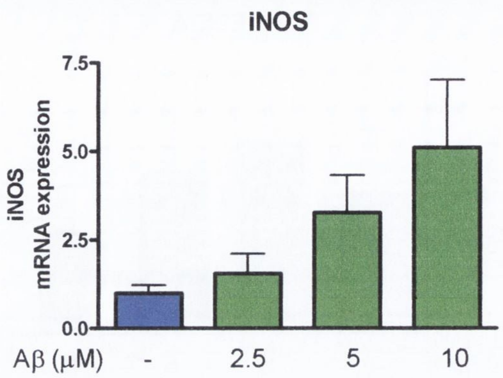
Figure 3.2.5: Aβ induces an inflammatory response in mixed glial cells

Aβ (2.5, 5, or 10 μM) was administered to mixed glial cells for 6 hours. A one-way ANOVA demonstrated a significant effect of Aβ treatment on (b) IL-1β [$F_{(3,20)}$=68.74, P<0.001], (c) TNFα [$F_{(3,20)}$=61.37, P<0.001], and (d) CD40 [$F_{(3,20)}$=93.30, P<0.001] mRNA expression. Aβ did not affect (a) iNOS, (e) VCAM-1, or (f) IP-10 mRNA.

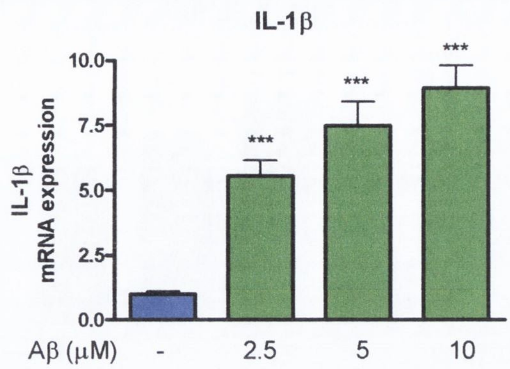
Data expressed as means ± SEM (n=5-6), ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 3.2.5:

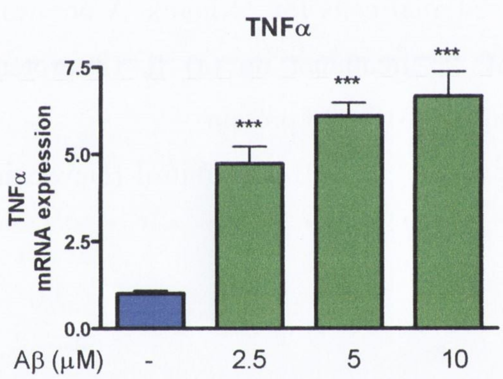
(a) iNOS mRNA



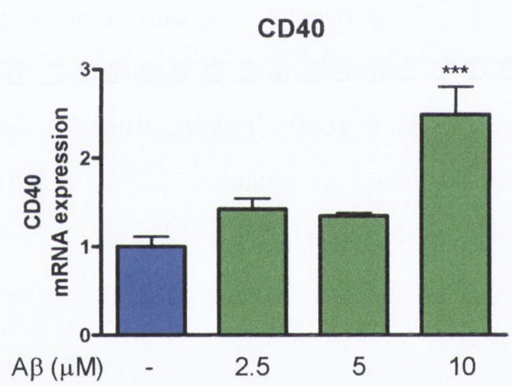
(b) IL-1β mRNA



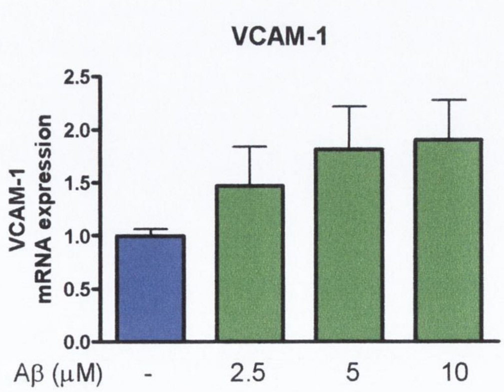
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

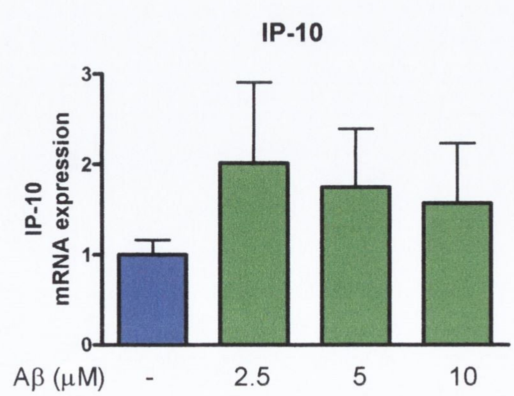


Figure 3.2.6:

(a) IL-1β protein (b) TNFα protein

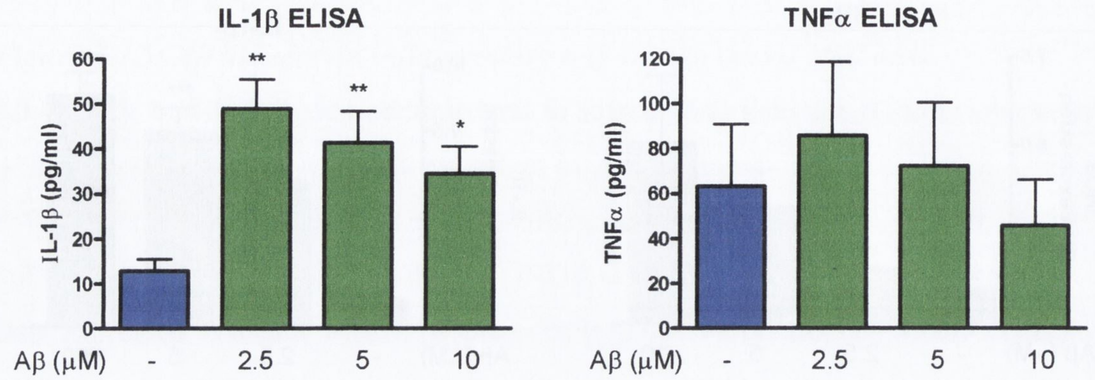


Figure 3.2.6: Aβ induces an inflammatory response in mixed glial cells

Aβ (2.5, 5, or 10 μM) was administered to mixed glial cells for 24 hours. A one-way ANOVA demonstrated a significant effect of Aβ treatment on (a) IL-1β protein [$F_{(3,18)}$=7.65, P=0.0017] expression. Aβ did not affect (b) TNFα protein.

Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control (Newman-Keuls post-hoc test).

3.3 LPS, thrombin, and Aβ induce an inflammatory response in microglia and astrocytes

The following studies were carried out to assess the ability of thrombin and Aβ to induce an inflammatory response in microglia and astrocytes. LPS (100 ng/ml), thrombin (20 U/ml), or Aβ (10 μM) was administered to cortical microglial or astrocyte cultures for 6 hours prior to the analysis of gene expression by RT-PCR. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Specifically, the mRNA expression of the inflammatory mediator iNOS, the pro-inflammatory cytokines IL-1β and TNFα, the co-stimulatory molecule CD40, and the inflammatory chemokine IP-10, were examined as they are reliable indicators of an inflammatory response.

LPS induces an inflammatory response in microglia and astrocytes: LPS was used as a positive control to show that the cultures were capable of producing an inflammatory response. LPS (100 ng/ml) significantly increased iNOS, IL-1β, TNFα, CD40, and IP-10 mRNA expression in microglia and astrocytes (Figure 3.3.1). This shows that LPS is capable of inducing an inflammatory response in separated glial cells.

Thrombin induces an inflammatory response in microglia and astrocytes: Thrombin (20 U/ml) significantly increased iNOS, IL-1β, TNFα, CD40, and IP-10 mRNA expression in microglia and astrocytes (Figure 3.3.1). This shows that thrombin is capable of inducing an inflammatory response in separated glial cells, largely similar in magnitude to that induced by LPS.

Aβ induces an inflammatory response in microglia and astrocytes: In microglia, Aβ (10 μM) significantly increased IL-1β, TNFα, CD40, and IP-10 mRNA expression, but not iNOS. In astrocytes, Aβ significantly increased iNOS, IL-1β, TNFα, CD40, and IP-10 mRNA expression (Figure 3.3.1). This shows that Aβ is capable of inducing an inflammatory response in separated glial cells, although it is much less robust than that induced by LPS or thrombin. Aβ induced a response in more of the markers tested in astrocytes than were induced in microglia, indicating that astrocytes also play a very important role in mediating the immune response to Aβ.

Figure 3.3.1: LPS, thrombin, and Aβ induce an inflammatory response in microglia and astrocytes

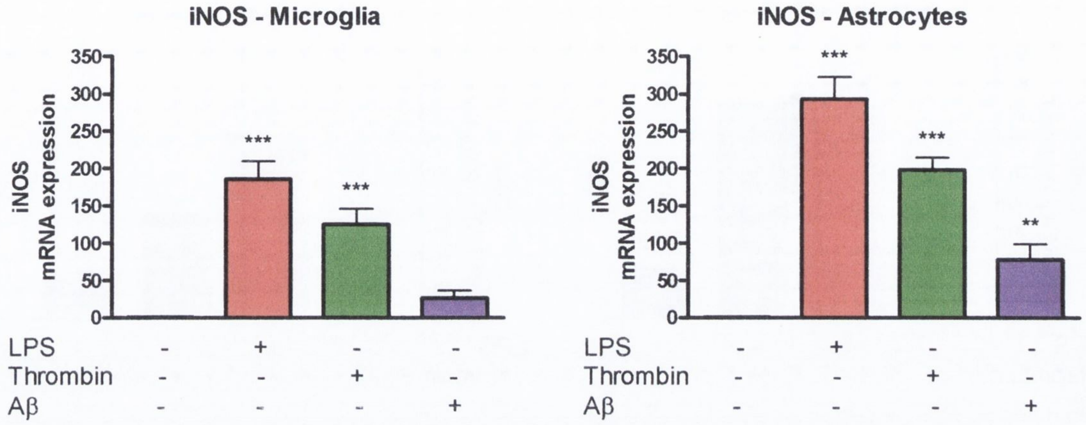
LPS (100 ng/ml), thrombin (20 U/ml), or Aβ (10 μM) was administered to cortical microglial or astrocyte cultures for 6 hours. A Student t-test demonstrated that in astrocytes, LPS (100 ng/ml) significantly increased (a) iNOS ($P<0.0001$), (b) IL-1β ($P<0.0001$), (c) TNFα ($P<0.0001$), (d) CD40 ($P<0.0001$), and (d) IP-10 ($P<0.0001$) mRNA expression, and in microglia it also significantly increased (a) iNOS ($P<0.0001$), (b) IL-1β ($P=0.0002$), (c) TNFα ($P<0.0001$), (d) CD40 ($P<0.0001$), and (d) IP-10 ($P<0.0001$) mRNA expression. A Student t-test demonstrated that in astrocytes, thrombin (20 U/ml) significantly increased (a) iNOS ($P<0.0001$), (b) IL-1β ($P<0.0001$), (c) TNFα ($P<0.0001$), (d) CD40 ($P<0.0001$), and (d) IP-10 ($P<0.0001$) mRNA expression, and in microglia it also significantly increased (a) iNOS ($P=0.0006$), (b) IL-1β ($P=0.0002$), (c) TNFα ($P=0.0002$), (d) CD40 ($P<0.0001$), and (d) IP-10 ($P<0.0001$) mRNA expression. A Student t-test demonstrated that in astrocytes, Aβ (10 μM) significantly increased (a) iNOS ($P=0.0084$), (b) IL-1β ($P<0.0001$), (c) TNFα ($P<0.0001$), (d) CD40 ($P=0.0039$), and (d) IP-10 ($P=0.0087$) mRNA expression, and in microglia it also significantly increased (b) IL-1β ($P=0.0003$), (c) TNFα ($P=0.0001$), (d) CD40 ($P=0.0001$), and (d) IP-10 ($P=0.0010$), but not (a) iNOS mRNA expression.

Data expressed as means ± SEM (n=5-6), *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. media control (Student's t-test).

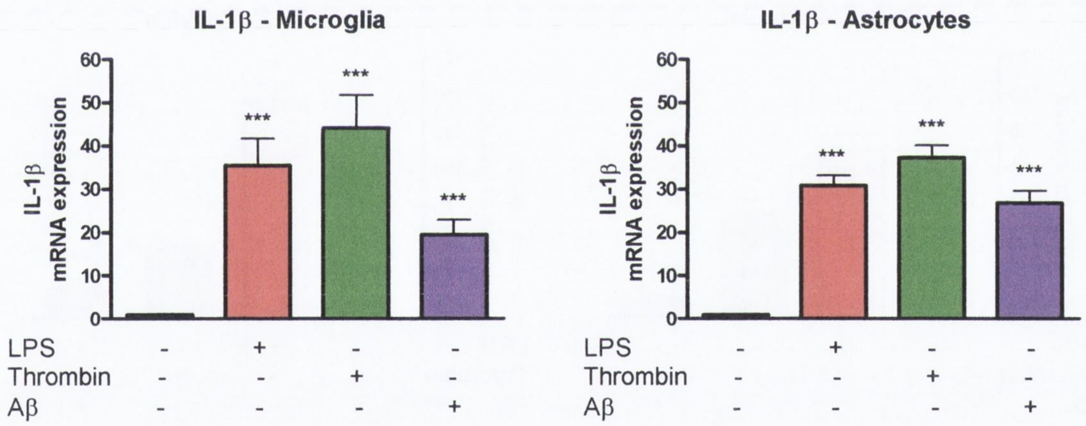
Figure 3.3.1:

Microglia **Astrocytes**

(a) iNOS mRNA



(b) IL-1β mRNA



(c) TNFα mRNA

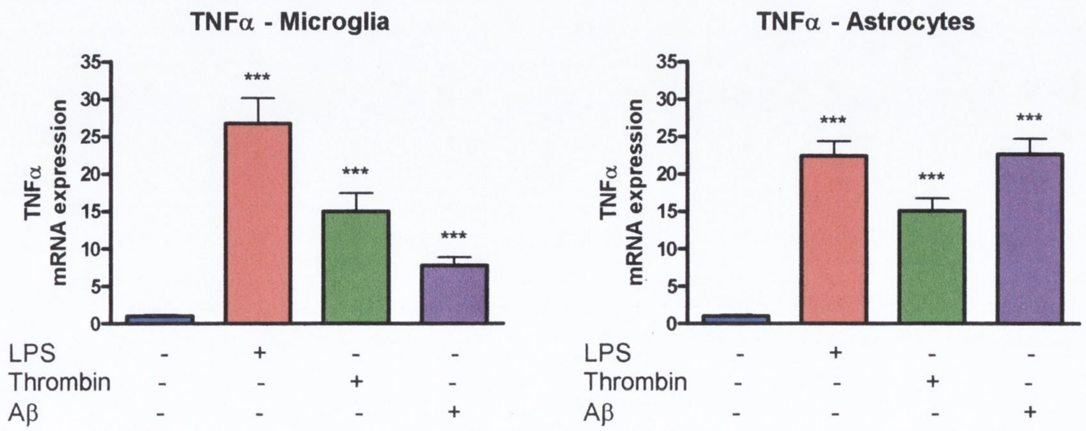
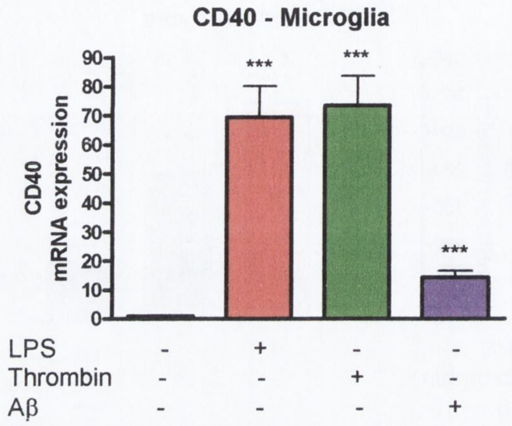
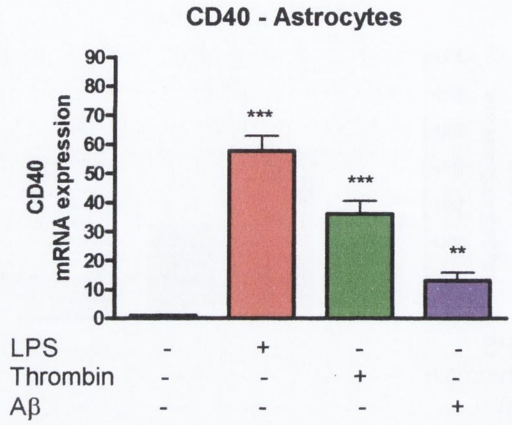


Figure 3.3.1:

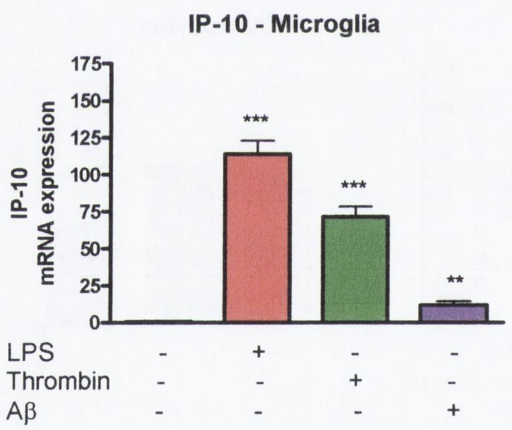
Microglia **Astrocytes**

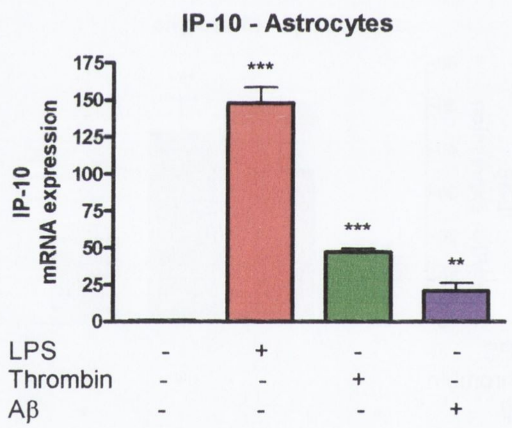
(d) CD40 mRNA





(d) IP-10 mRNA





3.4 NA attenuates the inflammatory response induced by thrombin and Aβ in mixed glial cells

As thrombin and Aβ had been shown to induce an immune response in mixed glial cells, the next step was to look at possible ways to reduce this inflammation. NA, a monoamine neurotransmitter, has been reported to elicit anti-inflammatory actions in the CNS. The following studies were carried out to assess the ability of NA to alter the inflammatory response induced by thrombin or Aβ in mixed glial cultures. Vehicle or NA (1 or 10 μM) was administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle, thrombin (20 U/ml), or Aβ (10 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. When thrombin was used as a treatment, the expression of iNOS, IL-1β, TNFα, CD40, IP-10, and VCAM-1 mRNA were examined. When Aβ was used as a treatment, the expression of IL-1β, TNFα, and CD40 mRNA only were examined as in the prior experiment these molecules had shown the most dramatic effect in response to Aβ. Alternatively cells were harvested after 24 hours and the cell-free supernatants were harvested for the assessment of cytokine production by ELISA. Specifically, the expression of TNFα protein was examined.

NA attenuates the inflammatory response induced by thrombin: Thrombin significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression. Both doses of NA (1 and 10 μM) significantly attenuated the thrombin-induced increase in TNFα, CD40, VCAM-1, and IP-10 mRNA expression. Only the 10 μM dose of NA significantly attenuated the thrombin-induced increase in iNOS and IL-1β mRNA expression (Figure 3.4.1). Thrombin also significantly increased TNFα protein expression and both doses of NA (1 and 10 μM) significantly attenuated the thrombin-induced increase (Figure 3.4.2).

NA attenuates the inflammatory response induced by Aβ: Aβ significantly increased IL-1β, TNFα, and CD40 mRNA expression. Both doses of NA (1 and 10μM) significantly attenuated the Aβ-induced increase in TNFα and CD40 mRNA expression. Neither dose of NA altered IL-1β mRNA expression (Figure 3.4.3).

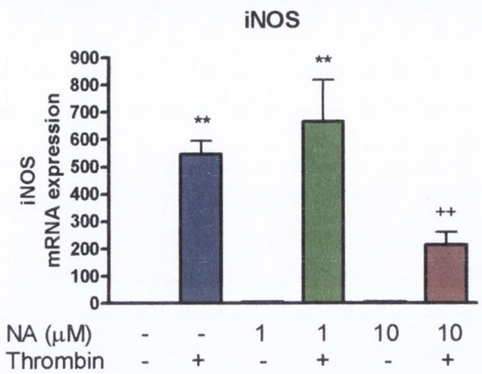
Figure 3.4.1: NA attenuates the inflammatory response induced by thrombin in mixed glial cells

NA (1 or 10 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between NA pre-treatment and thrombin treatment in (a) iNOS [$F_{(2,30)}$=5.94, P=0.0067], (b) IL-1β [$F_{(2,30)}$=9.68, P=0.0006], (c) TNFα [$F_{(2,30)}$=16.01, P<0.0001], (d) CD40 [$F_{(2,30)}$=8.76, P=0.001], (e) VCAM-1 [$F_{(2,30)}$=36.67, P<0.0001], and (f) IP-10 [$F_{(2,29)}$=81.52, P<0.0001] mRNA.

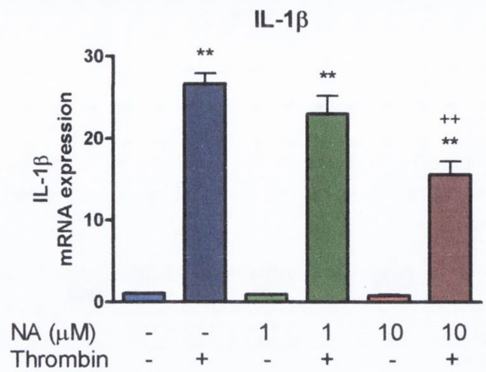
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

Figure 3.4.1:

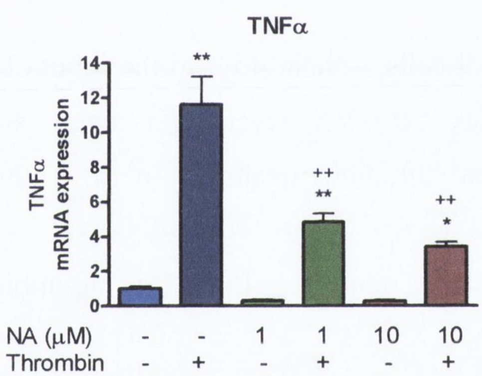
(a) iNOS mRNA



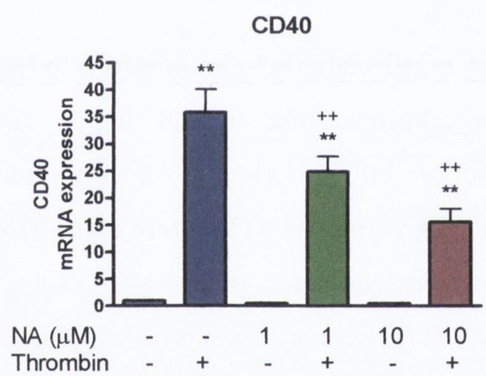
(b) IL-1β mRNA



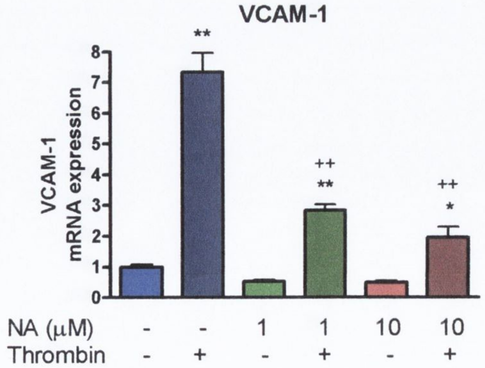
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

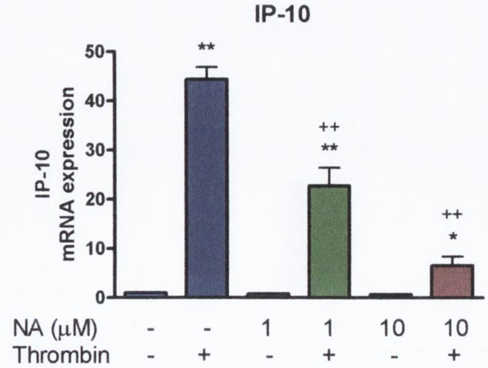


Figure 3.4.2:

(a) TNFα protein

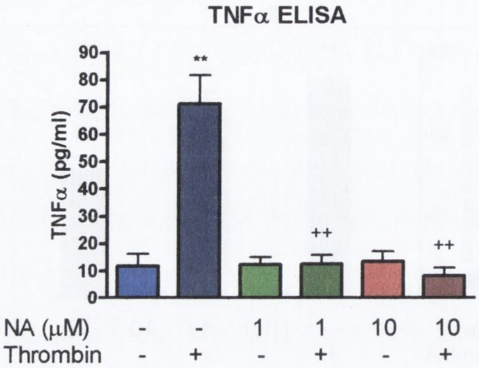


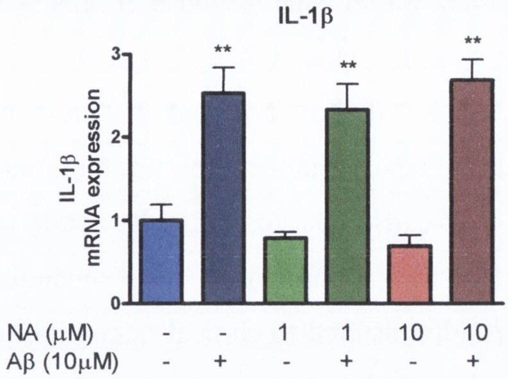
Figure 3.4.2: NA attenuates the inflammatory response induced by thrombin in mixed glial cells

NA (1 or 10 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 24 hours. A two-way ANOVA revealed a significant interaction effect between NA pre-treatment and thrombin treatment in (a) TNFα [$F_{(2,27)}$=19.54, P<0.0001] protein expression.

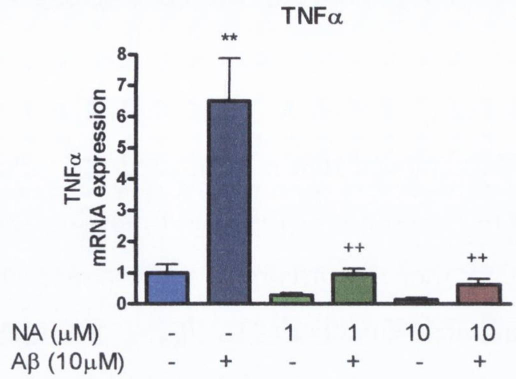
Data expressed as means ± SEM (n=5-6), **P<0.01 vs. control, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

Figure 3.4.3:

(a) IL-1β mRNA



(b) TNFα mRNA



(c) CD40 mRNA

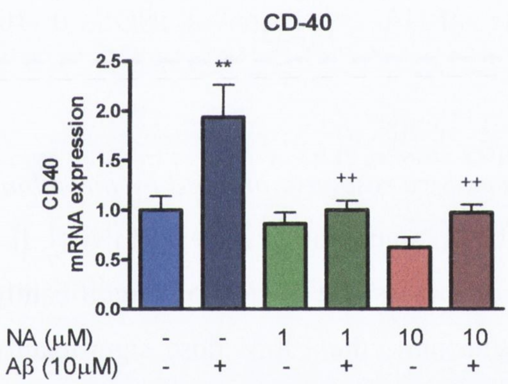


Figure 3.4.3: NA attenuates the inflammatory response induced by Aβ in mixed glial cells

NA (1 or 10 μM) was administered to mixed glial cells 2 hours prior to Aβ (10 μM) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between NA pre-treatment and Aβ treatment in (b) TNFα [$F_{(2,29)}$=11.34, P<0.001] and (c) CD40 [$F_{(2,28)}$=3.55, P=0.042] mRNA expression. There was a significant effect of Aβ treatment only in (a) IL-1β [$F_{(1,29)}$=51.21, P<0.0001].

Data expressed as means ± SEM (n=5-6), **P<0.01 vs. control, ++P<0.01 vs. Aβ (Newman-Keuls post-hoc test).

3.5 The effect of NA on the inflammatory response induced by thrombin, Aβ and LPS in microglia and astrocytes

As LPS, thrombin and Aβ had been shown to induce an immune response in separated glial cells, the next step was to look at possible ways to reduce this inflammation. NA has been reported to elicit anti-inflammatory actions in the CNS, and the previous studies showed that it attenuated the inflammatory response induced by thrombin and Aβ in mixed glial cells. The following studies were carried out to assess the ability of NA to alter the inflammatory response induced by LPS, thrombin or Aβ in separated glial cells. Vehicle or NA (0.1, 1 or 10 μM) was administered to cortical microglial or astrocyte cultures 2 hours prior to the administration of LPS (100 ng/ml), thrombin (20 U/ml), or Aβ (10 μM). This dose range of NA has been shown to decrease LPS-induced inflammation in mixed glial cells. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically, the mRNA expression of iNOS, IL-1β, TNFα, and CD40 were examined.

NA produces a modest attenuation of the inflammatory response induced by thrombin: In both microglia and astrocytes, thrombin (20 U/ml) significantly increased iNOS, IL-1β, TNFα, and CD40 mRNA. In microglia and astrocytes, the 10μM dose significantly attenuated CD40 mRNA expression. In microglia only, the 10μM dose significantly attenuated the thrombin-induced increase in TNFα mRNA expression. NA did not alter iNOS or IL-1β. In astrocytes only, both doses significantly attenuated the thrombin-induced increase in IL-1β mRNA, while only the 10μM dose significantly attenuated iNOS mRNA. NA did not alter TNFα mRNA (Figure 3.5.1).

NA produces a modest attenuation of the inflammatory response induced by Aβ: In both microglia and astrocytes, Aβ (10 μM) significantly increased IL-1β and TNFα mRNA expression, but not CD40 mRNA. NA did not significantly attenuate the Aβ effect in IL-1β, TNFα, or CD40 mRNA expression. In astrocytes only, NA (10 μM) combined with Aβ caused an increase in CD40 mRNA expression (Figure 3.5.2).

NA does not attenuate, and in some instances augments, the inflammatory response induced by LPS: LPS (100 ng/ml) significantly increased iNOS, IL-1β, TNFα, and CD40 mRNA expression in microglia and astrocytes. In microglia, NA (0.1 and 1 μM) significantly augmented the LPS-induced increase in iNOS mRNA expression. NA did not alter IL-1β, TNFα, or CD40 mRNA expression in microglia. In astrocytes, NA (0.1, 1, and 10 μM) significantly augmented the LPS-induced increase in iNOS, NA (0.1 and 1 μM) significantly augmented IL-1β and TNFα mRNA expression. NA did not alter CD40 mRNA expression in astrocytes (Figure 3.5.3).

Figure 3.5.1: NA produces a modest attenuation of the inflammatory response induced by thrombin in microglia and astrocytes

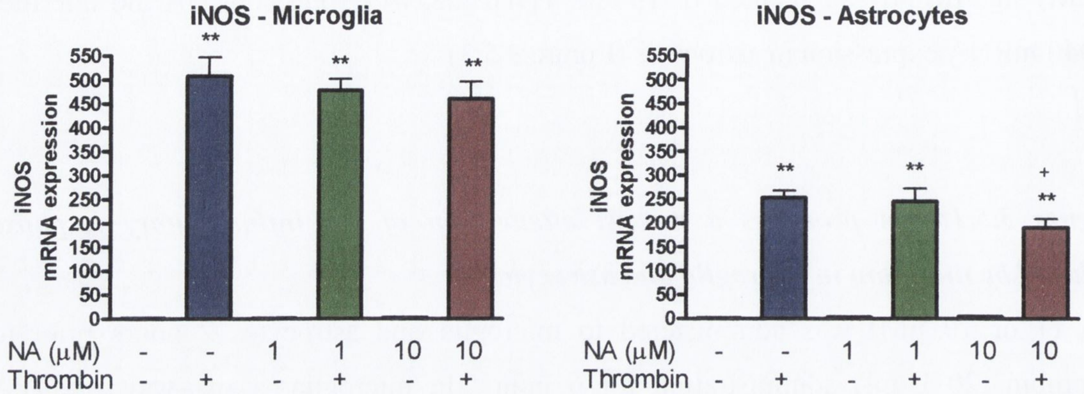
NA (1 or 10 μM) was administered to microglia and astrocytes 2 hours prior to thrombin (20 U/ml) administration for 6 hours. In microglia, a two-way ANOVA revealed a significant effect of thrombin treatment only in (a) iNOS [$F_{(1,24)}$=639.17, P<0.0001], (b) IL-1β [$F_{(1,23)}$=872.08, P<0.0001], (c) TNFα [$F_{(1,24)}$=376.63, P<0.0001], and (d) CD40 [$F_{(1,24)}$=314.82, P<0.0001] mRNA expression. In astrocytes, a two-way ANOVA revealed a significant interaction effect between NA pre-treatment and thrombin treatment in (b) IL-1β [$F_{(2,24)}$=9.86, P=0.0007] mRNA expression. There was a significant effect of thrombin treatment only in (a) iNOS [$F_{(1,24)}$=380.62, P<0.0001], (c) TNFα [$F_{(1,24)}$=327.54, P<0.0001], and (d) CD40 [$F_{(1,24)}$=177.48, P<0.0001] mRNA expression.

Data expressed as means ± SEM (n=4-5), *P<0.05, **P<0.01 vs. control, +P<0.05, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

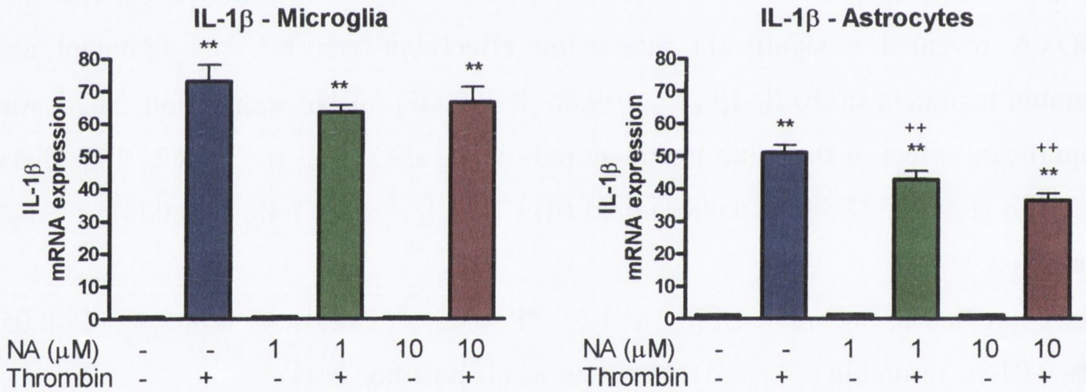
Figure 3.5.1:

Microglia **Astrocytes**

(a) iNOS mRNA



(b) IL-1β mRNA



(c) TNFα mRNA

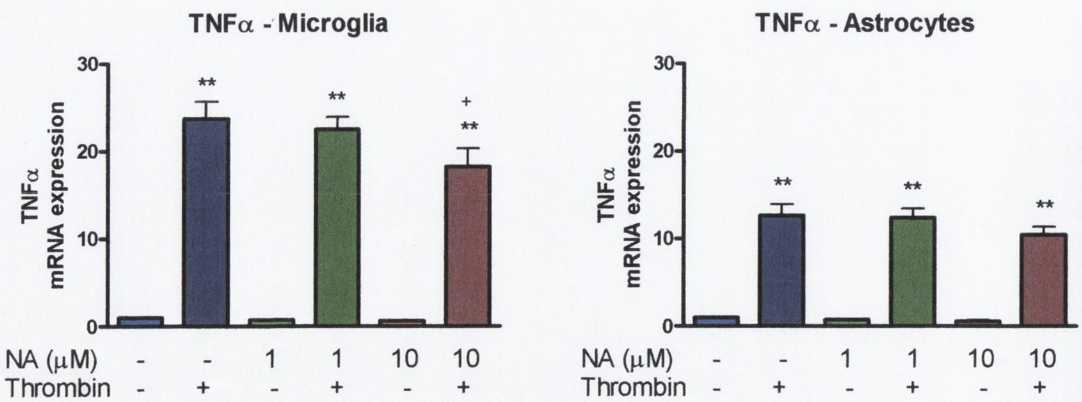


Figure 3.5.1:

Microglia **Astrocytes**

(d) CD40 mRNA

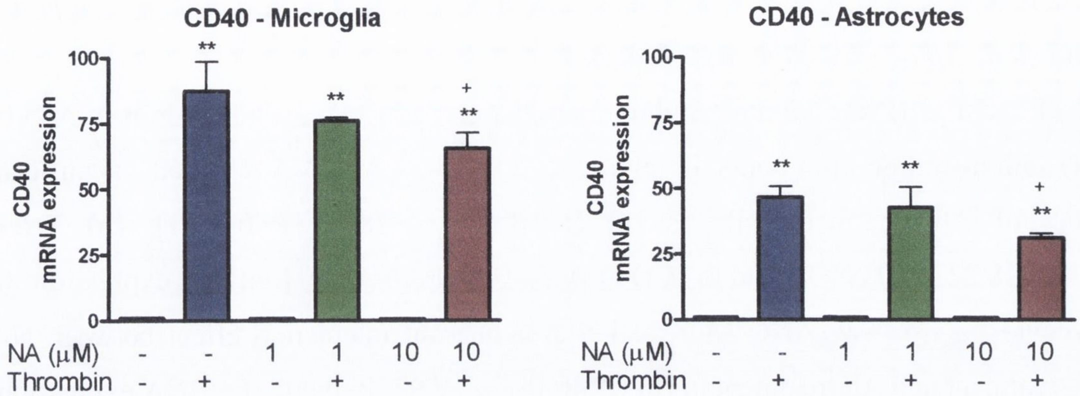


Figure 3.5.2: NA produces a modest attenuation of the inflammatory response induced by Aβ in microglia and astrocytes

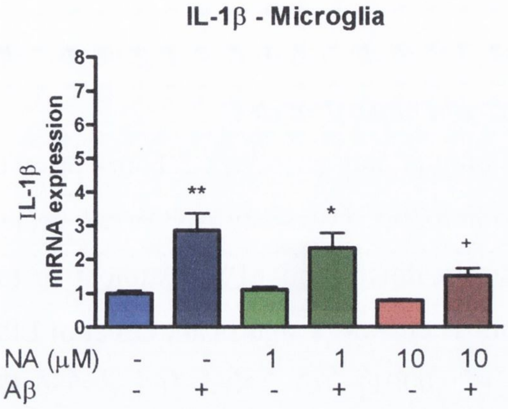
NA (1 or 10 μM) was administered to microglia and astrocytes 2 hours prior to Aβ (10 μM) administration for 6 hours. In microglia, a two-way ANOVA revealed a significant effect of Aβ treatment only in (a) IL-1β [$F_{(1,24)}$=28.30, P<0.0001], (b) TNFα [$F_{(1,24)}$=18.22, P=0.0003], and (c) CD40 [$F_{(1,24)}$=13.96, P=0.001] mRNA expression. In astrocytes, a two-way ANOVA revealed a significant interaction effect between NA pre-treatment and Aβ treatment in (c) CD40 [$F_{(2,24)}$=7.92, P=0.0023] mRNA expression. There was a significant effect of Aβ treatment only in (a) IL-1β [$F_{(1,24)}$=11.88, P=0.0021] and (b) TNFα [$F_{(1,24)}$=16.55, P=0.0004] mRNA expression.

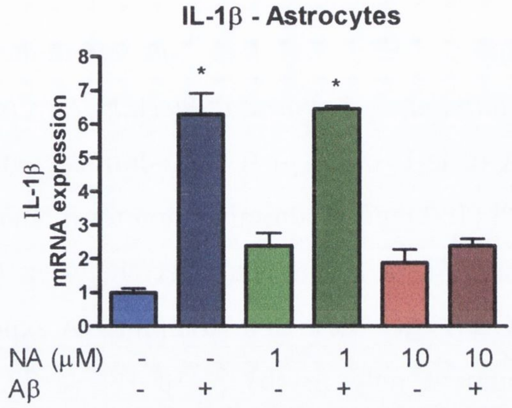
Data expressed as means ± SEM (n=4-5), *P<0.05, **P<0.01 vs. control, +P<0.05, ++P<0.01 vs. Aβ (10 μM) (Newman-Keuls post-hoc test).

Figure 3.5.2:

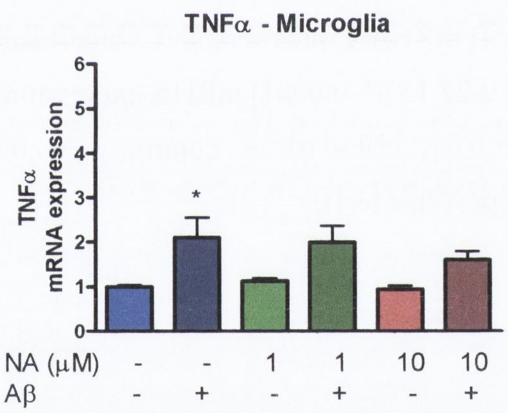
Microglia **Astrocytes**

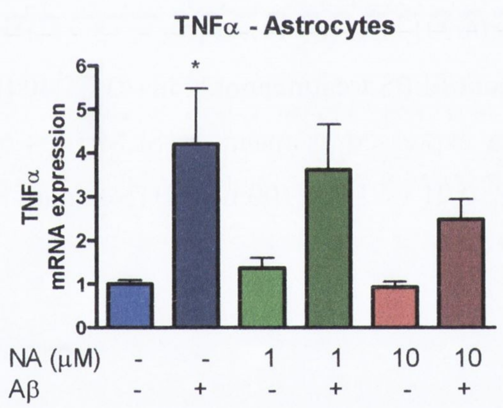
(a) IL-1β mRNA



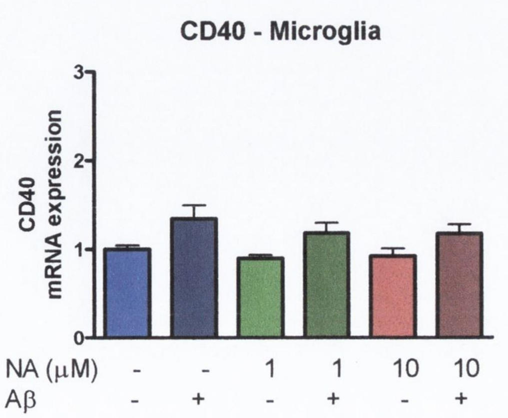


(b) TNFα mRNA





(c) CD40 mRNA



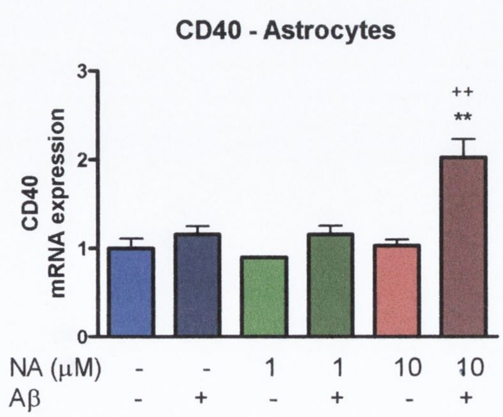


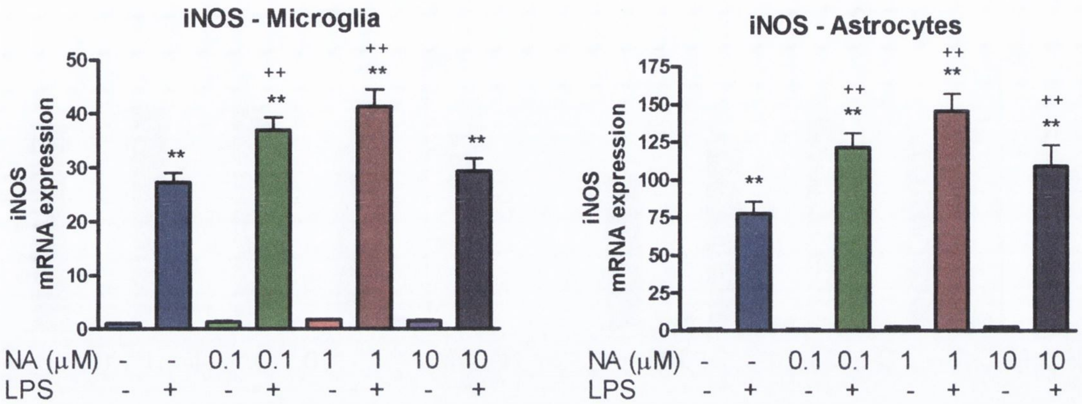
Figure 3.5.3: NA does not attenuate, and in some instances augments, the inflammatory response induced by LPS in microglia and astrocytes

NA (0.1, 1, or 10 μM) was administered to microglia and astrocytes 2 hours prior to LPS (100 ng/ml) administration for 6 hours. In microglia, a two-way ANOVA revealed a significant interaction effect between NA pre-treatment and LPS treatment in (a) iNOS [$F_{(3,39)}$=6.47, P=0.0012] mRNA expression. There was a significant effect of LPS treatment only in (b) IL-1β [$F_{(1,40)}$=272.34, P<0.0001], (c) TNFα [$F_{(1,40)}$=536.13, P<0.0001], and (d) CD40 [$F_{(1,40)}$=503.15, P<0.0001] mRNA expression. In astrocytes, a two-way ANOVA revealed a significant interaction effect between NA pre-treatment and LPS treatment in (a) iNOS [$F_{(3,38)}$=5.91, P=0.0021], (b) IL-1β [$F_{(3,38)}$=5.82, P=0.0022], and (c) TNFα [$F_{(3,38)}$=5.74, P=0.0024] mRNA expression, and a significant effect of LPS treatment only in (d) CD40 [$F_{(1,38)}$=627.13, P<0.0001] mRNA expression. Data expressed as means ± SEM (n=4-6), *P<0.05, **P<0.01 vs. control, +P<0.05, ++P<0.01 vs. LPS (100 ng/ml) (Newman-Keuls post-hoc test).

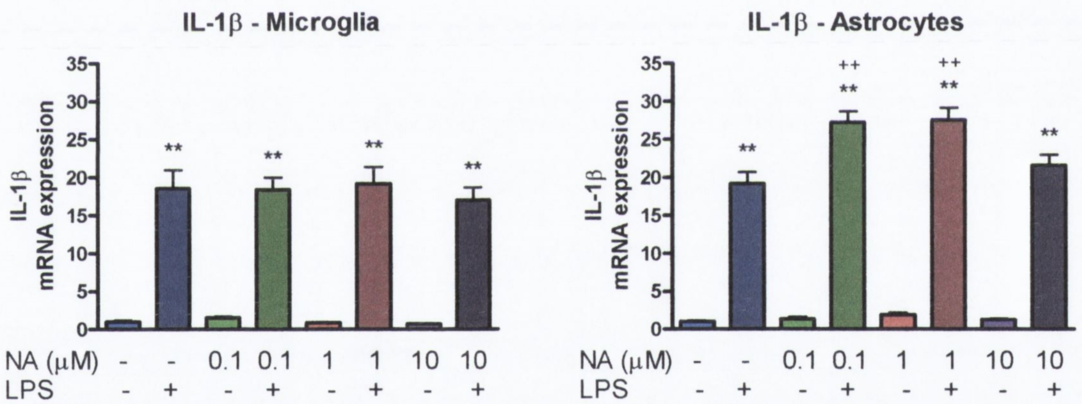
Figure 3.5.3:

Microglia | Astrocytes

(a) iNOS mRNA



(b) IL-1β mRNA



(c) TNFα mRNA

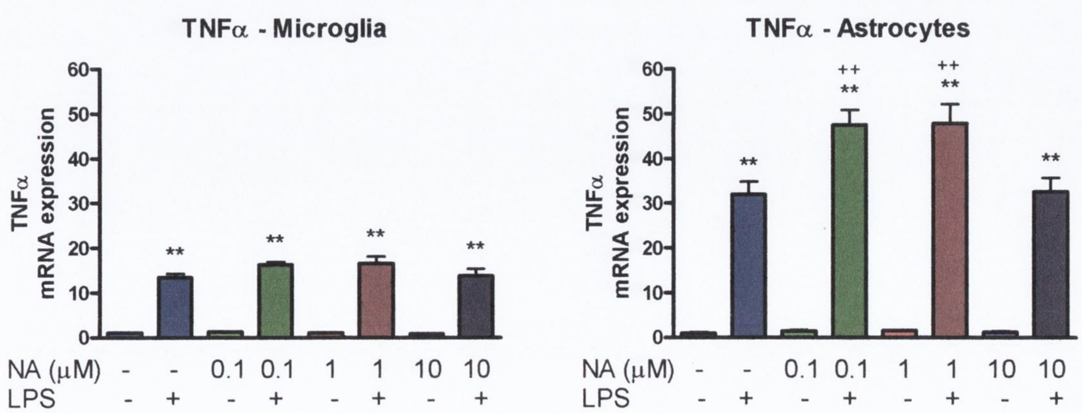
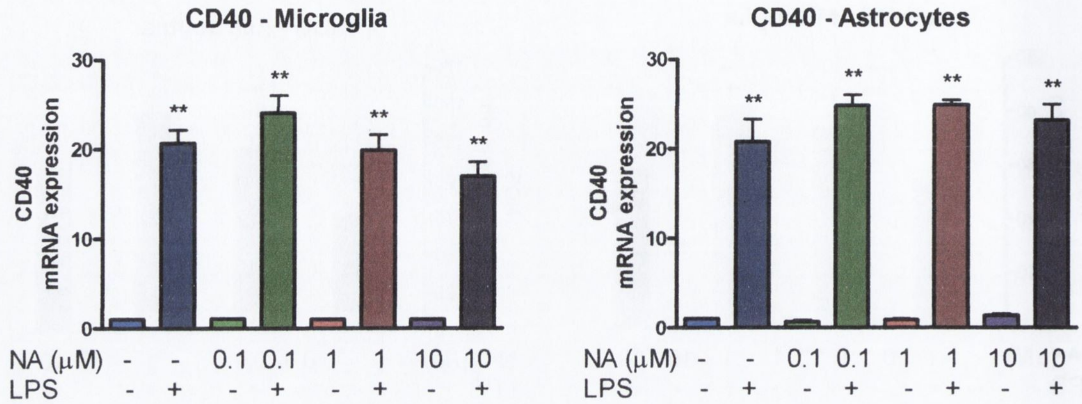


Figure 3.5.3:

Microglia Astrocytes

(d) CD40 mRNA



3.6 Astrocytes and microglia express β_1- and β_2-ARs

As the anti-inflammatory effects of NA are thought to be mediated via the β_2-AR, it was necessary to confirm that the β-ARs were being expressed on the glia in the mixed glial cultures. The following studies were carried out to assess the ability of astrocytes and microglia to express β_1- and β_2-ARs. Primary mixed glial cultures were grown on glass coverslips for up to 14 days. They were then fixed and stained using fluorescent immunocytochemistry, and imaged using a confocal microscope. An antibody specific to GFAP was used as a marker for astrocytes, and an antibody specific to CD11b was used as a marker for microglia. Antibodies specific to either the β_1- or the β_2-AR were also used. Hoescht was used to identify cell nuclei.

Astrocytes express β_1- and β_2-ARs: Astrocytes stained green for GFAP were found to co-localise with the red staining for β_1-ARs (Figure 3.6.1) or β_2-ARs (Figure 3.6.2). This co-localisation of the red and green staining indicates that the same cells express both GFAP and β-ARs, demonstrating that astrocytes express β-ARs. This means that NA and β-AR agonists are capable of exerting their effects on astrocytes.

Microglia express β_1- and β_2-ARs: Microglia stained green for CD11b were found to co-localise with the red staining for β_1-ARs (Figure 3.6.3) or β_2-ARs (Figure 3.6.4). This co-localisation of the red and green staining indicates that the same cells express both CD11b and β-ARs, demonstrating that microglia express β-ARs. This means that NA and β-AR agonists are capable of exerting their effects on microglia.

(a) (b)

(c) (d)

(e)

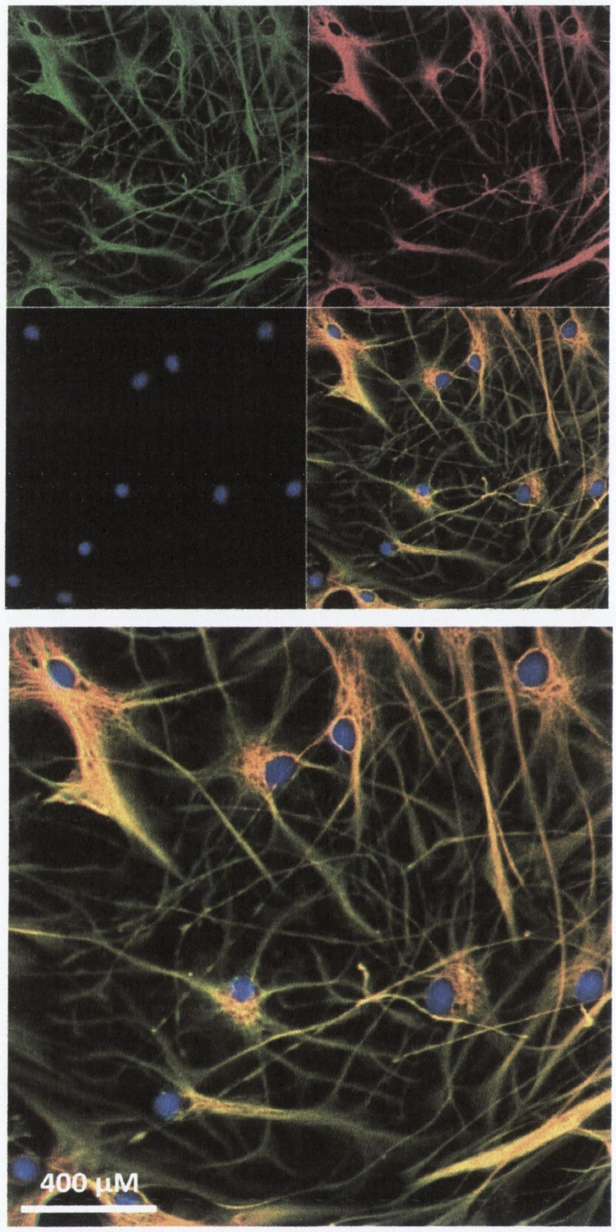


Figure 3.6.1: Astrocytes express the β_1-AR

Fluorescent immunocytochemistry was carried out on mixed glial cultures using antibodies against the astrocyte marker, GFAP (green), and the β_1-AR (red). The cells were imaged at 20X magnification. (a) is green staining indicating GFAP positive astrocytes, (b) is red staining indicating β_1-AR positive cells, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The green and red staining is colocalised indicating that the same cells express both GFAP and β_1-ARs, meaning that astrocytes express β_1-ARs. The white line represents a length of 400 µM.

(a) (b) (c) (d) (e)

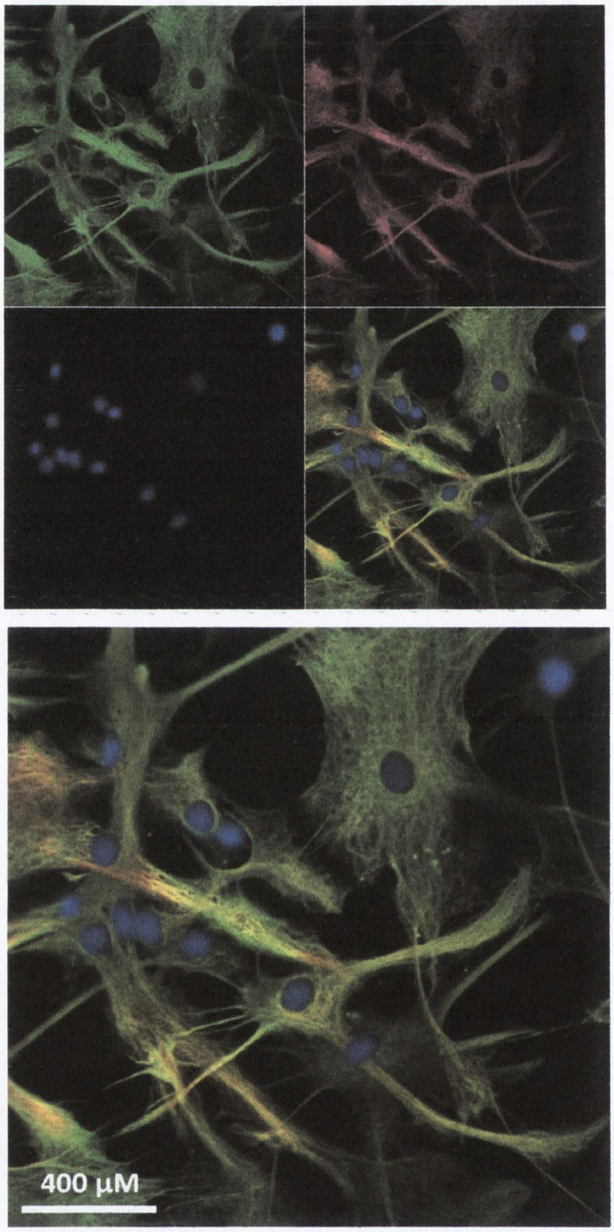


Figure 3.6.2: Astrocytes express the β_2-AR

Fluorescent immunocytochemistry was carried out on mixed glial cultures using antibodies against the astrocyte marker, GFAP (green), and the β_2-AR (red). The cells were imaged at 20X magnification. (a) is green staining indicating GFAP positive astrocytes, (b) is red staining indicating β_2-AR positive cells, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The green and red staining is colocalised indicating that the same cells express both GFAP and β_2-ARs, meaning that astrocytes express β_2-ARs. The white line represents a length of 400 μM.

(a) (b) (c) (d) (e)

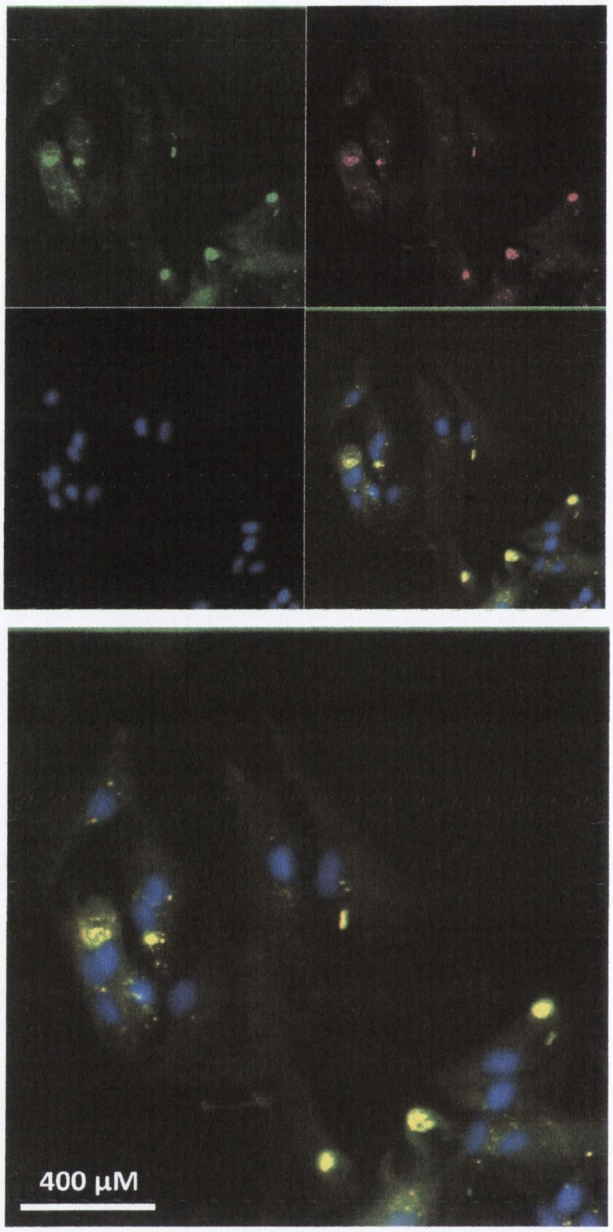


Figure 3.6.3: Microglia express the β_1-AR

Fluorescent immunocytochemistry was carried out on mixed glial cultures using antibodies against the microglia marker, CD11b (green), and the β_1-AR (red). The cells were imaged at 20X magnification. (a) is green staining indicating CD11b positive microglia, (b) is red staining indicating β_1-AR positive cells, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The green and red staining is colocalised indicating that the same cells express both CD11b and β_1-ARs, meaning that microglia express β_1-ARs. The white line represents a length of 400 μM.

(a) (b)

(c) (d)

(e)

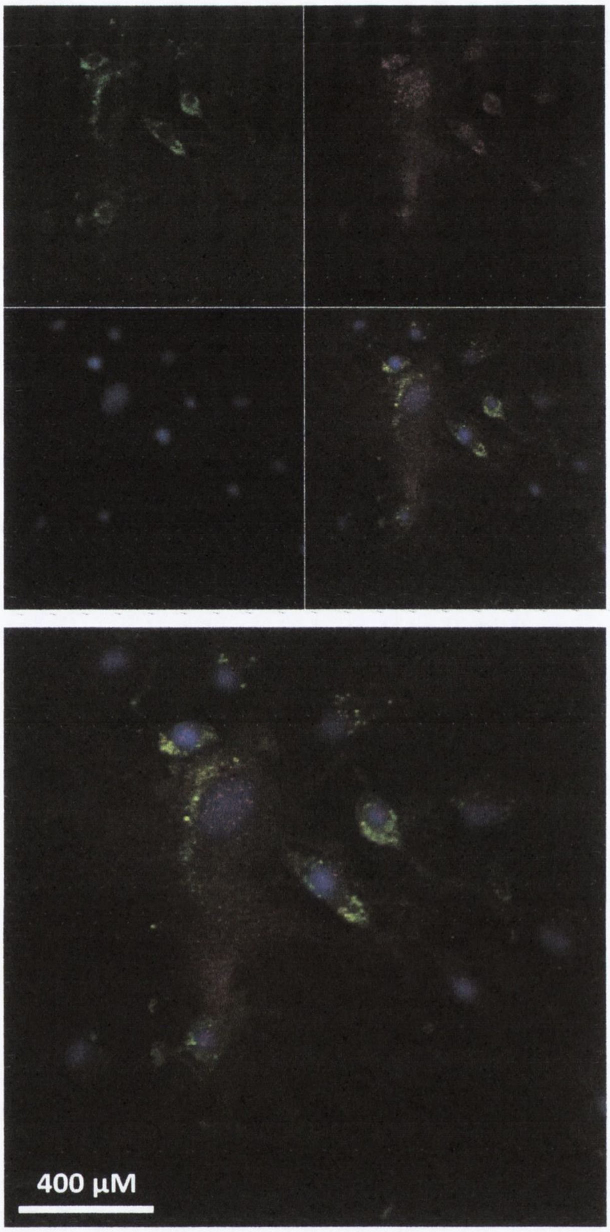


Figure 3.6.4: Microglia express the β_2-AR

Fluorescent immunocytochemistry was carried out on mixed glial cultures using antibodies against the microglia marker, CD11b (green), and the β_2-AR (red). The cells were imaged at 20X magnification. (a) is green staining indicating CD11b positive microglia, (b) is red staining indicating β_1-AR positive cells, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The green and red staining is colocalised indicating that the same cells express both CD11b and β_2-ARs, meaning that microglia express β_2-ARs. The white line represents a length of 400 µM.

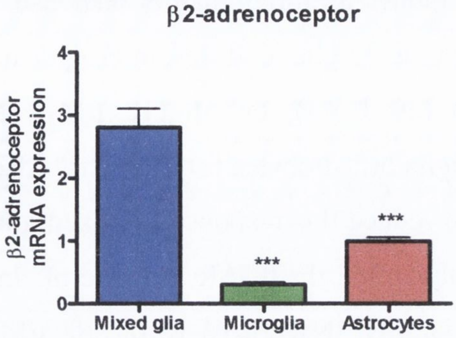
3.7 β_2-AR expression is reduced in separated microglial and astrocyte cultures compared to mixed glial cultures

The previous studies showed that astrocytes and microglia, when grown in mixed glial cultures, express β_1- and β_2-ARs. However the earlier studies showed that, in separated glial cultures, while NA modestly attenuated the inflammatory response induced by thrombin, it largely failed to attenuate the inflammatory response induced by Aβ (with the exception of IL-1β), and in some cases augmented the inflammatory response induced by LPS. This was an unexpected finding as many earlier studies in this and other laboratories, as well as the earlier studies in this section, have shown that NA potently suppressed the inflammatory response induced by LPS, thrombin and Aβ in mixed glial cultures. It was hypothesised that a possible reason for this discrepancy could be that expression of the β_2-AR might be reduced on glia when they are cultured separately instead of together. The following studies were carried out to assess whether growing glial cells separately instead of together altered the expression of β_2-AR expression. Cortical glial cells were either grown together as mixed glial cultures, or separated to form microglia or astrocyte cell cultures. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of β_2-AR gene expression by RT-PCR.

β_2-AR expression is dramatically reduced in microglia and astrocytes: Cultures of microglia or astrocytes, when grown separately, have significantly lower expression of β_2-AR mRNA compared to mixed glial cultures (Figure 3.7.1). This reduction in β_2-AR receptor expression is likely the explanation for the fact that NA did not have as potent an anti-inflammatory effect on the separated glial cultures as it did on the mixed glial cultures. For this reason the remaining experiments were carried out on mixed glial cultures only.

Figure 3.7.1:

(a) β_2-AR mRNA



(b) β-actin mRNA

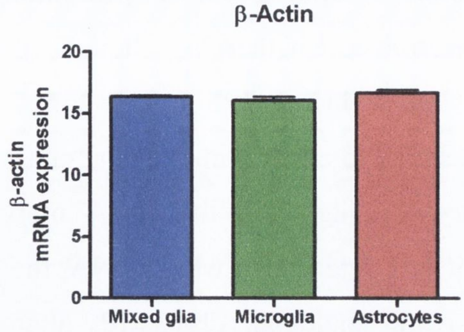


Figure 3.7.1: β_2-AR expression is dramatically reduced in microglia and astrocytes

A one-way ANOVA demonstrated that cultures of microglia or astrocytes, when grown separately, had significantly lower expression of (a) β_2-AR mRNA expression compared to mixed glial cultures [$F_{(2,13)}=44.02$, $P<0.0001$]. The expression of (b) β-actin mRNA was not altered.

Data expressed as means ± SEM (n=5-6), ***$P<0.001$ vs. mixed glia (Newman-Keuls post-hoc test).

3.8 LPS, thrombin, Aβ, NA, salbutamol, salmeterol, and db-cAMP do not alter cell viability

Following the finding that LPS, thrombin and Aβ induce an inflammatory response, it was determined whether they altered cell viability in mixed glial cultures, microglia and astrocytes. Vehicle, LPS (100 ng/ml), thrombin (20 U/ml), or Aβ (10 μM) was administered to cortical mixed glial cultures, microglia or astrocytes for 24 hours. Also, prior to assessing the ability of NA interventions to reduce this response in mixed glial cells, it was determined whether NA, the β-AR, salbutamol, the β_2-AR, salmeterol, and the c-AMP analogue, db-cAMP, altered cell viability. NA (0.1, 1, or 10 μM), salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to cortical mixed glial cultures for 24 hours. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. An Alamar Blue cell viability assay was carried out after 24 hours.

Table 3.8.1: LPS, thrombin, and Aβ do not alter cell viability in mixed glial cells, microglia or astrocytes:

n=8	Control	LPS	Thrombin	Aβ
Mixed glia	100±4.66	103.70±6.21	89.36±5.30	99.04±4.55

n=4	Control	LPS	Thrombin	Aβ
Microglia	100±1.16	97.52±3.49	93.71±2.37	98.33±0.77
Astrocytes	100±0.93	99.82±1.04	100.10±0.52	97.89±0.74

LPS (100 ng/ml), thrombin (20 U/ml), or Aβ (10 μM) was administered to mixed glia, microglia, or astrocytes for 24 hours and an Alamar Blue cell viability assay was carried out. One-way ANOVAs demonstrated that none of the treatments significantly altered cell viability. Data expressed as means ± SEM (n=4 or n=8).

Table 3.8.2: NA, the β-AR, salbutamol, the β_2-AR, salmeterol, and the c-AMP analogue, db-cAMP, do not alter cell viability in mixed glial cells:

n=6	Control	NA (0.1 μM)	NA (1 μM)	NA (10 μM)
Mixed glia	100±1.74	103.70±3.77	103.10±3.56	93.27±7.13

n=6	Control	Salbutamol	Salmeterol	db-cAMP
Mixed glia	100±14.59	76.38±15.27	127.30±4.72	112.30±8.33

NA (0.1, 1, or 10 μM), salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to mixed glia for 24 hours and an Alamar Blue cell viability assay was carried out. One-way ANOVAs demonstrated that none of the treatments significantly altered cell viability. Data expressed as means ± SEM (n=6).

3.9 The effect of the β-AR agonist, salbutamol, on the inflammatory response induced by thrombin and Aβ

The earlier studies showed that NA was capable of reducing the thrombin- and Aβ-induced immune response. Studies indicate that the anti-inflammatory actions of NA occur via activation of β_2-ARs on microglia and astrocytes. The following studies were carried out to assess the ability of the β-AR agonist, salbutamol, to alter the inflammatory response induced by thrombin or Aβ. Vehicle or salbutamol (10 μM) was administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle, thrombin (20 U/ml), or Aβ (2.5 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. When thrombin was used as a treatment, the expression of iNOS, IL-1β, TNFα, CD40, IP-10, and VCAM-1 mRNA were examined. When Aβ was used as a treatment, the expression of IL-1β and TNFα mRNA only were examined as in the prior experiment these molecules had shown the most dramatic response to Aβ.

The β-AR agonist, salbutamol, attenuates and augments the inflammatory response induced by thrombin: Thrombin significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression. Salbutamol (10 μM) significantly attenuated the thrombin-induced increase in TNFα, VCAM-1, and IP-10 mRNA expression, but it augmented the thrombin-induced increase in IL-1β. It did not alter iNOS or CD40 (Figure 3.9.1).

The β-AR agonist, salbutamol, augments the inflammatory response induced by Aβ: Salbutamol (10 μM) significantly augmented the Aβ-induced increase in IL-1β and TNFα mRNA expression (Figure 3.9.2).

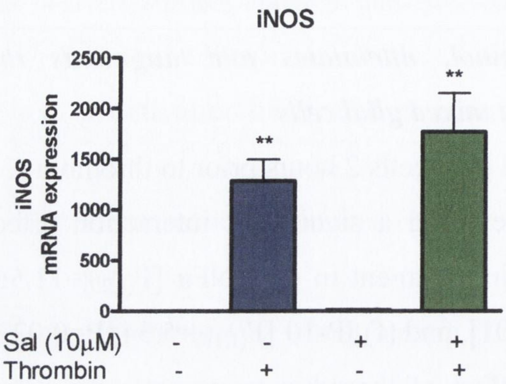
Figure 3.9.1: The β-AR agonist, salbutamol, attenuates and augments the inflammatory response induced by thrombin in mixed glial cells

Salbutamol (10 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration. A two-way ANOVA revealed a significant interaction effect between salbutamol pre-treatment and thrombin treatment in (c) TNFα [$F_{(1,20)}$=11.56, P=0.0028], (e) VCAM-1 [$F_{(1,20)}$=22.07, P=0.0001], and (f) IP-10 [$F_{(1,19)}$=5.34, P=0.032] mRNA expression. There was a significant effect of thrombin treatment only in (a) iNOS [$F_{(1,18)}$=59.29, P<0.0001], (b) IL-1β [$F_{(1,20)}$=122.75, P<0.0001], and (d) CD40 [$F_{(1,20)}$=168.68, P<0.0001] mRNA expression.

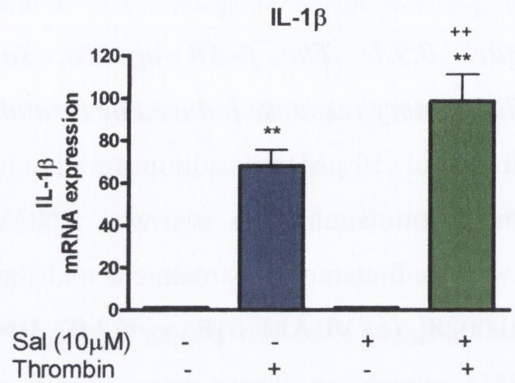
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

Figure 3.9.1:

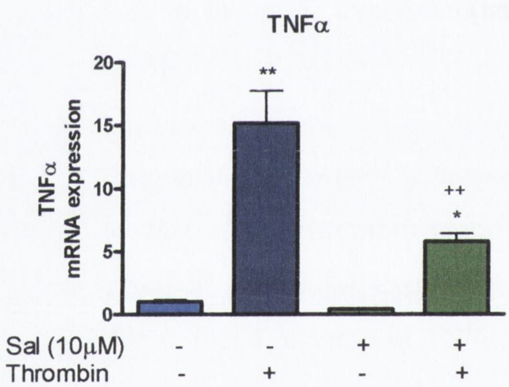
(a) iNOS mRNA



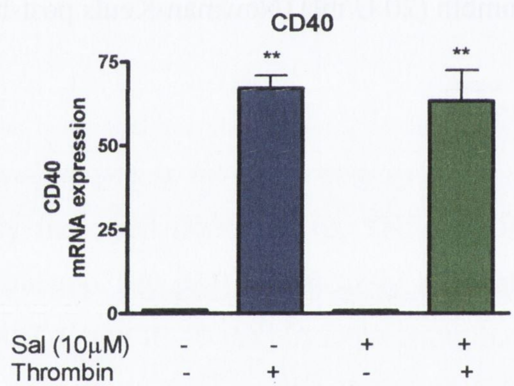
(b) IL-1β mRNA



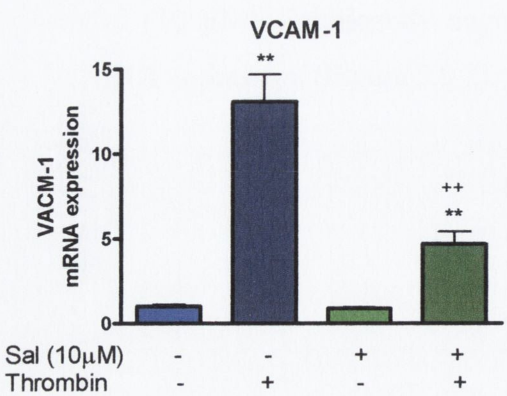
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

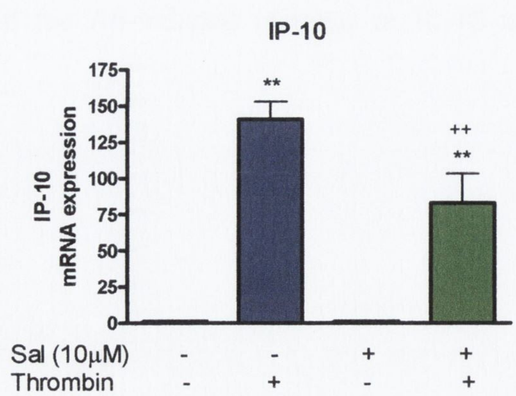


Figure 3.9.2:

(a) IL-1β mRNA (b) TNFα mRNA

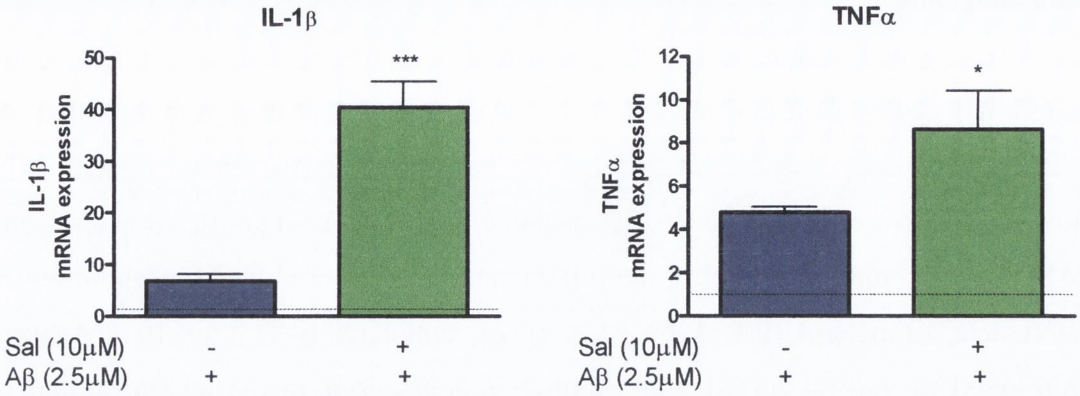


Figure 3.9.2: The β-AR agonist, salbutamol, augments the inflammatory response induced by Aβ in mixed glial cells

Salbutamol (10 μM) was administered to mixed glial cells 2 hours prior to Aβ (2.5 μM) administration. A student's t-test demonstrated a significant effect of salbutamol treatment on (a) IL-1β ($P<0.0001$) and (b) TNFα ($P=0.046$) mRNA expression.

Data expressed as means ± SEM (n=5-6), *$P<0.05$, ***$P<0.001$ vs. Aβ (2.5 μM) (student's t-test). Dotted line denotes level of mRNA expression for control group.

3.10 The effect of the β_2-AR agonist, salmeterol, and β_1-AR agonist, xamoterol, on the inflammatory response induced by thrombin or Aβ, and on unstimulated mixed glial cells

The β-AR agonist salbutamol both attenuated and augmented the inflammatory response induced by thrombin and Aβ. It was hypothesised that the reason for this could be due to opposing actions induced by the activation of β_1- versus β_2-ARs. The following studies were carried out to assess the ability of β_2-AR agonist, salmeterol, and β_1-AR agonist, xamoterol, to alter the inflammatory response induced by thrombin or Aβ. Vehicle, salmeterol (0.1, 1 or 10 μM), or xamoterol (0.1, 1 or 10 μM) were administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle, thrombin (20 U/ml), or Aβ (2.5 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically, the expression of iNOS, IL-1β, TNFα, CD40, IP-10, and VCAM-1 mRNA were examined. As the anti-inflammatory effects were mediated by β_2-AR, the ability of salmeterol to alter protein expression was also examined. Vehicle or salmeterol (0.1, 1 or 10 μM) was administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle, thrombin (20 U/ml), or Aβ (2.5 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 24 hours and the cell-free supernatants were harvested for the assessment of cytokine production by ELISA. Specifically, the expression of TNFα and IL-1β protein was examined.

The β_2-AR agonist, salmeterol, attenuates whereas the β_1-AR agonist, xamoterol, augments the inflammatory response induced by thrombin: All doses of salmeterol (0.1. 1, or 10 μM) significantly attenuated the thrombin-induced effect in TNFα and CD40 mRNA and TNFα protein expression, but only the highest dose (10 μM) significantly decreased the thrombin-induced effect in VCAM-1 and IP-10 mRNA and IL-1β protein expression. Salmeterol did not alter iNOS or IL-1β. All doses of xamoterol (0.1, 1, and 10μM) significantly augmented the thrombin-induced effect in IL-1β mRNA expression, but only the two lower doses (0.1 and 1μM) significantly augmented iNOS and IP-10. Xamoterol did not alter TNFα, CD40, or VCAM-1 mRNA (Figure 3.10.1). All doses of salmeterol (0.1. 1, or 10 μM) significantly attenuated the thrombin-induced effect in TNFα protein expression, but only the highest dose (10 μM) significantly decreased the thrombin-induced effect in IL-1β protein expression (Figure 3.10.2).

The β_2-AR agonist, salmeterol, and the β_1-AR agonist, xamoterol, augment the inflammatory response induced by Aβ: Salmeterol (1 μM) and xamoterol (0.1 and 1 μM) augmented the Aβ-induced increase in IL-1β mRNA expression, but did not alter TNFα mRNA (Figure 3.10.3). Salmeterol (0.1 and 1 μM) augmented IL-1β protein, but did not alter TNFα protein (Figure 3.10.4).

The β_2-AR agonist, salmeterol, decreases whereas the β_1-AR agonist, xamoterol, increases the inflammatory response in unstimulated mixed glial cells: Salmeterol (0.1 and 1 μM) significantly decreased CD40 mRNA expression. Salmeterol did not alter iNOS, IL-1β, TNFα, VCAM-1, or IP-10 mRNA. Xamoterol (1 and 10μM) significantly increased TNFα mRNA expression, and the highest dose (10 μM) significantly increased iNOS, IL-1β, CD40, VCAM-1, and IP-10 mRNA (Figure 3.10.5). Salmeterol did not alter TNFα protein, but xamoterol (10 μM) significantly increased TNFα protein (Figure 3.10.6).

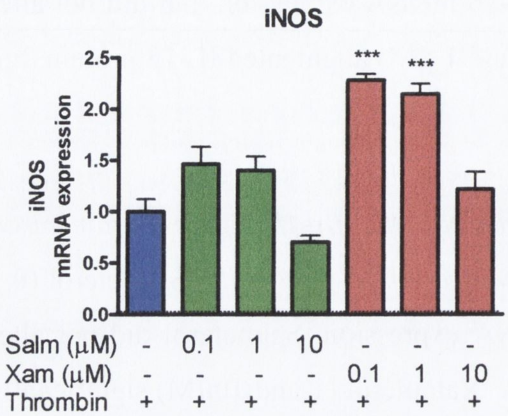
Figure 3.10.1: The β_2-AR agonist, salmeterol, attenuates, whereas the β_1-AR agonist, xamoterol, augments the inflammatory response induced by thrombin in mixed glial cells

Salmeterol (0.1, 1, or 10 μM) or xamoterol (0.1, 1, or 10 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) iNOS [$F_{(6,35)}$=21.74, P<0.0001], (b) IL-1β [$F_{(6,35)}$=6.92, P<0.0001], (c) TNFα [$F_{(6,35)}$=28.72, P<0.0001], (d) CD40 [$F_{(6,35)}$=7.91, P<0.001], (e) VCAM-1 [$F_{(6,35)}$=9.99, P<0.0001], and (f) IP-10 [$F_{(6,35)}$=33.44, P<0.0001] mRNA. The β_2-AR agonist, salmeterol attenuated the thrombin-induced increase in (c) TNFα, (d) CD40, (e) VCAM-1, and (f) IP-10 mRNA expression. There was no change in (a) iNOS or (b) IL-1β mRNA expression. The β_1-AR agonist, xamoterol, augmented the thrombin-induced increase in (a) iNOS, (b) IL-1β, and (f) IP-10 mRNA expression. There was no change in (c) TNFα, (d) CD40, or (e) VCAM-1.

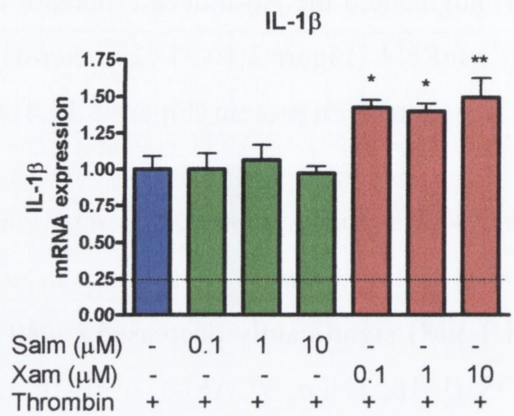
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01, ***P<0.001 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.10.1:

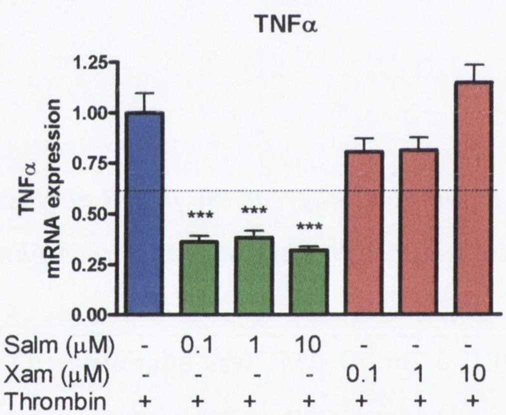
(a) iNOS mRNA



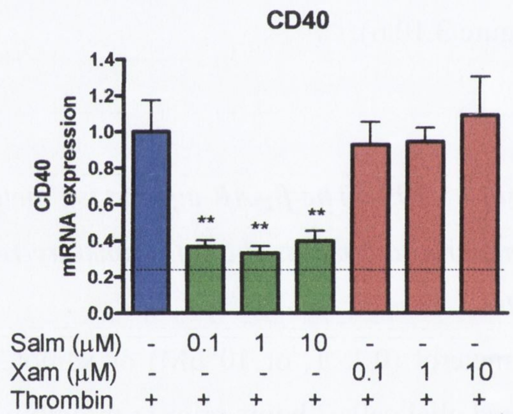
(b) IL-1β mRNA



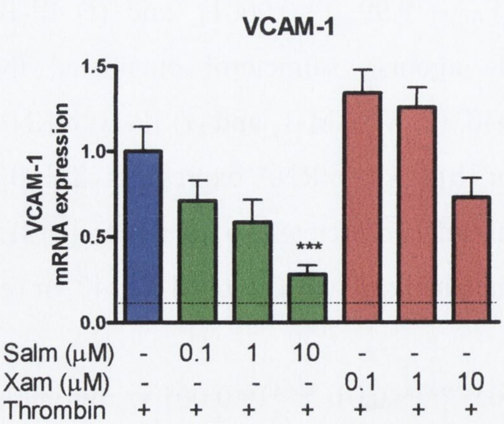
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

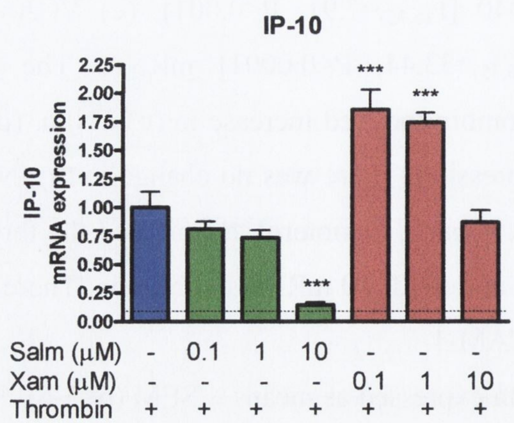


Figure 3.10.2:

(a) IL-1β protein (b) TNFα protein

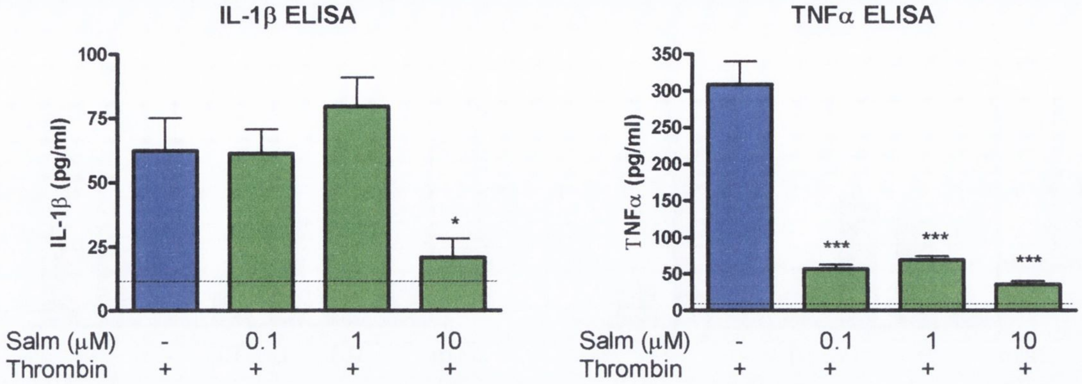


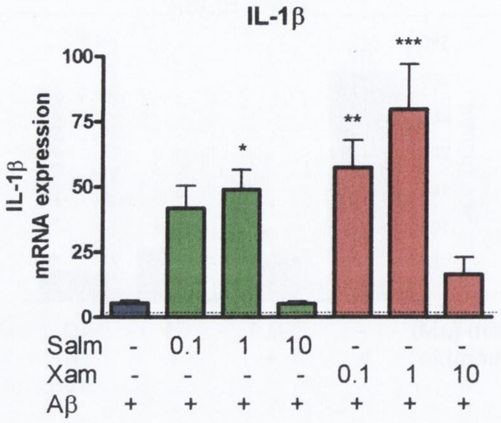
Figure 3.10.2: The β_2-AR agonist, salmeterol, attenuates the inflammatory response induced by thrombin in mixed glial cells

Salmeterol (0.1, 1, or 10 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 24 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) IL-1β [$F_{(3,18)}$=5.58, P=0.0069], and (b) TNFα [$F_{(3,20)}$=61.07, P<0.0001] protein expression. The β_2-AR agonist, salmeterol attenuated the thrombin-induced increase in (a) IL-1β and (b) TNFα protein expression.

Data expressed as means ± SEM (n=5-6), *P<0.05, ***P<0.001 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test). Dotted line denotes level of protein expression for control group.

Figure 3.10.3:

(a) IL-1β mRNA



(b) TNFα mRNA

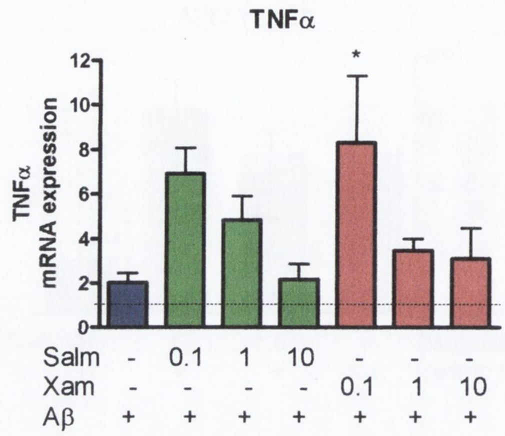


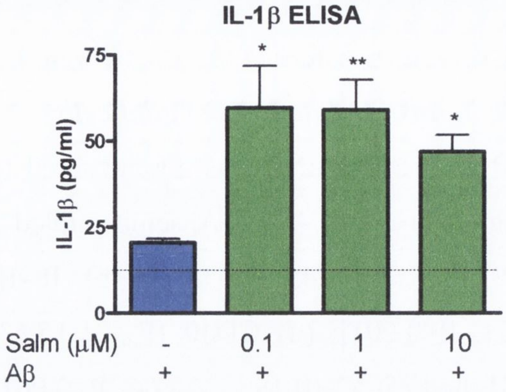
Figure 3.10.3: The β_2-AR agonist, salmeterol, and the β_1-AR agonist, xamoterol, augment the inflammatory response induced by Aβ in mixed glial cells

Salmeterol (0.1, 1, or 10 μM) or xamoterol (0.1, 1, or 10 μM) was administered to mixed glial cells 2 hours prior to Aβ (2.5 μM) administration for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) IL-1β [$F_{(6,31)}$=8.03, P<0.0001] and (b) TNFα [$F_{(6,29)}$=2.93, P=0.023] mRNA. Salmeterol augmented the Aβ-induced increase in (a) IL-1β but not (b) TNFα mRNA. Xamoterol augmented the Aβ-induced increase in (a) IL-1β and (b) TNFα mRNA.

Data expressed as means ± SEM (n=4-6), *P<0.05, **P<0.01, ***P<0.001 vs. Aβ (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.10.4:

(a) IL-1β protein



(b) TNFα protein

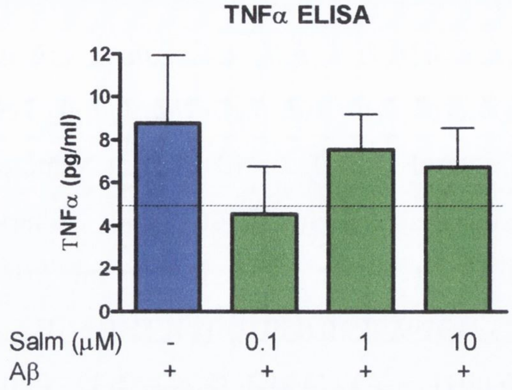


Figure 3.10.4: The β₂-AR agonist, salmeterol, augments the inflammatory response induced by Aβ in mixed glial cells

Salmeterol (0.1, 1, or 10 μM) was administered to mixed glial cells 2 hours prior to Aβ (2.5 μM) administration for 24 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) IL-1β protein [$F_{(3,20)}$=5.44, P=0.0067]. Salmeterol augmented the Aβ-induced increase in (a) IL-1β but not (b) TNFα protein.

Data expressed as means ± SEM (n=6), *P<0.05, **P<0.01 vs. Aβ (Newman-Keuls post-hoc test). Dotted line denotes level of protein expression for control group.

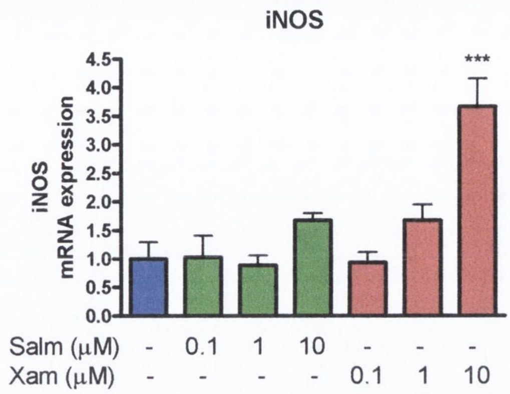
Figure 3.10.5: The β_2-AR agonist, salmeterol, decreases whereas the β_1-AR agonist, xamoterol, increases the inflammatory response in unstimulated mixed glial cells

Salmeterol (0.1, 1, or 10 μM) or xamoterol (0.1, 1, or 10 μM) was administered to mixed glial cells for 6 hours prior to harvesting. A one-way ANOVA demonstrated a significant effect of treatment on (a) iNOS [$F_{(6,33)}$=11.24, P<0.001], (b) IL-1β [$F_{(6,35)}$=3.39, P=0.0097], (c) TNFα [$F_{(6,33)}$=8.54, P<0.001], (d) CD40 [$F_{(6,35)}$=15.63, P<0.001], (e) VCAM-1 [$F_{(6,35)}$=4.32, P=0.0023], and (f) IP-10 [$F_{(6,35)}$=8.64, P<0.001] mRNA expression. The β_2-AR agonist, salmeterol, decreased (d) CD40 mRNA expression. There was no change in (a) iNOS (b) IL-1β (c) TNFα, (e) VCAM-1, or (f) IP-10 mRNA expression. The β_1-AR agonist, xamoterol, increased (a) iNOS, (b) IL-1β, (c) TNFα, (d) CD40, (e) VCAM-1 and (f) IP-10 mRNA expression.

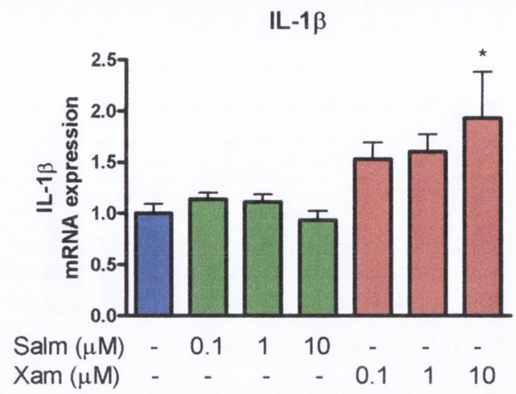
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01, ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 3.10.5:

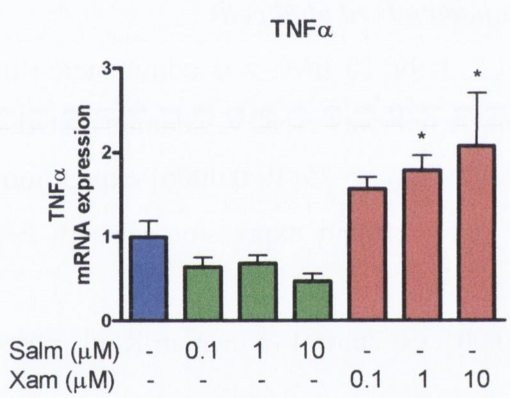
(a) iNOS mRNA



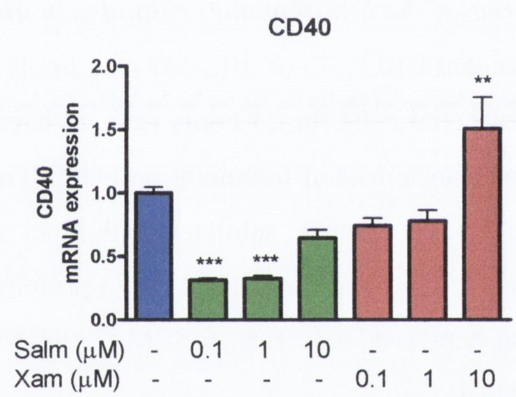
(b) IL-1β mRNA



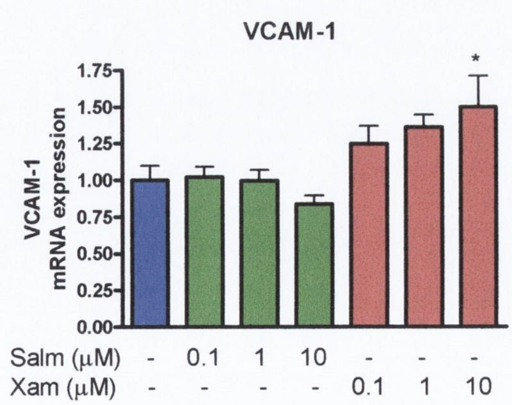
(c) TNFα mRNA



(d) CD40 mRNA



(e) VCAM-1 mRNA



(f) IP-10 mRNA

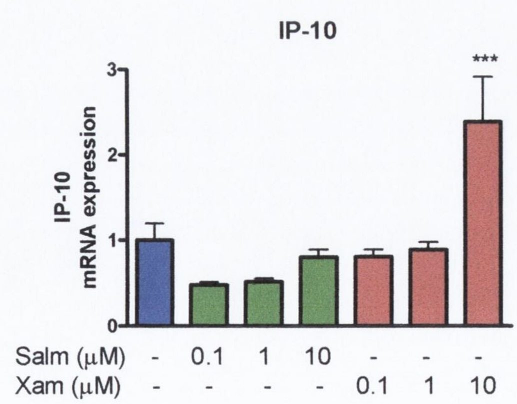


Figure 3.10.6:

(a) TNFα protein

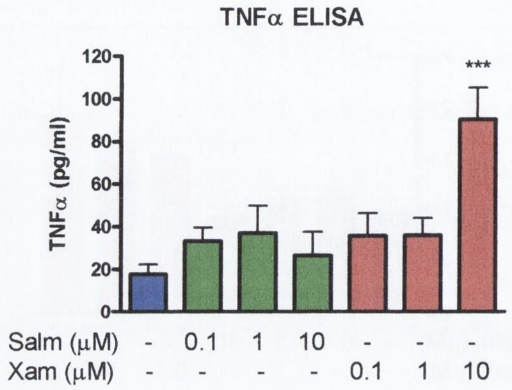


Figure 3.10.6: The β_1-AR agonist, xamoterol, but not the β_2-AR agonist, salmeterol, increases the inflammatory response in unstimulated mixed glial cells

Salmeterol (0.1, 1, or 10 μM) or xamoterol (0.1, 1, or 10 μM) was administered to mixed glial cells for 24 hours prior to harvesting. A one-way ANOVA demonstrated a significant effect of treatment on (a) TNFα protein [$F_{(6,35)}=5.25$, $P=0.0006$] expression. The β_2-AR agonist, salmeterol, did not alter TNFα protein expression. The β_1-AR agonist, xamoterol, increased TNFα protein expression.

Data expressed as means ± SEM (n=6), ***$P<0.001$ vs. control (Newman-Keuls post-hoc test).

3.11 The effect of a cAMP analogue, a PKA activator, and an EPAC activator on the inflammatory response induced by thrombin and Aβ

β_2-ARs signal by increasing intracellular cAMP concentrations which results in downstream activation of PKA and exchange protein directly activated by cAMP (EPAC). The following studies were carried out to assess the ability of c-AMP analogue (db-cAMP), PKA activator (BNZ), or EPAC activator (pCPT), to alter the inflammatory response induced by thrombin or Aβ. Vehicle, db-cAMP (100, 300, or 500 μM), BNZ (300 μM), or pCPT (300 μM) was administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle, thrombin (20 U/ml), or Aβ (2.5 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Alternatively, cells were harvested after 24 hours and the cell-free supernatants were harvested for the assessment of cytokine production by ELISA. Specifically, the expression of TNFα protein was examined.

Db-cAMP attenuates the inflammatory response induced by thrombin: Thrombin significantly increased IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression. Both doses of db-cAMP (100 and 500μM) significantly attenuated the thrombin-induced increase in TNFα, CD40, and VCAM-1 mRNA expression. Only the higher dose attenuated IP-10 mRNA. Db-cAMP did not alter IL-1β (Figure 3.11.1).

The PKA activator, BNZ, and the EPAC activator, pCPT, attenuate the inflammatory response induced by thrombin: Db-cAMP (300 μM), BNZ (300μM), and pCPT (300μM) significantly attenuated the thrombin-induced effect in TNFα, CD40, VCAM-1 and IP-10 mRNA expression. pCPT also significantly attenuated IL-1β mRNA (Figure 3.11.2). Db-cAMP, BNZ, and pCPT also significantly attenuated the thrombin-induced effect in TNFα protein (Figure 3.11.3)

The effect of db-cAMP, the PKA activator, BNZ, and the EPAC activator, pCPT, on the inflammatory response induced by Aβ: Db-cAMP (300 μM) and BNZ (300 μM) significantly augmented the Aβ-induced effect in IL-1β, but attenuated it in TNFα mRNA expression. There was no effect of pCPT (Figure 3.11.4).

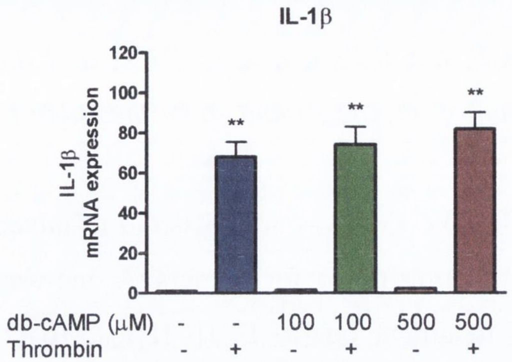
Figure 3.11.1: The c-AMP analogue, db-cAMP, attenuates the inflammatory response induced by thrombin in mixed glial cells

Db-cAMP (100 or 500 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between db-cAMP pre-treatment and thrombin treatment in (b) TNFα [$F_{(2,30)}$=24.66, P<0.0001], (c) CD40 [$F_{(2,30)}$=4.75, P=0.02], and (d) VCAM-1 [$F_{(2,30)}$=15.54, P<0.0001]. There was a significant effect of thrombin treatment only in (a) IL-1β [F(1,30)=236.36, P<0.0001] and (e) IP-10 [$F_{(1,29)}$=248.20, P<0.0001] mRNA expression. Db-cAMP (100 and 500 μM) attenuates the thrombin-induced increase in (b) TNFα, (c) CD40, and (d) VCAM-1 mRNA expression. Db-cAMP (500 μM only) attenuates the thrombin-induced increase in (e) IP-10 mRNA expression. There is no change in (b) IL-1β.

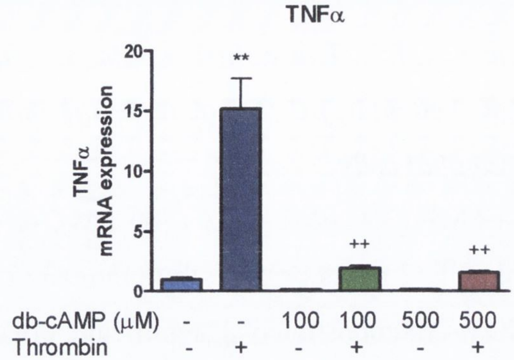
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control, +P<0.5, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

Figure 3.11.1:

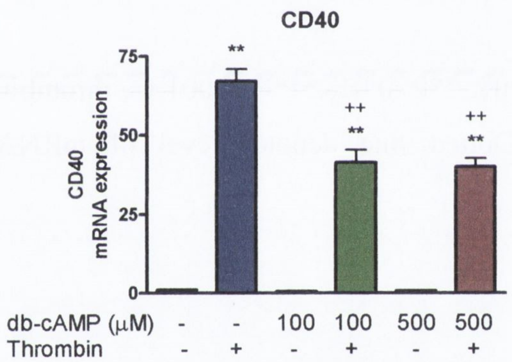
(a) IL-1β mRNA



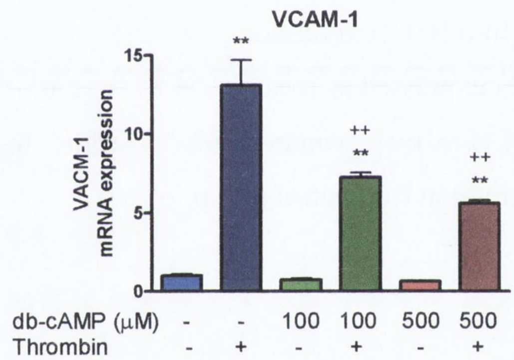
(b) TNFα mRNA



(c) CD40 mRNA



(d) VCAM-1 mRNA



(e) IP-10 mRNA

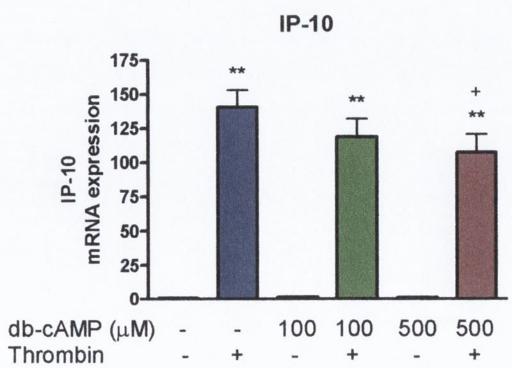


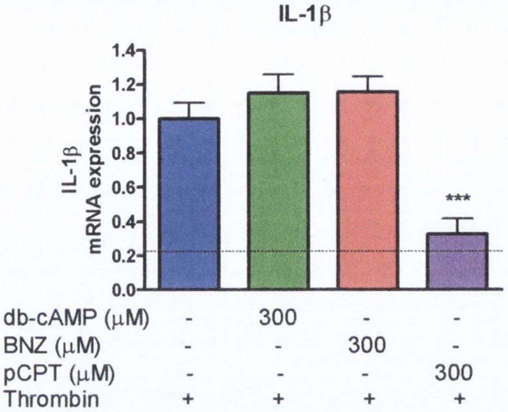
Figure 3.11.2: The c-AMP analogue, db-cAMP, the PKA activator, BNZ, and the EPAC activator, pCPT, attenuate the inflammatory response induced by thrombin in mixed glial cells

Db-cAMP (300 μM), BNZ (300 μM), or pCPT (300 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) IL-1β [$F_{(3,20)}$=16.41, P<0.0001], (b) TNFα [$F_{(3,20)}$=30.83, P<0.0001], (c) CD40 [$F_{(3,20)}$=19.37, P<0.0001], (d) VCAM-1 [$F_{(3,20)}$=40.54, P<0.0001], and (e) IP-10 [$F_{(3,20)}$=24.46, P<0.0001] mRNA expression. Db-cAMP, BNZ, and pCPT significantly attenuated (c) TNFα, (d) CD40, (e) VCAM-1, and (f) IP-10 mRNA expression. Only pCPT significantly attenuated (b) IL-1β mRNA expression.

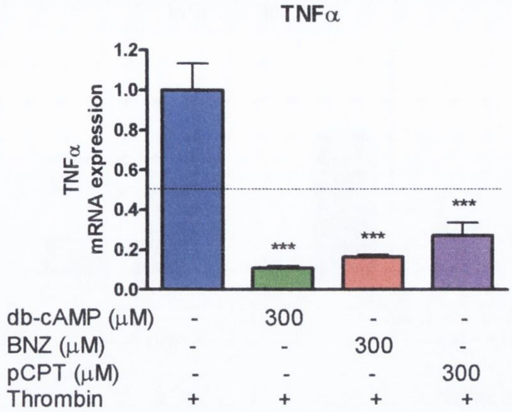
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01, ***P<0.001 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.11.2:

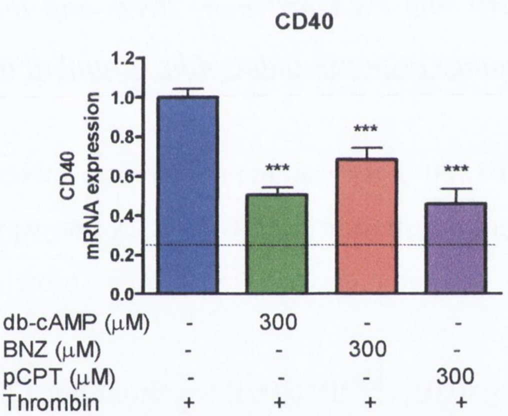
(a) IL-1β mRNA



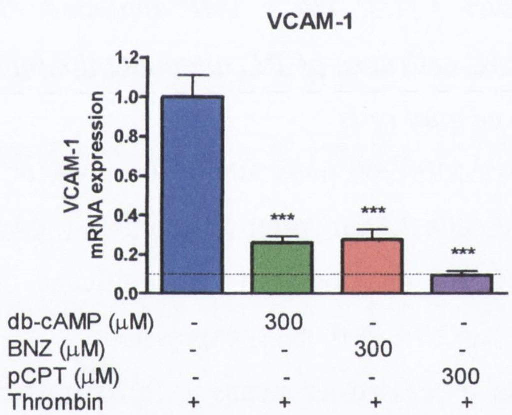
(b) TNFα mRNA



(c) CD40 mRNA



(d) VCAM-1 mRNA



(e) IP-10 mRNA

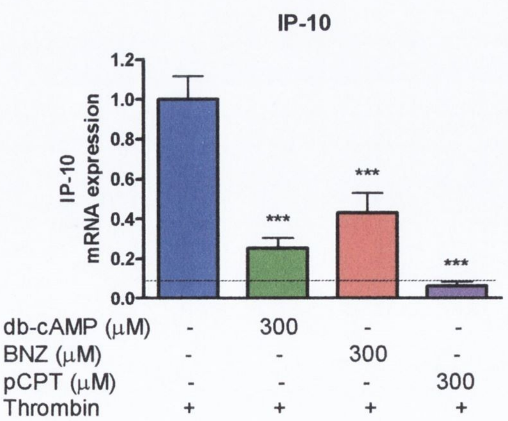


Figure 3.11.3:

(a) TNFα protein

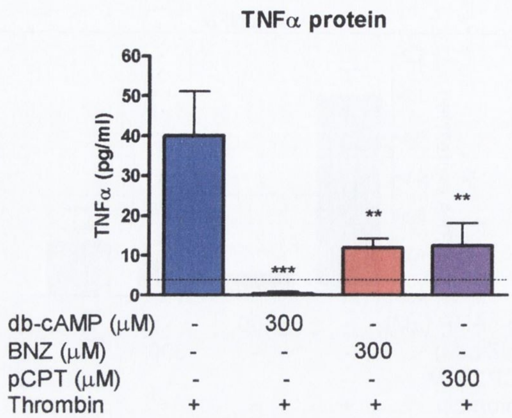


Figure 3.11.3: The c-AMP analogue, db-cAMP, the PKA activator, BNZ, and the EPAC activator, pCPT, attenuate the inflammatory response induced by thrombin in mixed glial cells

Db-cAMP (300 μM), BNZ (300 μM), or pCPT (300 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 24 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) TNFα protein [$F_{(3,19)}$=7.95, P=0.0012] expression.

Data expressed as means ± SEM (n=5-6), **P<0.01, ***P<0.001 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test). Dotted line denotes level of protein expression for control group.

Figure 3.11.4:

(a) IL-1β mRNA (b) TNFα mRNA

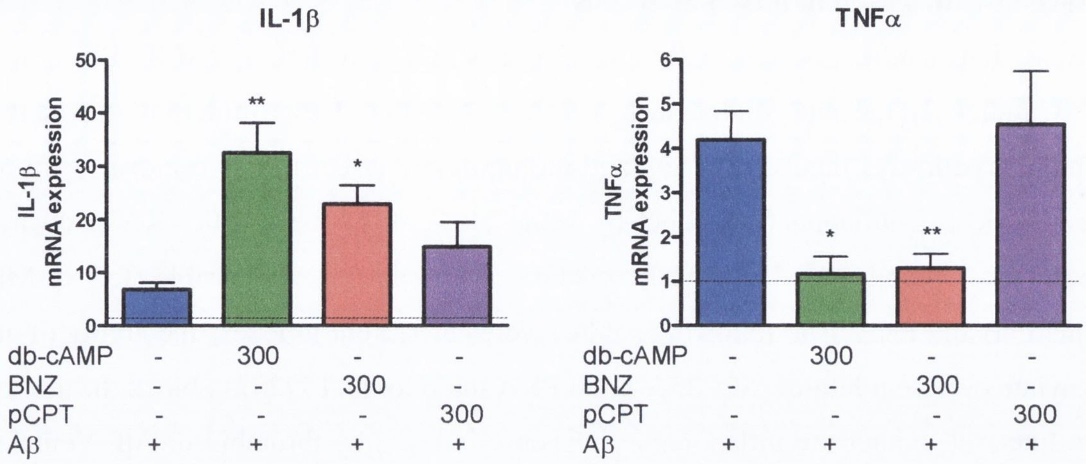


Figure 3.11.4: The effect of db-cAMP, the PKA activator, BNZ, and the EPAC activator, pCPT, on the inflammatory response induced by Aβ in mixed glial cells

Db-cAMP (300 μM), BNZ (300 μM), or pCPT (300 μM) was administered to mixed glial cells 2 hours prior to Aβ (2.5 μM) administration for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (a) IL-1β [$F_{(3,18)}$=7.46, P=0.0019] and (b) TNFα [$F_{(3,19)}$=7.41, P=0.0017] mRNA expression. Db-cAMP and BNZ significantly augmented (b) IL-1β, but attenuated (a) TNFα mRNA expression. There was no effect of pCPT.

Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. Aβ (2.5 μM) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

3.12 Adenylate cyclase and PKA inhibitors do not block, and in some cases augment, the salmeterol induced attenuation of the inflammatory response induced by thrombin in mixed glial cells

The previous studies showed that β_2-AR agonists and activators of cAMP, PKA, and EPAC mimicked the anti-inflammatory actions of NA. In order to further investigate the signalling pathways involved in this anti-inflammatory effect it was decided to try to prevent the anti-inflammatory effect by using blockers of the cAMP-PKA pathway, specifically inhibitors of adenylate cyclase (the enzyme responsible for cAMP formation) and PKA. The following studies were carried out to assess the ability of an adenylate cyclase inhibitor (SQ22536) or a PKA inhibitor (KT5720) to block the ability of salmeterol to alter the inflammatory response induced by thrombin or Aβ. Vehicle, SQ22536 (10 μM), or KT5720 (10 μM) was administered to cortical mixed glial cultures 30 min prior to the administration of vehicle or salmeterol (10 μM), which was administered 2 hours prior to the administration of vehicle or thrombin (20 U/ml). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically the mRNA expression of TNFα, CD40, VCAM-1, and IP-10 were examined as these molecules showed the greatest anti-inflammatory effect when treated with β-AR agonists.

The adenylate cyclase inhibitor, SQ22536, does not alter, while the PKA inhibitor, KT5720, augments the salmeterol-induced attenuation: Salmeterol significantly decreased the thrombin-induced effect in CD40, TNFα, VCAM-1, and IP-10 mRNA expression. SQ22536 and KT5720 had no effect on CD40, TNFα, VCAM-1, or IP-10 mRNA expression. Salmeterol, with SQ22536 or KT5720, significantly decreased the thrombin-induced effect in CD40, TNFα, VCAM-1, and IP-10 mRNA expression. SQ22536 did not alter the Salmeterol-induced effects. KT5720 augmented the Salmeterol-induced effects in VCAM-1 and IP-10, but it had no effect on TNFα or CD40 (Figure 3.12.1).

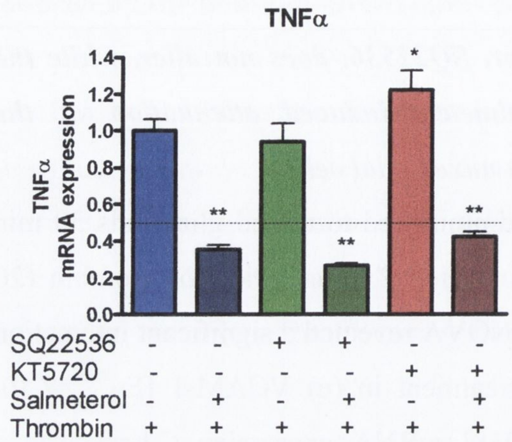
Figure 3.12.1: The adenylate cyclase inhibitor, SQ22536, does not alter, while the PKA inhibitor, KT5720, augments the salmeterol-induced attenuation of the inflammatory response induced by thrombin in mixed glial cells

SQ22536 (10 μM) or KT5720 (10 μM) was administered to mixed glial cells 30 min prior to salmeterol (10 μM), which was administered 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between pre-treatment and salmeterol treatment in (c) VCAM-1 [$F_{(2,29)}$=7.10, P=0.0031] and (d) IP-10 [$F_{(2,28)}$=3.70, P=0.038] mRNA expression. There was a significant effect of salmeterol treatment only in (a) TNFα [$F_{(1,29)}$=165.48, P<0.0001] and (b) CD40 [$F_{(1,29)}$=42.51, P<0.0001]. KT5720 significantly augmented the salmeterol-induced attenuation in (c) VCAM-1 and (d) IP-10, but not (a) TNFα or (b) CD40. SQ22536 had no effect.

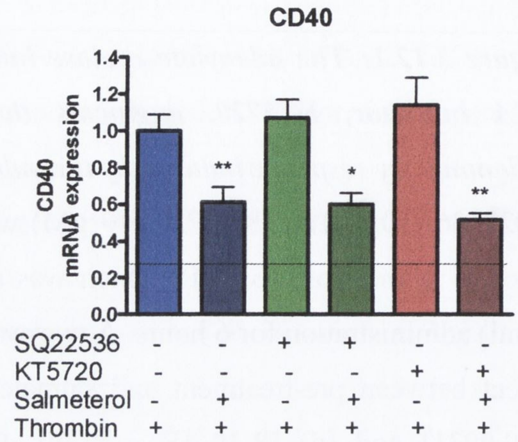
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01, vs. thrombin (20 U/ml), ++P<0.01 vs. thrombin with salmeterol (10μM) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.12.1:

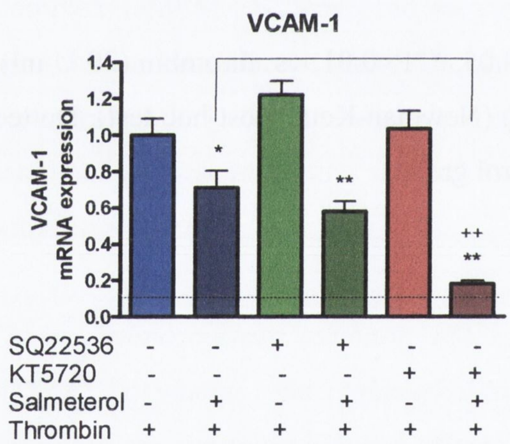
(a) TNFα mRNA



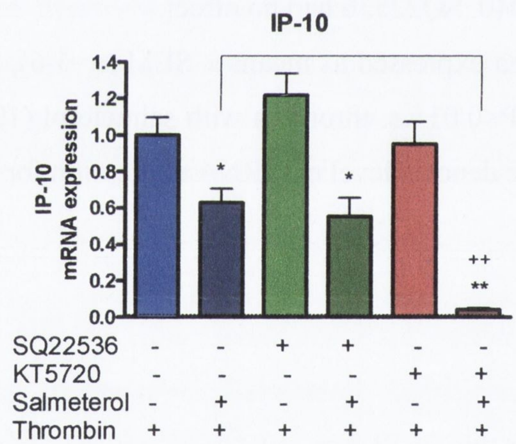
(b) CD40 mRNA



(c) VCAM-1 mRNA



(d) IP-10 mRNA



3.13 The effect of the β-AR antagonist, propranolol, on the NA-induced attenuation of the inflammatory response induced by thrombin and Aβ

The previous studies showed that the anti-inflammatory actions of NA were mimicked by β_2-AR agonists. In order to further investigate the receptors involved in this anti-inflammatory effect, it was decided to try to block the anti-inflammatory effect by using a β-AR antagonist. The following studies were carried out to assess to ability of the β-AR antagonist, propranolol, to block the ability of NA to attenuate the inflammatory response induced by thrombin or Aβ. Vehicle or propranolol (1 μM) was administered to cortical mixed glial cultures 30 min prior to the administration of vehicle or NA (10 μM), which was administered 2 hours prior to the administration of vehicle, thrombin (20 U/ml) or Aβ (2.5 μM). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR.

The β-AR antagonist, propranolol, augments the NA-induced attenuation of the inflammatory response induced by thrombin: Thrombin significantly increased TNFα, CD40, VCAM-1, and IP-10 mRNA expression. NA significantly attenuated the thrombin-induced increase in TNFα and VCAM-1 mRNA expression, but not CD40 or IP-10. NA with propranolol significantly decreased the thrombin-induced increase in TNFα, CD40, VCAM-1, and IP-10 mRNA expression. Propranolol alone augmented the thrombin-induced increase in TNFα mRNA expression only. Propranolol augmented the NA-induced attenuation in VCAM-1 and IP-10, but it did not alter TNFα or CD40 (Figure 3.13.1). Propranolol did not alter the expression of TNFα, CD40, VCAM-1, or IP-10 mRNA in cells which were not treated with thrombin (data not shown).

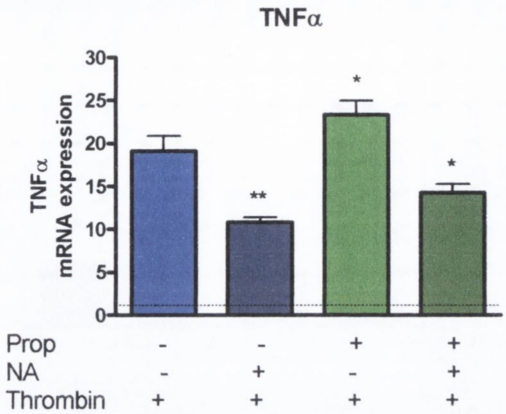
The β-AR antagonist, propranolol, does not alter the NA-induced attenuation of the inflammatory response induced by Aβ: NA and propranolol, alone or in combination, significantly attenuated the Aβ-induced effect in TNFα mRNA expression. Propranolol did not alter the NA-induced effects in TNFα. None of the treatments altered IL-1β mRNA (Figure 3.13.2). Propranolol did not alter the expression of TNFα or IL-1β mRNA in cells which were not treated with Aβ (data not shown).

Figure 3.13.1: The β-AR antagonist, propranolol, augments the NA-induced attenuation of the inflammatory response induced by thrombin in mixed glial cells

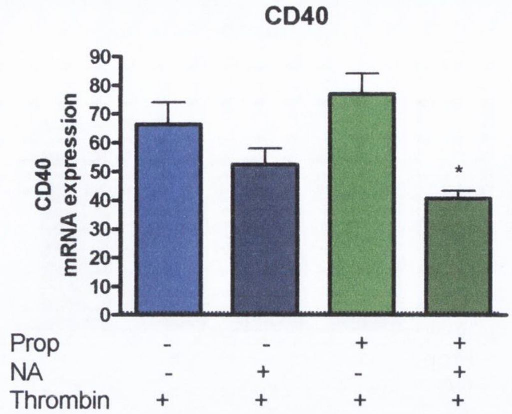
Propranolol (1 μM) was administered to mixed glial cells 30 min prior to NA (10 μM), which was administered 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between propranolol pre-treatment and NA treatment in (d) IP-10 [$F_{(1,20)}$=18.13, P=0.0004] mRNA expression. There was a significant effect of NA treatment in (a) TNFα [$F_{(1,20)}$=42.91, P<0.0001], (b) CD40 [$F_{(1,20)}$=17.74, P=0.0004], and (c) VCAM-1 [$F_{(1,20)}$=26.02, P<0.0001]. There was a significant effect of propranolol treatment only in (a) TNFα [$F_{(1,20)}$=8.25, P=0.0094]. Propranolol significantly augmented the NA-induced attenuation in (c) VCAM-1 and (d) IP-10 mRNA expression, but it did not affect (a) TNFα or (b) CD40. Data expressed as means ± SEM (n=6), *P<0.05, **P<0.01, ***P<0.001 vs. thrombin (20 U/ml), +P<0.05, ++P<0.01, +++P<0.001 vs. thrombin with NA (10μM) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.13.1:

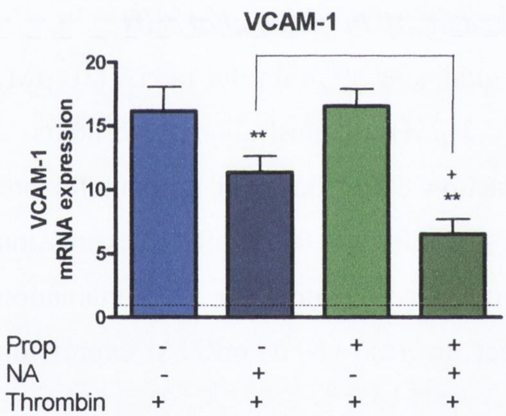
(a) TNFα mRNA



(b) CD40 mRNA



(c) VCAM-1 mRNA



(d) IP-10 mRNA

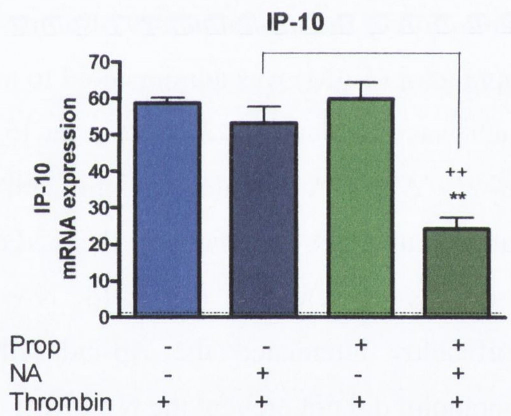


Figure 3.13.2:

(a) IL-1β mRNA (b) TNFα mRNA

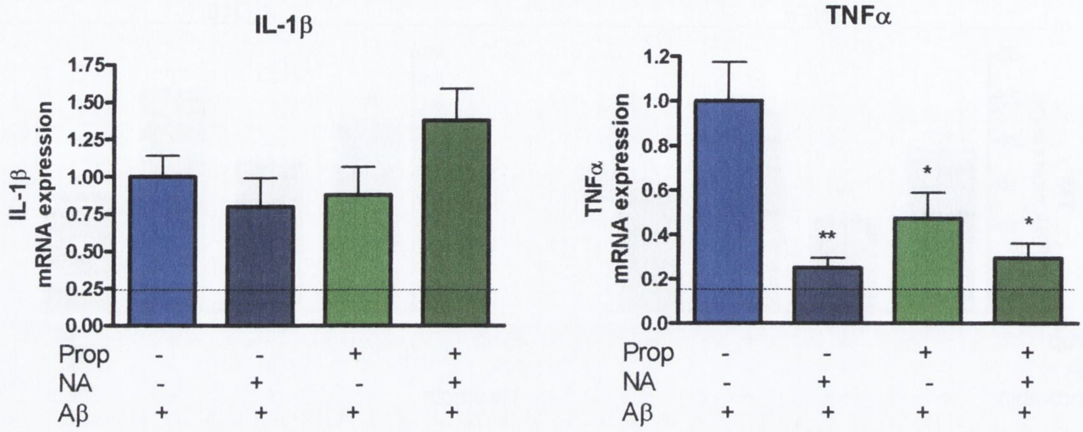


Figure 3.13.2: The β-AR antagonist, propranolol, does not alter the NA-induced attenuation of the inflammatory response induced by Aβ in mixed glial cells

Propranolol (1 μM) was administered to mixed glial cells 30 min prior to NA (10 μM), which was administered 2 hours prior to Aβ (2.5 μM) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between propranolol pre-treatment and NA treatment in (b) TNFα [$F_{(1,20)}$=6.80, P=0.017] mRNA expression. There was no effect in (a) IL-1β. NA and propranolol, alone or in combination, significantly attenuated the Aβ-induced effect in (a) TNFα mRNA expression. Propranolol did not prevent the NA effect.

Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. Aβ (2.5 μM) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

3.14 The effect of the selective β_1-AR antagonist, metoprolol, and the selective β_2-AR antagonist, butoxamine, on the NA-induced attenuation of the inflammatory response induced by thrombin

The previous study showed that the β-AR antagonist, propranolol, augmented the ability of NA to attenuate the thrombin-, but not the Aβ-, induced inflammatory response. It was hypothesised that this could be due to the blockade of the pro-inflammatory actions that had been perceived to be mediated via the β_1-AR. As the anti-inflammatory actions of NA are mediated via β_2-AR it was decided to examine the effects of selective β_1-AR and β_2-AR antagonists. The following studies were carried out to assess to ability of the β_1-AR antagonist metoprolol and the β_2-AR antagonist butoxamine to block the ability of NA to attenuate the inflammatory response induced by thrombin or Aβ. Vehicle, metoprolol (10 μM), or butoxamine (10 μM) was administered to cortical mixed glial cultures 30 min prior to the administration of vehicle or NA (10 μM), which was administered 2 hours prior to the administration of vehicle or thrombin (20 U/ml). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR.

The selective β_1-AR antagonist, metoprolol, and the selective β_2-AR antagonist, butoxamine, attenuate the NA-induced attenuation of the inflammatory response induced by thrombin: NA significantly reduced TNFα, CD40, VCAM-1 and IP-10 mRNA expression. The β_1-AR antagonist metoprolol and the β_2-AR antagonist butoxamine significantly increased VCAM-1 and IP-10 mRNA expression. Butoxamine also significantly decreased TNFα mRNA. Metoprolol attenuated the NA-induced decrease in VCAM-1 and IP-10 mRNA expression. Butoxamine also attenuated the NA-induced decrease in IP-10 (Figure 3.14.1).

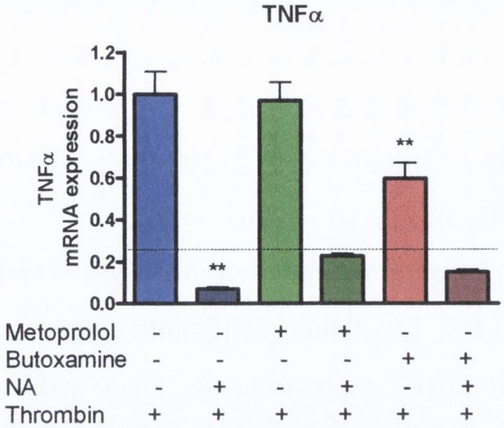
Figure 3.14.1: The selective β_1-AR antagonist, metoprolol, augments, and the selective β_2-AR antagonist, butoxamine, augments and attenuates the NA-induced attenuation of the inflammatory response induced by thrombin in mixed glial cells

Metoprolol (10 μM) or butoxamine (10 μM) was administered to mixed glial cells 30 min prior to NA (10 μM), which was administered 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA revealed a significant interaction effect between pre-treatment and NA treatment in (a) TNFα [$F_{(2,28)}$=7.68, P=0.0022], (c) VCAM-1 [$F_{(2,30)}$=8.13, P=0.0015], and (d) IP-10 [$F_{(2,29)}$=10.15, P=0.0005] mRNA expression. There was a significant effect of NA treatment in (b) CD40 [$F_{(1,29)}$=34.51, P<0.0001] mRNA expression. There was also a significant effect of metoprolol or butoxamine treatment in (b) CD40 [$F_{(2,29)}$=3.62, P=0.039] mRNA expression. NA significantly reduced mRNA expression in all targets. Metoprolol and butoxamine significantly increased (c) VCAM-1 and (d) IP-10 mRNA expression. Butoxamine also significantly decreased (a) TNFα mRNA. Metoprolol attenuated the NA-induced attenuation in (c) VCAM-1 and (d) IP-10 mRNA expression. Butoxamine also attenuated the NA-induced attenuation in (d) IP-10.

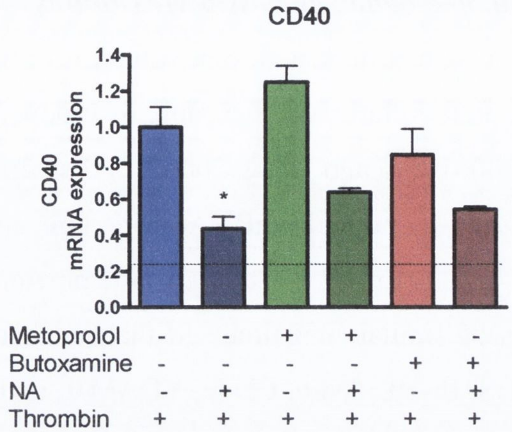
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. thrombin (20 U/ml), +P<0.05, ++P<0.01 vs. thrombin with NA (10μM) (Newman-Keuls post-hoc test). Dotted line denotes level of mRNA expression for control group.

Figure 3.14.1

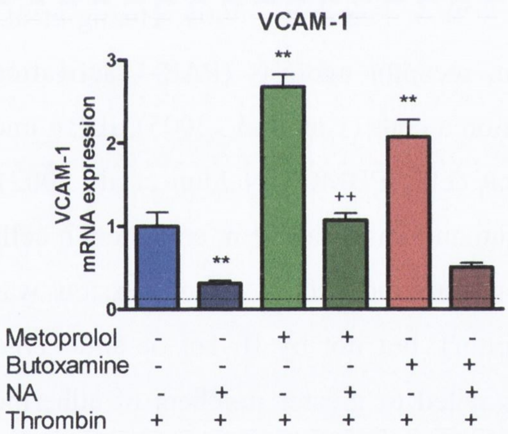
(a) TNFα mRNA



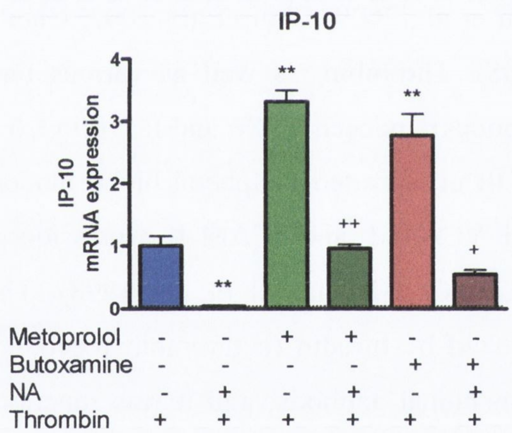
(b) CD40 mRNA



(c) VCAM-1 mRNA



(d) IP-10 mRNA



3.15 Discussion

3.15.1 LPS, thrombin, and Aβ induce an inflammatory response in primary mixed glial, microglial, and astrocyte cultures

Consistent with other studies, LPS was used as a positive control to show that the glial cultures were capable of producing an inflammatory response (Szczepanik et al., 2001, Liu and Hong, 2003). LPS significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression, and TNFα and IL-1β protein expression.

Thrombin induced an inflammatory response in all three culture types, which was of similar magnitude to that induced by LPS. Thrombin significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA expression, and TNFα and IL-1β protein expression. This fits with other *in vitro* studies on thrombin that showed that it results in activation of microglia (Lee et al., 2005, Katsuki et al., 2006), and astrocytes (Ishida et al., 2006), and that it induces iNOS and increases expression of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 in glial cells (Moller et al., 2000, Ryu et al., 2000, Choi et al., 2003, Choi et al., 2005, Lee et al., 2005, Huang et al., 2008). Thrombin, as well as various thrombin receptor agonists (PAR-1 activating peptides), induced TNFα and IL-1β in C6 rat glioma cells (Fan et al., 2005), IL-1α and IL-1β in activated peripheral blood mononuclear cells (PBMC) (Naldini et al., 2002), and VCAM-1 and ICAM-1 expression on human umbilical vein endothelial cells (HUVECs) (Kaplanski et al., 1998). This thrombin-induced CAM expression was blocked by hirudin (a thrombin receptor inhibitor), but not by IL-1ra or anti-TNFα monoclonal antibody, and it was functional as it led to greater numbers of adhering THP-1 macrophagic cells, PBMCs, or purified monocytes (Kaplanski et al., 1998). This appears to be the first study to report that thrombin induces IP-10 production, although it has been shown that thrombin can induce other chemokines such as IL-8 and MCP-1 (Marin et al., 2001, Yoshida et al., 2001, Szaba and Smiley, 2002).

Aβ also induced an inflammatory response in all three culture types, although it was less robust than the response induced by LPS or thrombin. Aβ significantly increased IL-1β and TNFα mRNA expression, while only the highest dose (10 μM) significantly increased CD40. There was a tendency towards an increase in iNOS but it was not significant, possibly due to variation in the samples. There was no effect on VCAM-1 or IP-10 mRNA. Aβ also significantly increased IL-1β protein expression, but not TNFα protein expression. However this may have been due to a high control reading for TNFα protein and high variability in the other samples. This Aβ-induced inflammatory response fits with other studies which showed that exposure of microglia cultures to Aβ resulted in an increase in the secretion of the pro-inflammatory cytokines IL-1β and TNFα (Szczepanik et al., 2001, Rogers et al., 2002, McGeer and McGeer, 2003), and with a study which showed that astrocyte cultures treated with Aβ produced iNOS, IL-1β, and TNFα (Akama and Van Eldik, 2000). From this point on, when Aβ was used as a treatment, the expression of IL-1β and TNF-α only were examined, as these molecules showed the most dramatic effect in response to Aβ.

These results show that astrocytes are capable of producing an inflammatory response just as well as microglia, indicating an important role for these cells in the brains immune response. This is consistent with other findings which have implicated astrocytes in the inflammatory response (Forloni et al., 1997, Liberto et al., 2004, Nicholson et al., 2004, Iwai et al., 2006), and is particularly important as astrocytes are the most numerous cells in the CNS and greatly outnumber microglia (Stevens, 2003).

3.15.2 NA attenuates the inflammatory response in mixed glial cultures much more potently than in enriched microglial and astrocyte cultures

As LPS, thrombin, and Aβ had been shown to induce an immune response in glial cells, the next step was to look at possible ways to reduce this inflammation. NA, as expected, dramatically attenuated the inflammatory response induced by thrombin and Aβ in mixed glial cells. NA (1 and 10 μM) attenuated the thrombin-induced increase in TNFα, CD40, VCAM-1, and IP-10 mRNA expression and TNFα protein expression, but only the 10 μM dose of NA attenuated iNOS and IL-1β mRNA expression. No IL-1β protein data is available for this experiment as there was a problem with the IL-1β ELISA kit. NA significantly attenuated the Aβ-induced increase in TNFα and CD40 mRNA expression, but not IL-1β mRNA. This NA-induced attenuation of the inflammatory response is consistent with numerous other studies which have used microglia, astrocytes, and brain endothelial cells to demonstrate that NA, by binding to β_2-ARs and activating cAMP signalling pathways, can inhibit the expression of many inflammatory genes including iNOS, IL-1β, TNFα, VCAM-1, ICAM-1, and MHC-class-II induced by cytokines, LPS, and Aβ (Ballestas and Benveniste, 1997, Feinstein et al., 2002, Madrigal et al., 2005). However we report for the first time that NA also reduces the inflammatory response induced by thrombin.

In microglial and astrocyte cultures, NA significantly attenuated of the inflammatory response induced by thrombin and Aβ, however the magnitude of this attenuation was much less than the attenuation induced by NA in mixed glial cultures. Only the 10 μM dose of NA produced an attenuation in the separated glia whereas both the 1 μM and 10 μM doses had attenuated the inflammatory response in mixed glial cells. NA (10 μM) attenuated the thrombin-induced increase in iNOS and IL-1β in astrocytes only, in TNFα expression in microglia only, and in CD40 mRNA expression in both astrocytes and microglia. NA (10 μM) attenuated the Aβ-induced increase in iNOS expression in microglia only. However in TNFα mRNA expression, while it did not produce a significant attenuation, it did prevent the Aβ-induced increase from becoming significant. The combination of Aβ and NA produced a significant increase in CD40 mRNA expression in astrocytes, however this is an individual results and likely due to contamination of the sample. Also NA did not attenuate, and in some instances augmented, the inflammatory response induced by LPS. NA (0.1 μM and 1 μM) significantly augmented the LPS-induced increase in iNOS in microglia and astrocytes, and in IL-1β and TNFα in astrocytes only. It did not alter CD40 mRNA expression. Interestingly the 10 μM dose of NA did not produce any effect. This was an unexpected finding as many previous studies in this laboratory, as well as the earlier studies in this section, have shown that NA potently suppressed the inflammatory response induced by LPS in mixed glial cultures (Ballestas and Benveniste, 1997, Feinstein et al., 2002, Madrigal et al., 2005). As the anti-inflammatory actions of NA are thought to be mediated via the β_2-AR (Borger et al., 1998, Feinstein et al., 2002, De Keyser et al., 2004b), it was hypothesised that a potential reason for this unusual result could be that expression of the β_2-AR might be reduced on glia when they are cultured separately instead of together. Consistent with this hypothesis, it was shown that enriched cultures of microglia or astrocytes had significantly lower expression of β_2-AR mRNA expression compared to mixed glial cultures (65% reduction in astrocytes and 89% reduction in microglia). This result was unexpected as the immunocytochemistry conducted in this section, in concurrence with other studies, showed that astrocytes and microglia in mixed glial cultures express β_1- and β_2-ARs (Tanaka et al., 2002, De Keyser et al., 2004). However, the finding that β_2-AR expression is dramatically reduced in microglial and astrocyte cultures is likely the explanation for the unusual NA-induced responses that had been shown. This is also consistent with other studies from this laboratory which showed that LPS and inflammatory cytokines reduce β_2-AR expression and responsiveness in glial cells (Ryan, 2009).

In addition to this, MCSF and GMCSF, which are used as part of the process when growing glial cultures for separation, have been shown to induce an inflammation (Cook et al., 2004, De Ciuceis et al., 2005, Fleetwood et al., 2007). Interestingly, a number of studies by Van Oosterhout and colleagues showed that pre-incubation of peripheral blood mononuclear cells (PBMC) for 20 hours with GM-CSF decreased β-AR agonist induced cAMP production (Van Oosterhout et al., 1990, Van Oosterhout et al., 1992). For these reasons the remaining experiments were carried out on mixed glial cultures only. Also mixed glial cultures are closer to the natural environment of glial distribution in the brain and therefore would give a better indication of how the treatments used could react *in vivo*.

3.15.3 Thrombin-induced inflammation and NA interventions

The β-AR agonist salbutamol, the β_2-AR agonist salmeterol, the c-AMP analogue db-cAMP, the PKA activator BNZ, and the EPAC activator pCPT, all attenuated the thrombin-induced inflammatory response in TNFα, CD40, IP-10, and VCAM-1 mRNA. Salbutamol and salmeterol also attenuated the thrombin-induced increase in iNOS mRNA, however the data for iNOS with db-cAMP was conflicting so it was removed from this study. Salmeterol, db-cAMP, BNZ, and pCPT also attenuated the thrombin-induced increase in TNFα protein. Only the highest dose of salmeterol (10 μM) attenuated the thrombin-induced inflammatory response in IL-1β protein. No IL-1β protein data is available for the other experiments as there was a problem with the IL-1β ELISA kit. This attenuation is consistent with a study showing that the thrombin-induced increase in MAPK phosphorylation and CD40L expression in platelets was inhibited by the cAMP elevating PGE1 (Schwarz et al., 2000), and that the thrombin-induced effects on airway smooth muscle cells were inhibited by salbutamol and other cAMP increasing agents such as forskolin and PGE1 (Tomlinson et al., 1994 1995, Stewart et al., 1997). Furthermore, one study showed that thrombin results in a decrease in cAMP (Bogatcheva et al., 2002), therefore it is logical that increasing cAMP inhibits the effects of thrombin.

Interestingly, salbutamol also augmented the thrombin-induced increase in IL-1β mRNA, and the β_1-AR agonist xamoterol augmented iNOS, IL-1β, and IP-10 mRNA. These results indicate that the β_2-AR mediates an anti-inflammatory effect, while the β_1-AR has modest pro-inflammatory effect. The response of TNFα or IL-1β protein expression to xamoterol was not investigated as this study focused on the anti-inflammatory actions of NA. This is consistent with other studies which have shown that anti-inflammatory effects are mediated solely via the β_2-AR and not the β_1-AR (Frohman et al., 1988, Borger et al., 1998, Nakamura et al., 1998), however none of these papers has proposed a theory as to why this is the case.

When the mixed glia were not treated with an inflammatory stimulus, the β_2-AR agonist, salmeterol, significantly decreased CD40 mRNA expression, but not iNOS, IL-1β, TNFα, VCAM-1, or IP-10 mRNA, or TNFα protein. However, the β_1-AR agonist, xamoterol, significantly increased iNOS, IL-1β, TNFα, CD40, VCAM-1, and IP-10 mRNA, and TNFα protein expression. No IL-1β protein data is available for this experiment as there was a problem with the IL-1β ELISA kit. This shows that even under basal conditions the β_2-AR agonist salmeterol decreased expression of inflammatory molecules, whereas a β_1-AR agonist xamoterol increased expression.

The adenylate cyclase inhibitor SQ22536 did not alter the salmeterol-induced inhibition of the thrombin-induced inflammatory response CD40, TNFα, VCAM-1, and IP-10 mRNA expression, whereas the PKA inhibitor KT5720 augmented the salmeterol-induced effects in VCAM-1 and IP-10 mRNA expression. This means that inhibition of adenylate cyclase or PKA is insufficient to prevent the anti-inflammatory actions induced by β_2-AR activation, and can in fact increase them. Although this result was not expected, it is consistent with a study on microglia which showed that NA inhibited LPS-induced iNOS expression, but that KT5720 did not reverse the effects of NA while SQ22536 actually potentiated NA inhibitory effects (Dello Russo et al., 2004). Another study showed that salmeterol activated microglia and increased ROS production via the selective phosphorylation of ERK, a process that was independent of PKA (Qian et al., 2009). In contrast to this, the salbutamol- and cAMP-induced inhibition of the thrombin-induced proliferation of cultured airway smooth muscle cells was attenuated by a cAMP antagonist (Tomlinson et al., 1995). These findings suggest that, although PKA is a primary target for activation by cAMP, other pathways, such as the EPAC or ERK pathways, may also play a role in mediating the NA-induced inhibition of glial activation.

It had been hypothesised that AR antagonists would be capable of attenuating the NA-induced inhibition of thrombin-induced inflammation. The β-AR antagonist propranolol augments, while the selective β_1-AR antagonist metoprolol and the selective β_2-AR antagonist butoxamine attenuate, the NA-induced inhibition of the thrombin-induced expression of VCAM-1 and IP-10. These results are not consistent with studies which show that propranolol prevents the anti-inflammatory effects of NA (Tanaka et al., 2002, Madrigal et al., 2009, McNamee et al., 2010), prevents the NA-induced inhibition of the ATP-induced increase in the TNFα (Morioka et al., 2009), and inhibits NA-induced VCAM-1 expression (Chen et al., 2005). Propranolol, metoprolol, and butoxamine did not have any effect on unstimulated cells (data not shown). Interestingly propranolol alone increased while butoxamine alone decreased the thrombin-induced expression of TNFα. Also, metoprolol and butoxamine increased VCAM-1 and IP-10 indicating that β-AR antagonists can have a pro-inflammatory effect on their own. These results are not consistent with reports that propranolol, metoprolol, and butoxamine do not alter TNFα concentrations induced by subarachnoid haemorrhage (Kato et al., 2009), or that metoprolol either did not alter (Dorenkamp et al., 2005, Wolf et al., 2007) or decreased (Liang et al., 2009) VCAM-1 expression. A potential explanation for this is that there is basal expression of NA in glial cultures which provides a low level tonic inhibition of the inflammatory response and that blocking the β-ARs prevents this.

3.15.4 Aβ-induced inflammation and NA interventions

In contrast to the earlier finding that NA attenuated the inflammatory response induced by Aβ, salbutamol and xamoterol augmented the Aβ-induced increase in TNFα and IL-1β, while salmeterol only augmented the Aβ-induced increase in IL-1β. The β-AR antagonist propranolol, either alone or in combination with NA, significantly attenuated the Aβ-induced effect in TNFα but had no effect on IL-1β. This shows that β-AR agonists, unlike NA itself, can augment the Aβ-induced inflammation while β-AR antagonists can have an anti-inflammatory effect similar to that induced by NA. This finding could be explained by other studies which show that Aβ increased cAMP formation, an increase that was inhibited by β-AR antagonists (Igbavboa et al., 2006), and Aβ up-regulates the cAMP-specific phosphodiesterase PDE4B (Sebastiani et al., 2006), implicating cAMP as an important mediator of Aβ-induced microglial activation.

Interestingly, when molecules downstream of β-AR activation are used, the results are again similar to those induced by NA. The cAMP analogue db-cAMP and the PKA activator BNZ significantly attenuated the Aβ-induced increase in TNFα mRNA expression. However db-cAMP augmented Aβ-induced IL-1β mRNA. These findings are consistent with studies showing that increasing cAMP levels protected against Aβ-induced developmental abnormalities (Buznikov et al., 2008) and inhibition of LTP

(Wang et al., 2009), and that the Aβ-induced increase in endozepine release in cultured rat astrocytes was blocked by an adenylate cyclase inhibitor and a PKA inhibitor (Tokay et al., 2008). Db-cAMP inhibited the LPS-induced release of TNFα and IL-1β from microglial cultures (Schubert et al., 2000), but it augmented Aβ-induced iNOS expression and NO release (Pyo et al., 1999). The contradictory effect of db-cAMP on IL-1β and TNFα could be accounted for by a study showing that db-cAMP can induce glial activation and increase IL-1β in astrocyte cultures (LaDu et al., 2000, LaDu et al., 2001).

The β-AR antagonist propranolol did not block the NA-induced attenuation in TNFα mRNA expression. However propranolol alone significantly attenuated the Aβ-induced effect in TNFα mRNA. None of the treatments altered IL-1β mRNA expression. This means that AR antagonists are not sufficient to prevent the anti-inflammatory effect induced by NA. Again this is not consistent with studies which show that propranolol prevents the anti-inflammatory effects of NA (Tanaka et al., 2002, Madrigal et al., 2009, McNamee et al., 2010).

3.15.5 Conclusion

The first finding of this section is that astrocyte, microglial, and mixed glial cultures are capable of exhibiting an inflammatory response following stimulation with LPS, thrombin or Aβ. This is an important finding as it emphasises the vital role that astrocytes play in the brain's immune response. It is also important in regard to the inflammatory environment that is found in the brain following a stroke or during PD and AD. If suitable treatments are to be developed which will modulate this inflammatory response they must target astrocyte-induced inflammation as well as microglial-induced inflammation.

The second finding of this section is that NA has a predominantly anti-inflammatory effect which is mimicked by activation of the β_2-AR pathway and downstream molecules such as cAMP, PKA, and EPAC. However it can also have a small pro-inflammatory effect, caused by β_1-AR activation. Interestingly, β-AR antagonists and inhibitors of adenylate cyclase and PKA are not capable of preventing the NA-induced effects, indicating that there may be additional pathways by which NA can exert its anti-inflammatory effects in glial cells. Further research is required in order to elucidate other pathways which may be involved in mediating the anti-inflammatory actions of NA. One such target for research could be the ERK pathway (Qian et al., 2009).

These studies showed that the induced inflammatory response and the subsequent attenuation of this response occurred both in the mRNA and in the protein of TNFα and IL-1β, indicating that the effects took place at a transcriptional level and that they were maintained through the translational level to protein expression. These molecules were chosen to be investigated at the protein level as they are among the first pro-inflammatory mediators to be released in response to glial activation (Liberto et al., 2004, Lucas et al., 2006) and are potent modulators of the immune response, causing the induction of cytokines, chemokines, adhesion molecules, and inflammatory enzymes such as iNOS (Laflamme et al., 1999, Akama and Van Eldik, 2000, Aggarwal, 2003). This means that they are good indicators of the state of the inflammatory response. However the other molecules were only examined at the mRNA level, therefore future studies could examine the effect of thrombin and β-AR activation on the levels of protein expression of iNOS, CD40, IP-10, and VCAM-1.

4. The response of neurons to cytokines and β-AR activation

4.1 Introduction

The studies in Chapter 3 of this thesis showed that thrombin and Aβ are capable of directly activating glial cells to induce production of the cytokines IL-1β and TNFα, and that β-AR activation is capable of dramatically reducing this inflammatory response. It was hypothesised that these cytokines and β-AR activation could have a direct effect on neurons. The studies in this chapter were carried out to assess the effect of the pro-inflammatory cytokines IL-1β, TNFα, and IFNγ on neuronal cytokine receptor expression and neuronal viability, and to examine the ability of β_2-ARs and the downstream signalling molecule cAMP to alter neuronal cytokine receptor expression and to protect against cytokine-induced neurotoxicity.

Cytokines have been shown to be neurotoxic: For example TNFα can induce apoptosis in a number of cell types including neurons (Yang et al., 2002, Buntinx et al., 2004a, Buntinx et al., 2004b, Nakashima et al., 2005), however it can also be neuroprotective (Yang et al., 2002). The effects are dose and receptor dependent: high doses are toxic and are mediated via TNFR1, while lose doses can be trophic and are mediated via TNFR2 (Yang et al., 2002, Bernardino et al., 2005). IFNγ has also been shown to induce apoptotic cell death (Buntinx et al., 2004b). The effects of these cytokines are amplified when given together, for example when IFNγ was given in conjunction with TNFα they induced greater amounts of cell death than when administered individually (Buntinx et al., 2004b). Although there is substantial evidence from many *in vivo* studies indicating that endogenous IL-1β is an important mediator of neuronal injury (Allan et al., 2005), *in vitro* studies have found it difficult to establish the role of IL-1β in cell death. Some studies show that IL-1β alone can cause neuronal death (Bielarczyk et al., 2005, Abcouwer et al., 2008), while others say that it is only capable of exacerbating cell death that is induced by excitotoxic agents such as NMDA (Viviani et al., 2003). Still other studies have shown IL-1β to be neuroprotective, although this effect appears to be time dependent as the protective actions involved a 24 hour treatment with IL-1β, while enhanced injury was seen when cells were only treated with IL-1β for a few minutes (Allan et al., 2005).

IL-1β binds to IL-1Rs. NPCs express IL-1R1 and IL-1R2 (Wang et al., 2007), and neurons express IL-1R1 (Friedman, 2001, Wang et al., 2006a, Wang et al., 2006b), but IL-1R2 is commonly expressed on immune cells in the brain (Lucas et al., 2006). IL-1R1 mediates the effects of IL-1 through activation of NFκB and members of the MAPK family, ultimately resulting in inflammatory gene expression (Fitzgerald and O'Neill, 2000, Liew et al., 2005). In contrast to this, IL-1R2 functions as a decoy receptor, competing with IL-1R1 for IL-1β, thus acting as a negative regulator of the IL-1 system and eliciting an anti-inflammatory action (Colotta et al., 1994, Fitzgerald and O'Neill, 2000). In neuronal cultures, IL-1R1 associates with the cytosolic adaptor protein MYD88 which leads to phosphorylation of the tyrosine kinase Src (Davis et al., 2006, Tsakiri et al., 2008a), and the IL-1β-induced production of chemokines from neurons is dependent on IL-1R1 signalling (Tsakiri et al., 2008b). These studies indicate that IL-1R1 is extremely important in mediating the pro-apoptotic and pro-inflammatory effects of IL-1β. However some studies have shown that IL-R1 signalling is also important for neuronal survival. For instance, IL-1β protects against retinal ganglion cell death via activation of IL-1R1 (Diem et al., 2003), and cultured cerebellar granule cells from IL-1R1 KO mice were more vulnerable to NMDA treatment compared to the wild type controls (Pelidou et al., 2002).

TNFα signals through TNFR1 and TNFR2 which are expressed on neurons (Neumann et al., 1997a, McGuire et al., 2001, Bette et al., 2003, Dziewulska and Mossakowski, 2003, Yan et al., 2003). TNFR1 contains a death domain which activates caspase pathways leading to apoptosis while TNFR2 does not contain a death domain and is responsible for cell proliferation (Aggarwal, 2003, Marchetti et al., 2004). For example the apoptosis of motoneurons is regulated by TNFR1 which is transiently expressed during a short period corresponding to that of apoptosis, and over expression of TNFR1 is sufficient to induce apoptosis (Mahul-Mellier et al., 2008). Numerous studies have used cultures created from KO mice to show that neurons from TNFR1 KO mice were protected from TNFα- or glutamate-induced apoptosis, while neurons from TNFR2 KO mice showed increased cell death even at low doses (Yang et al., 2002, Marchetti et al., 2004). Similar results are seen with *in vivo* studies, for example TNFR1 KO mice showed a strong reduction of neurodegeneration following ischemia, while TNFR2 KO mice suffered increased neurodegeneration (Fontaine et al., 2002), and KO mice for TNFR1 or TNFR2 alone showed no effect on motoneuron cell death, but a dual TNFR1 and TNFR2 KO showed an almost complete prevention of cell loss (Raivich et al., 2002). TNFR1 is also required for Aβ-induced neuronal death (He et al., 2007). The pro-apoptotic signals of TNFR1 are such that the removal of these signals results in a large increase in the number of new, mature hippocampal neurons as demonstrated in TNFR1 KO and TNFR1/TNFR2 KO mice, but not TNFR2 KO mice (Iosif et al., 2006). However, some studies found increased neuronal damage after injury in TNFR1 KO mice (Gary et al., 1998, Lambertsen et al., 2009). In contrast to TNFR1, TNFR2 activation is anti-apoptotic and results in long term NFκB activation leading to new protein synthesis which is essential for neuronal survival (Aggarwal, 2003, Marchetti et al., 2004). TNFα has been shown to be neuroprotective against excitotoxic damage in primary cortical neurons via a process dependent on TNFR2 and NFκB activation (Dolga et al., 2008a), and cultured neurons that over-express TNFR2 are protected against TNFα induced apoptosis (Williams et al., 2005). Similarly, use of antisense oligonucleotides against TNFR2 in a neuroblastoma cell line inhibited TNFR2 expression, leading to significantly increased cell death following hypoxic conditions or treatment with TNFα or Aβ (Shen et al., 1997).

IFNγ acts via the IFNgR which signals via the JAK-STAT signalling pathway leading to gene transcription. The IFNgR1 subunit has high ligand binding affinity, while the IFNgR2 subunit is necessary for signalling (Bach et al., 1997, Chen et al., 2006, Gough et al., 2008). Neurons have also been shown to express IFNgR1 and IFNgR2 *in vitro* (Neumann et al., 1997a, Neumann et al., 1997b), and IFNgRs are found in motoneurons (Linda et al., 1998) and at pre- and postsynaptic sites in the spinal cord (Vikman et al., 1998, Tsuda et al., 2009). IFNgRs are important in mediating IFNγ induced toxicity: For example, in mixed glial-neuronal cultures if the microglia, but not the neurons, were deficient in IFNgR there was a decrease in IFNγ induced dopaminergic cell death (Mount et al., 2007). However, a study investigating neuropathogenesis following viral infection showed that IFNgR KO mice had much higher levels of neuronal death than wild type mice (Keogh et al., 2003).

The ability of cytokines to alter cytokine production is well documented. For example, TNFα and IL-1β have the ability to induce themselves and each other (Zetterstrom et al., 1998, Blond et al., 2002, Dunn et al., 2002, Buntinx et al., 2004a), and IFNγ can induce TNFα secretion in microglia co-cultured with astrocytes (Yamamoto et al., 2007). Cytokines can also alter expression of their receptors, for example IL-1β down-regulates IL-1R1 mRNA in perivascular cells, but not in neuronal cell groups (Ericsson et al., 1995), while peritoneal IL-1β stimulation up-regulates expression of IL-1R1 in the rat carotid body (Zhang et al., 2007).

Chapter 3 of this thesis showed that NA and β-AR activation is capable of dramatically reducing the thrombin- and Aβ-induced inflammatory response. It was hypothesised that β-AR activation could have a direct effect on neurons as β_1- and β_2-ARs are expressed on neurons (Morin et al., 1997) and NA can protect against glial-induced neuronal cell death (Madrigal et al., 2005, Madrigal et al., 2006). If β-AR activation can alter cytokine receptor expression on neurons it could make the neurons more or less susceptible to the actions of cytokines and the associated inflammatory response. This hypothesis is supported by many studies which have shown that various molecules including hormones, statins, and pathogenic factors are capable of altering cytokine receptor expression and thereby influencing cell death. For example, increasing TNFR1 signalling can be detrimental: the pathogenic factor trimethyltin induced TNFR1 causing cell death in almost 90% of neurons in mixed neuronal-glial cultures (Figiel and Dzwonek, 2007) and progesterone doubled TNFR1 expression and thus TNFα-induced cell death in differentiated PC12 cells (Koski et al., 2004). Meanwhile inhibition of the TNFR1 system can be beneficial: dexamethasone inhibited up-regulation of TNFR1 preventing TNFα induced toxicity *in vitro* (Dinh et al., 2008a, Dinh et al., 2008b), estradiol abolished TNFR1 expression and decreased TNFα induced cell death in differentiated PC12 cells (Koski et al., 2004), pre-treatment with soluble TNFR1 reduced glutamate-induced neuronal cell death (Kogo et al., 2006), and the prosaptide neurotrophin TX14(A) decreased TNFR1 expression leading to prevention of hyperalgesia (Wagner et al., 1998). Induction of TNFR2 signalling can also be beneficial. For example lovastatin increases neuronal TNFR2 expression and protects cortical neurons from glutamate-induced excitotoxicity in primary cortical neurons from wild type and TNFR1 KO but not TNFR2 KO mice (Dolga et al., 2008b).

The objective of this section was to investigate the ability of the inflammatory cytokines IL-1β, TNFα, and IFNγ to alter neuronal cytokine receptor expression and to investigate the ability of β-AR agonists and db-cAMP to protect against cytokine-induced neurotoxicity.

4.2 Neurons express β_1- and β_2-ARs

As the experiments on glia in Chapter 3 had shown that the effects of NA were mediated via the β-ARs it was necessary to determine whether β-ARs were expressed in primary neuronal cultures. The following studies were carried out to assess the ability of neurons to express β_1- and β_2-ARs. Primary neuronal cultures were grown on glass coverslips for up to 6 days. They were then fixed and stained using fluorescent immunocytochemistry, and imaged using a confocal microscope. An antibody specific to Neu-N was used as a marker for neurons, and antibodies specific to either the β_1- or the β_2-AR were also used. Hoescht was used to identify cell nuclei.

Neurons express β_1- and β_2-ARs: Neurons stained red for Neu-N were found to co-localise with the green staining for β_1-ARs (Figure 4.2.1) or β_2-ARs (Figure 4.2.2). This co-localisation of the red and green staining indicates that the same cells express both Neu-N and β-ARs, demonstrating that neurons express β-ARs. This means that NA and β-AR agonists are capable of exerting their effects on neurons.

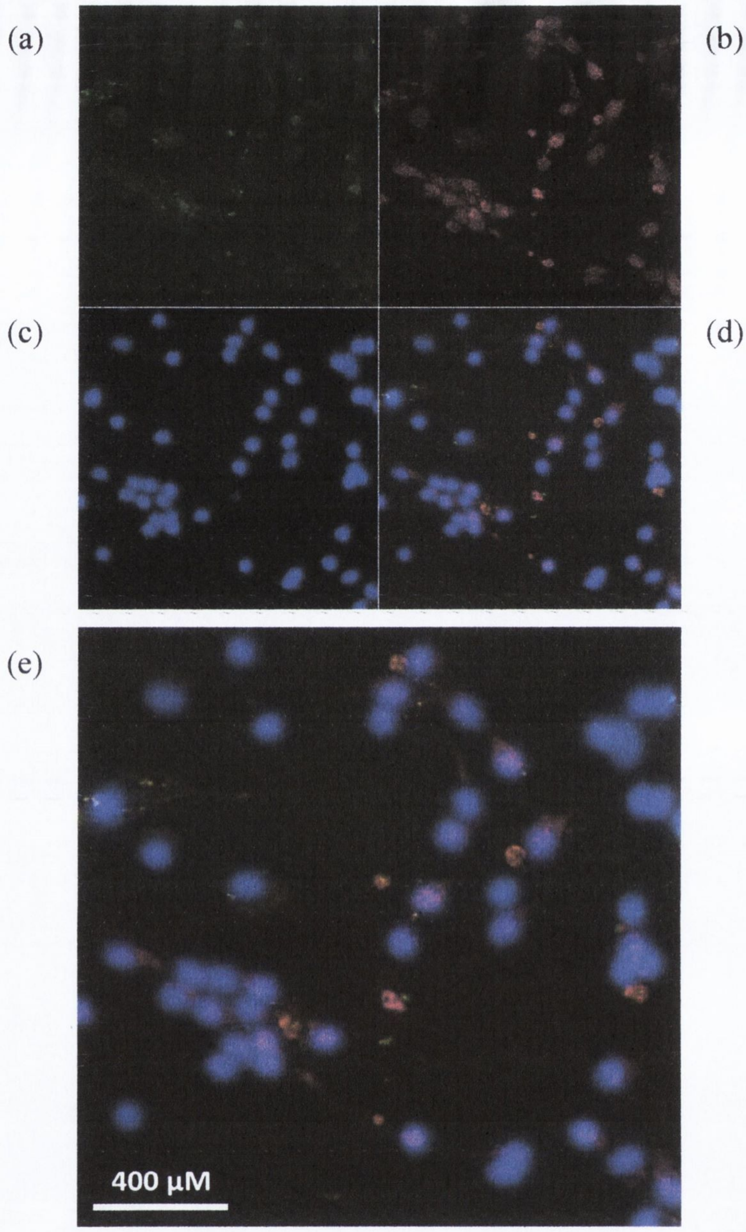


Figure 4.2.1: Neurons express the β_1-AR

Fluorescent immunocytochemistry was carried out on primary neuronal cultures using antibodies against the marker of neuronal nuclei, Neu-N, and the β_1-AR. The cells were imaged at 20X magnification. (a) is green staining indicating β_1-AR positive cells, (b) is red staining indicating Neu-N positive neurons, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The red and green staining are colocalised indicating that the same cells express both Neu-N and β_1-ARs, meaning that neurons express β_1-ARs. The white line represents a length of 400 μM.

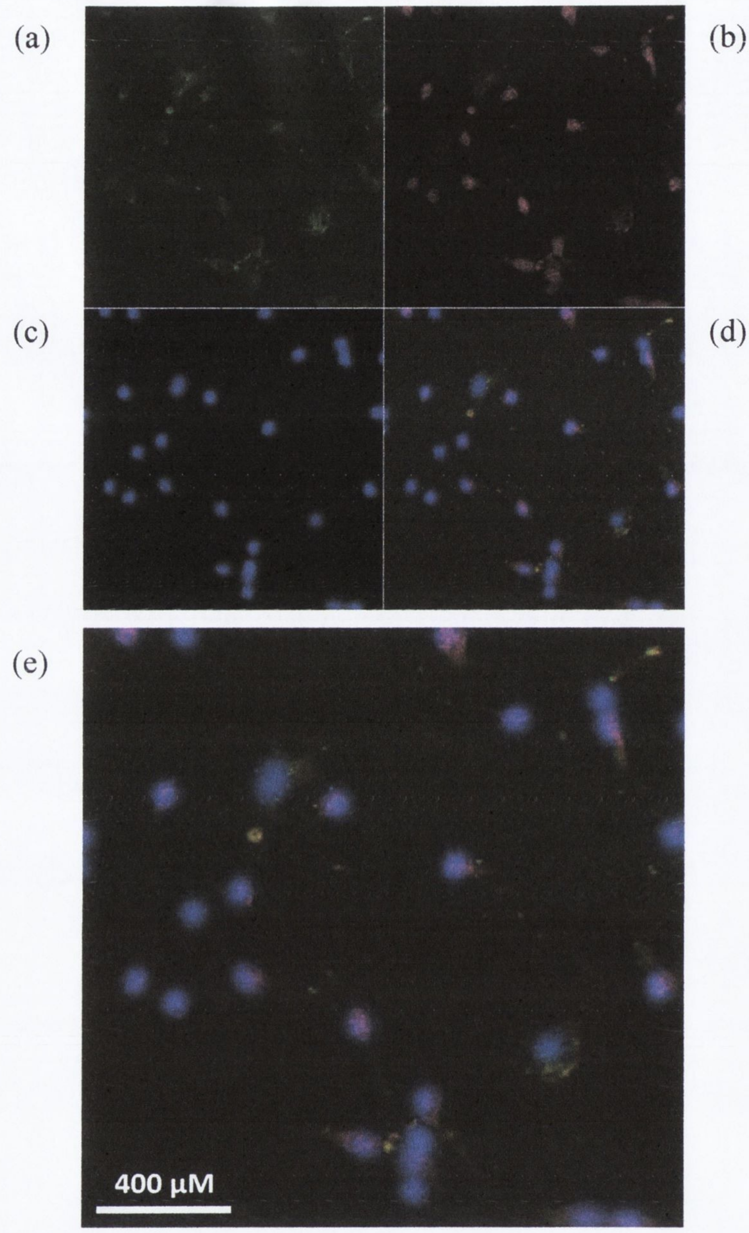


Figure 4.2.2: Neurons express the β_2-AR

Fluorescent immunocytochemistry was carried out on primary neuronal cultures using antibodies against the marker of neuronal nuclei, Neu-N, and the β_2-AR. The cells were imaged at 20X magnification. (a) is green staining indicating β_2-AR positive cells, (b) is red staining indicating Neu-N positive neurons, (c) is blue staining indicating cell nuclei stained with Hoescht, (d) shows the colocalisation of (a), (b) and (c), and (e) is an enlarged copy of (d). The red and green staining are colocalised indicating that the same cells express both Neu-N and β_2-ARs, meaning that neurons express β_2-ARs. The white line represents a length of 400 μM.

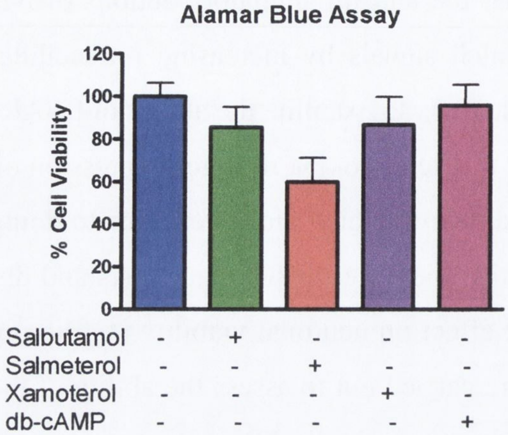
4.3 The β-AR agonists, salbutamol, salmeterol and xamoterol, and the cAMP analogue, db-cAMP, do not alter neuronal viability

The studies on mixed glial cultures showed that the anti-inflammatory actions of NA occur via activation of the β_2-AR pathway which signals by increasing intracellular cAMP concentrations. The major focus of this study is to examine the ability of β_2-ARs and the downstream signalling molecule cAMP to alter cytokine receptor expression on neurons and to protect against cytokine-induced neurotoxicity. However prior to doing these experiments it was necessary to confirm that the doses of β-AR agonists and db-cAMP being employed did not have any direct effect on neuronal viability i.e. did not induce neurotoxicity. The following studies were carried out to assess the ability of the β-AR agonist salbutamol, the β_2-AR agonist salmeterol, the β_1-AR agonist xamoterol, and the cAMP analogue db-cAMP, to alter neuronal viability. Vehicle, salbutamol (10 μM), salmeterol (10 μM), xamoterol (10 μM), or db-cAMP (300 μM) was administered to primary neuronal cultures for 24 hours. Complete-NBM was used as a control treatment for unstimulated cells. An Alamar Blue cell viability assay and an LDH assay were carried out after 24 hours.

Salbutamol, salmeterol, xamoterol, and db-cAMP do not alter neuronal viability: When an Alamar blue assay or an LDH assay was conducted the β-AR agonist, salbutamol, the β_2-AR agonist, salmeterol, the β_1-AR agonist, xamoterol, and the cAMP analogue, db-cAMP, did not significantly alter cell viability or LDH release (Figure 4.3.1).

Figure 4.3.1:

(a) Alamar Blue Assay



(b) LDH Assay

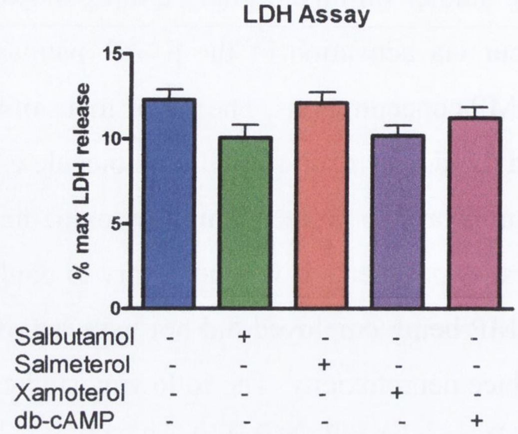


Figure 4.3.1: Salbutamol, salmeterol, xamoterol, and db-cAMP do not alter neuronal viability

Salbutamol (10 μM), salmeterol (10 μM), xamoterol (10 μM), or db-cAMP (300 μM) was administered to primary neuronal cultures and (a) an Alamar Blue cell viability assay or (b) an LDH assay was carried out after 24 hours. A one-way ANOVA demonstrated that none of the treatments altered (a) cell viability or (b) LDH release. Data expressed as means ± SEM (n=7-8).

4.4 Effect of the β-AR agonists, salbutamol, salmeterol, and xamoterol, and the cAMP analogue, db-cAMP, on expression of IL-1β, TNFα, and IFNγ receptors in neurons

The following studies were carried out to assess the ability of salbutamol, salmeterol, xamoterol, and db-cAMP to alter IL-1β, TNFα, and IFNγ receptor expression on neurons. Vehicle, salbutamol (10 μM), salmeterol (1 or 10 μM), xamoterol (1 or 10 μM), or db-cAMP (300 μM) was administered to primary neuronal cultures. Complete-NBM was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically the mRNA expression of the pro-inflammatory IL-1β receptor IL-1R1, the anti-inflammatory IL-1β receptor IL-1R2, the pro-inflammatory TNFα receptor TNFR1, the anti-inflammatory TNFα receptor TNFR2, and the IFNγ receptors IFNgR1 and IFNgR2, were examined.

Salbutamol increases neuronal expression of IL-1R2, TNFR2, and IFNgR1: The β-AR agonist salbutamol (10 μM) significantly increased neuronal IL-1R2, TNFR2, and IFNgR1 mRNA expression, without altering IL-1R1, TNFR1, or IFNgR2 mRNA expression (Figure 4.4.1).

Salmeterol, but not xamoterol, increases neuronal expression of IL-1R2 and TNFR2: The selective β_2-AR agonist salmeterol (1 and 10 μM) significantly increased neuronal IL-1R2 and TNFR2 mRNA expression, without altering IL-1R1, TNFR1, IFNgR1, or IFNgR2 mRNA expression. In contrast, the selective β_1-AR agonist xamoterol (1 and 10 μM) failed to alter neuronal IL-1R1, IL-1R2, TNFR1, TNFR2, IFNgR1, or IFNgR2 mRNA expression (Figure 4.4.2).

Db-cAMP increases neuronal expression of IL-1R2, TNFR2, and IFNgR1, and decreases expression of IL-1R1: Db-cAMP significantly decreased neuronal IL-1R1 mRNA expression, and significantly increased neuronal IL-1R2, TNFR2, and IFNgR1 mRNA expression, without altering TNFR1 or IFNgR2 mRNA expression (Figure 4.4.3).

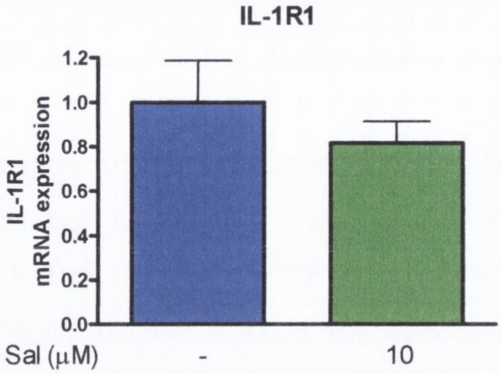
Figure 4.4.1: Salbutamol increases neuronal expression of IL-1R2, TNFR2, and IFNgR1

Salbutamol (10 μM) was administered to primary neuronal cultures for 6 hours. A Student t-test demonstrated that salbutamol (10 μM) significantly increased (b) IL-1R2 (P=0.0013), (d) TNFR2 (P=0.019), and (e) IFNgR1 (P=0.048) mRNA expression. It did not alter (a) IL-1R1, (c) TNFR1, or (f) IFNgR2 mRNA.

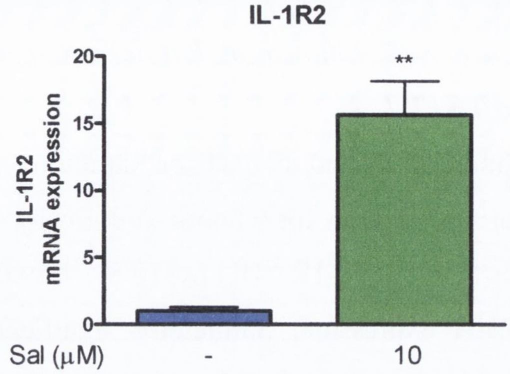
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01, vs. control (Student t-test).

Figure 4.4.1:

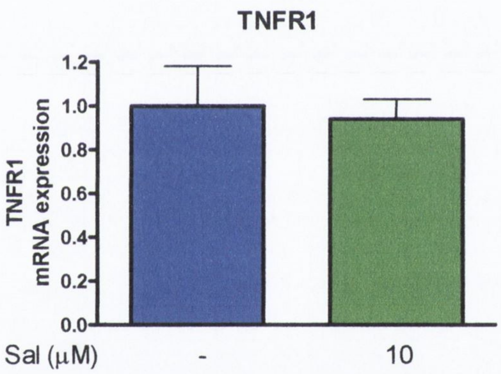
(a) IL-1R1 mRNA



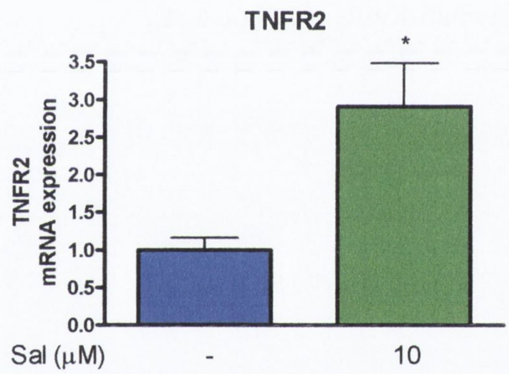
(b) IL-1R2 mRNA



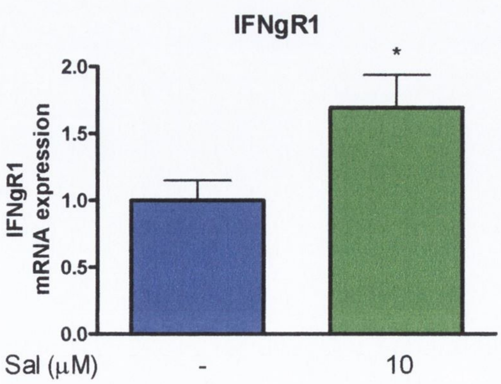
(c) TNFR1 mRNA



(d) TNFR2 mRNA



(e) IFNgR1 mRNA



(f) IFNgR2 mRNA

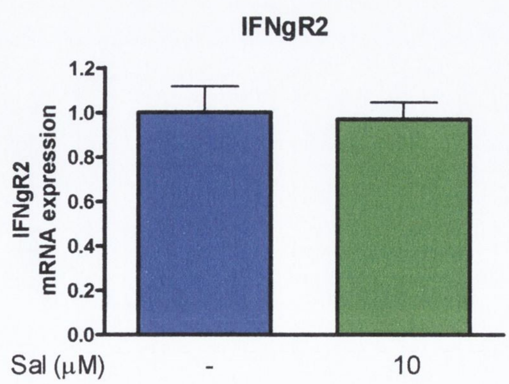


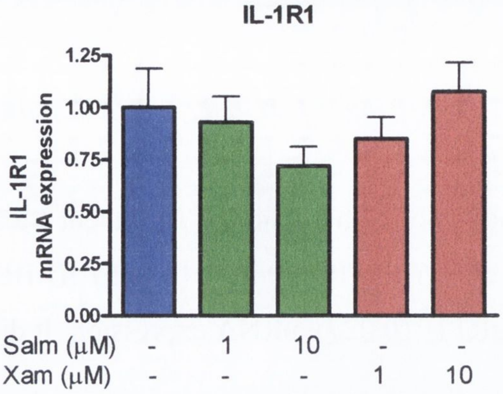
Figure 4.4.2: Salmeterol, but not xamoterol, increases neuronal expression of IL-1R2 and TNFR2

Salmeterol (1 and 10 μM) or xamoterol (1 and 10 μM) was administered to primary neuronal cultures for 6 hours. A one-way ANOVA demonstrated a significant treatment effect on (b) IL-1R2 [$F_{(4,18)}$=10.81, P=0.0001] and (d) TNFR2 [$F_{(4,19)}$=3.04, P=0.043] mRNA expression. Salmeterol significantly increased (b) IL-1R2 and (d) TNFR2 mRNA expression. It did not alter (a) IL-1R1, (c) TNFR1, (e) IFNgR1, or (f) IFNgR2. Xamoterol did not alter (a) IL-1R1, (b) IL-1R2, (c) TNFR1, (d) TNFR2, (e) IFNgR1, or (f) IFNgR2 mRNA expression.

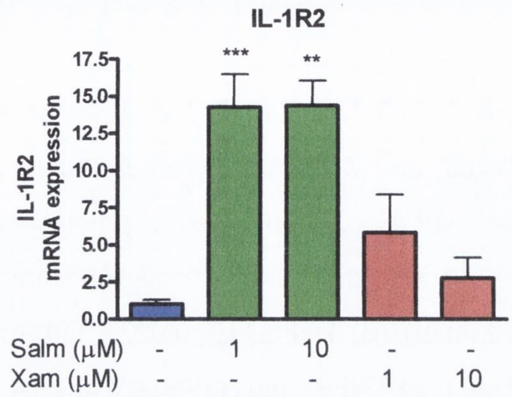
Data expressed as means ± SEM (n=4-6), *P<0.05, **P<0.01, ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 4.4.2:

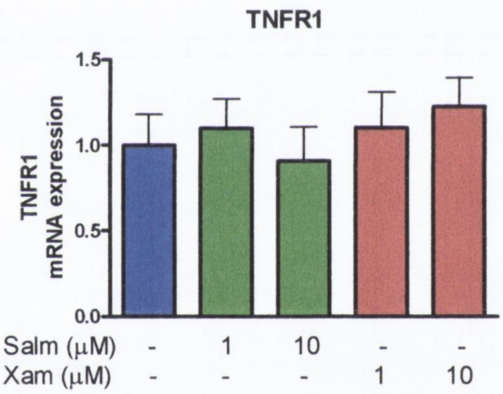
(a) IL-1R1 mRNA



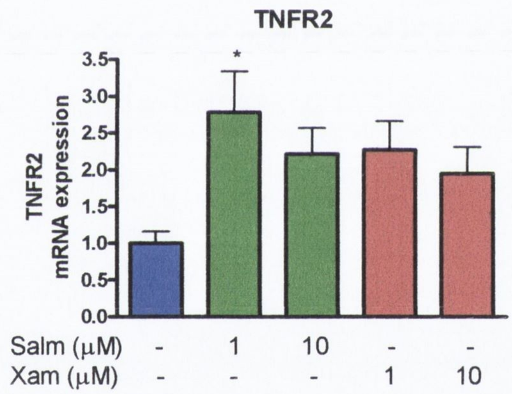
(b) IL-1R2 mRNA



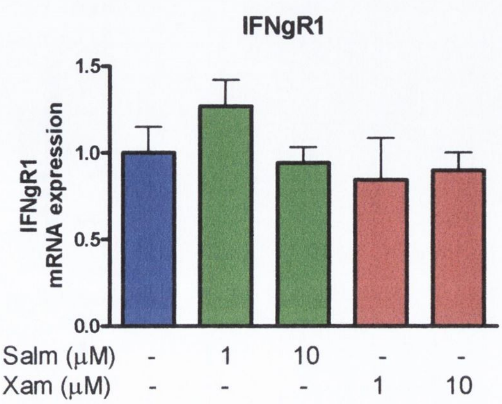
(c) TNFR1 mRNA



(d) TNFR2 mRNA



(e) IFNgR1 mRNA



(f) IFNgR2 mRNA

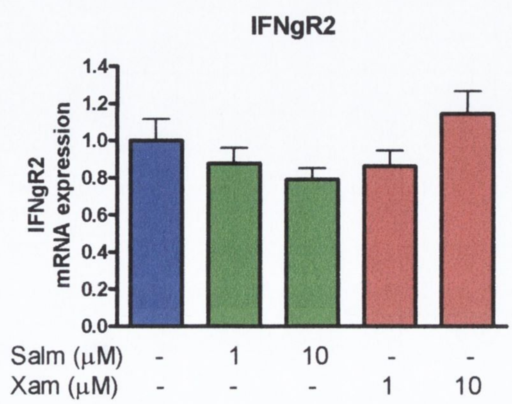


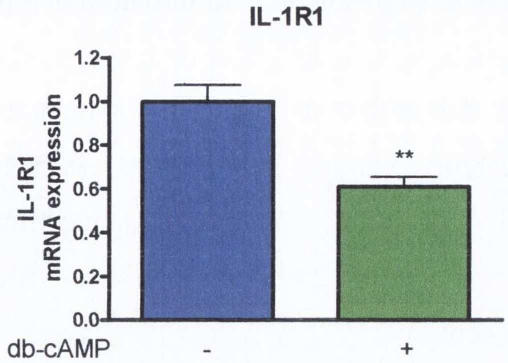
Figure 4.4.3: Db-cAMP increases neuronal expression of IL-1R2, TNFR2, and IFNgR1, and decreases expression of IL-1R1

Db-cAMP was administered to primary neuronal cultures for 6 hours. A Student t-test demonstrated that db-cAMP significantly altered (a) IL-1R1 (P=0.0011), (b) IL-1R2 (P=0.0039), (d) TNFR2 (P=0.022), and (e) IFNgR1 (P=0.027) mRNA expression. It did not alter (c) TNFR1 or (f) IFNgR2 mRNA.

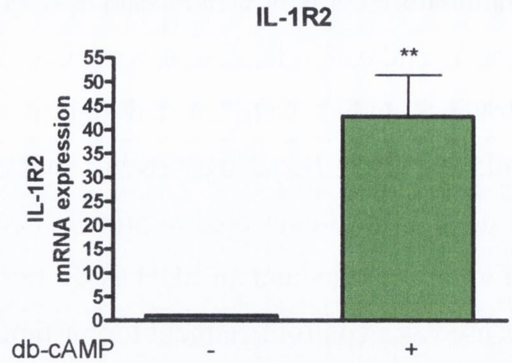
Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control (Student t-test).

Figure 4.4.3:

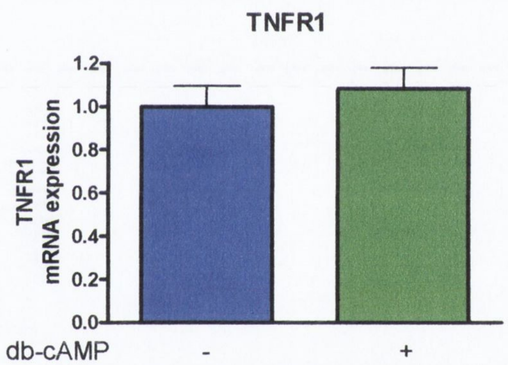
(a) IL-1R1 mRNA



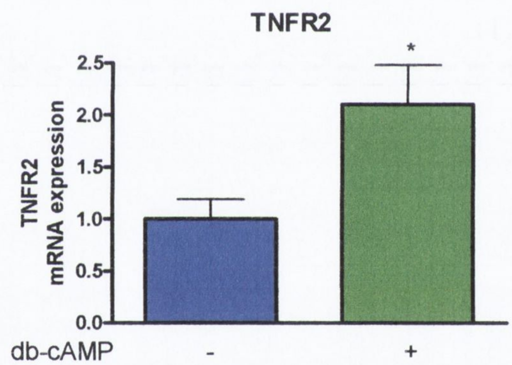
(b) IL-1R2 mRNA



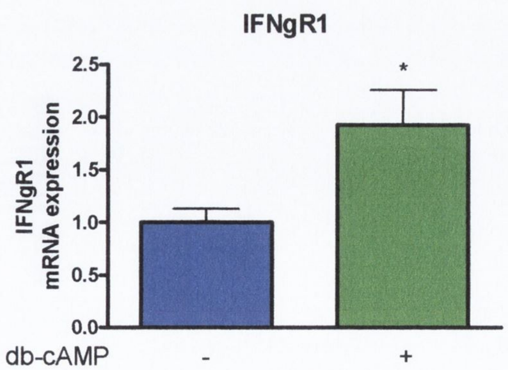
(c) TNFR1 mRNA



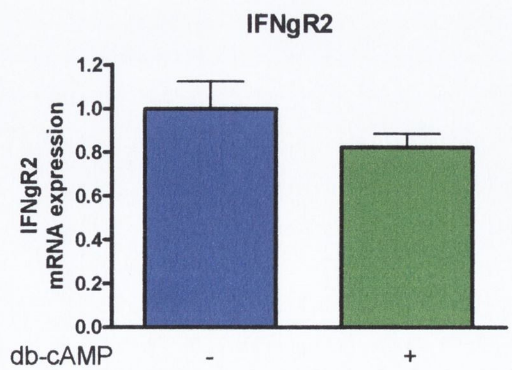
(d) TNFR2 mRNA



(e) IFNgR1 mRNA



(f) IFNgR2 mRNA



4.5 Cytokines do not alter neuronal viability

Studies from Chapter 3 of this thesis showed that glial cells produced the pro-inflammatory cytokines TNFα and IL-1β in response to treatment with thrombin or Aβ. It was hypothesised that these cytokines may have a detrimental effect on neurons. The following studies were carried out to assess the ability of the pro-inflammatory cytokines TNFα, IL-1β and IFNγ to alter cell viability. Vehicle, TNFα, IL-1β and IFNγ (50 μM) were administered to primary neuronal cultures for 24 hours. An Alamar Blue cell viability assay and an LDH assay were carried out after 24 hours. Complete-NBM was used as a control treatment for unstimulated cells.

Cytokines do not alter neuronal viability: When an Alamar blue assay was conducted the cytokines TNFα, IL-1β and IFNγ did not significantly alter cell viability. Similarly, when an LDH assay was conducted none of the treatments altered LDH release (Figure 4.5.1).

Figure 4.5.1:

(a) Alamar Blue Assay (b) LDH Assay

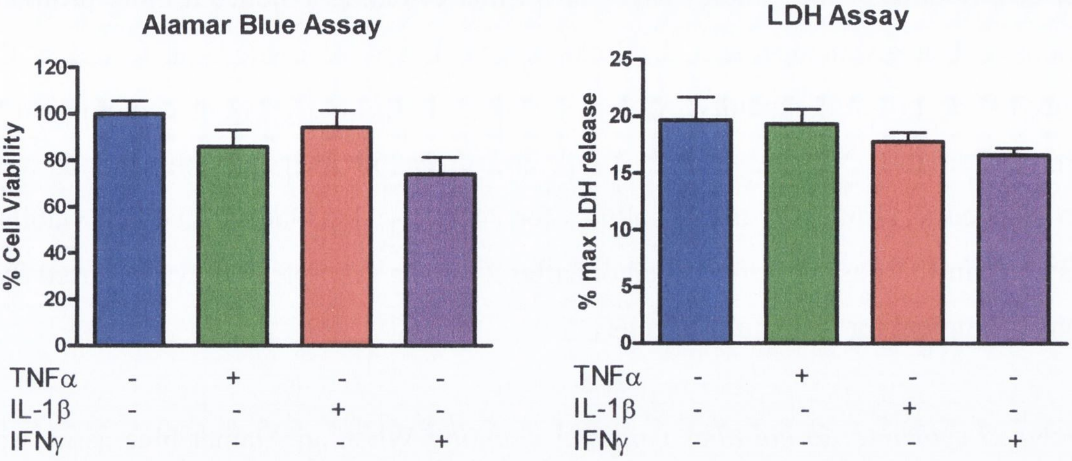


Figure 4.5.1: Cytokines do not alter neuronal viability

TNFα, IL-1β, or IFNγ (50 μM) was administered to primary neuronal cultures and (a) an Alamar Blue cell viability assay or (b) an LDH assay was carried out after 24 hours. A one-way ANOVA demonstrated that none of the treatments significantly altered (a) cell viability or (b) LDH release.

Data expressed as means ± SEM (n=7-8).

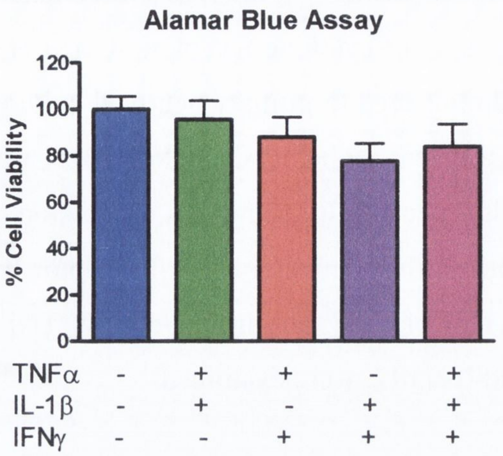
4.6 Combined cytokines do not alter neuronal viability

The previous study showed that the individual cytokines IL-1β, TNFα and IFNγ did not alter cell viability. Other studies have shown that cytokines produce a more profound response when given together. The following studies were carried out to assess the ability of the pro-inflammatory cytokines TNFα, IL-1β, and IFNγ in combination to alter cell viability. Vehicle, TNFα, IL-1β, and IFNγ (50 μM), in combination, were administered to primary neuronal cultures for 24 hours. An Alamar Blue cell viability assay and an LDH assay were carried out after 24 hours. Complete-NBM was used as a control treatment for unstimulated cells.

Combined cytokines do not alter neuronal viability: When an Alamar blue assay was conducted the combination of the cytokines TNFα, IL-1β, and IFNγ did not significantly alter cell viability. Similarly, when an LDH assay was conducted none of the treatments altered LDH release (Figure 4.6.1).

Figure 4.6.1:

(a) Alamar Blue Assay



(b) LDH Assay

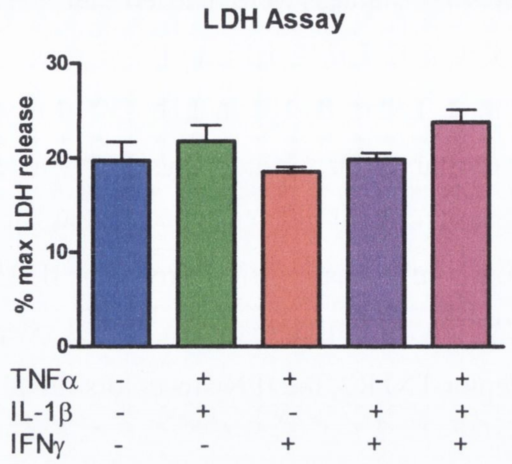


Figure 4.6.1: Combined cytokines do not alter neuronal viability

TNFα, IL-1β and IFNγ (50 μM), in combination, were administered to primary neuronal cultures and (a) an Alamar Blue cell viability assay or (b) an LDH assay was carried out after 24 hours. A one-way ANOVA demonstrated that none of the treatments significantly altered (a) cell viability or (b) LDH release.

Data expressed as means ± SEM (n=7-8).

4.7 Cytokines alter expression of cytokine receptors in neurons

Cytokines alter the expression of other cytokines and their receptors in the body. The following studies were carried out to assess the ability of the pro-inflammatory cytokines TNFα, IL-1β, and IFNγ to alter expression of cytokine receptors in neurons. Vehicle, TNFα, IL-1β, or IFNγ (50 μM) was administered to primary neuronal cultures for 6 hours prior to the analysis of gene expression by RT-PCR. Complete-NBM was used as a control treatment for unstimulated cells. Specifically the mRNA expression of the pro-inflammatory IL-1β receptor IL-1R1, the anti-inflammatory IL-1β receptor IL-1R2, the pro-inflammatory TNFα receptor TNFR1, the anti-inflammatory TNFα receptor TNFR2, the IFNγ receptors IFNgR1 and IFNgR2, were examined.

Cytokines alter expression of cytokine receptors in neurons: TNFα significantly increased IFNgR1 and IFNgR2 mRNA expression. IFNγ significantly increased TNFR1, and TNFR2 mRNA expression. IL-1β failed to alter mRNA expression and there was no effect on IL-1R1 or IL-1R2 mRNA (Figure 4.7.1).

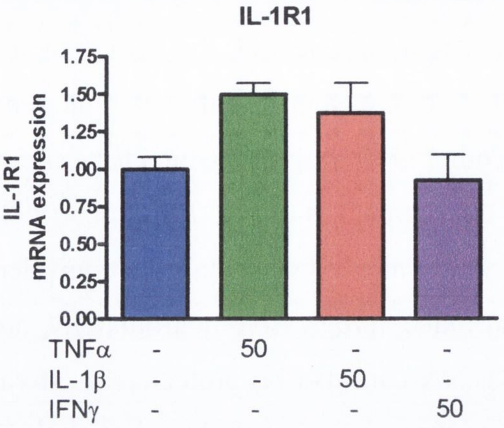
Figure 4.7.1: Cytokines alter expression of cytokine receptors in neurons

TNFα, IL-1β, or IFNγ (50 μM) was administered to primary neuronal cultures for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (c) TNFR1 [$F_{(3,18)}$=13.40, P<0.0001], (d) TNFR2 [$F_{(3,18)}$=3.50, P=0.037], (e) IFNgR1 [$F_{(3,18)}$=3.56, P=0.035], and (f) IFNgR2 [$F_{(3,18)}$=9.56, P=0.0005] mRNA expression. There was no effect on (a) IL-1R1 or (b) IL-1R2 mRNA. TNFα significantly increased (e) IFNgR1 and (f) IFNgR2 only. IFNγ significantly increased (c) TNFR1 and (d) TNFR2 mRNA only. IL-1β had no effect.

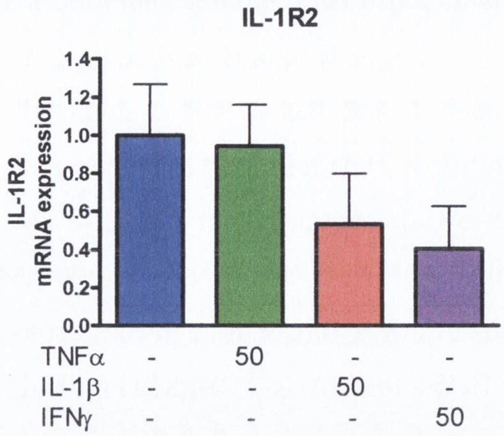
Data expressed as means ± SEM (n=3-6), *P<0.05, **P<0.01, ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 4.7.1:

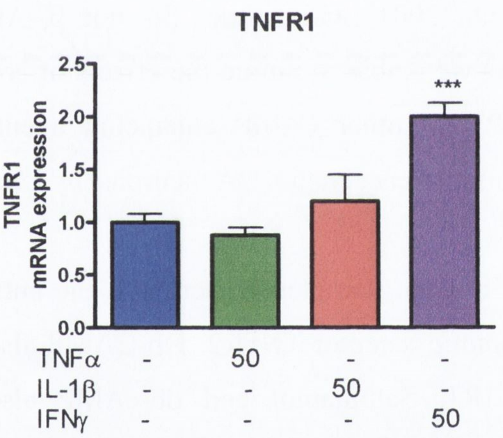
(a) IL-1R1 mRNA



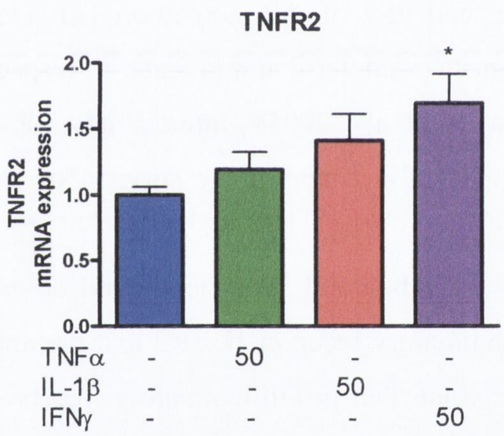
(b) IL-1R2 mRNA



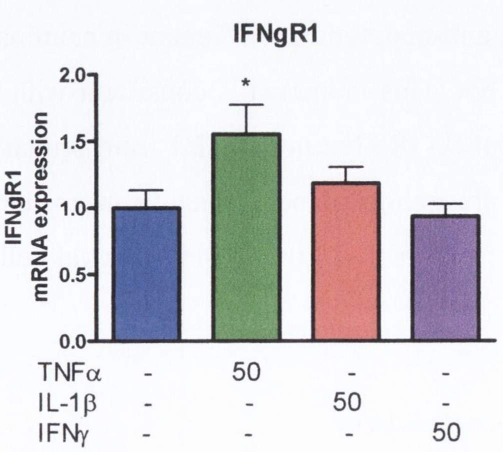
(c) TNFR1 mRNA



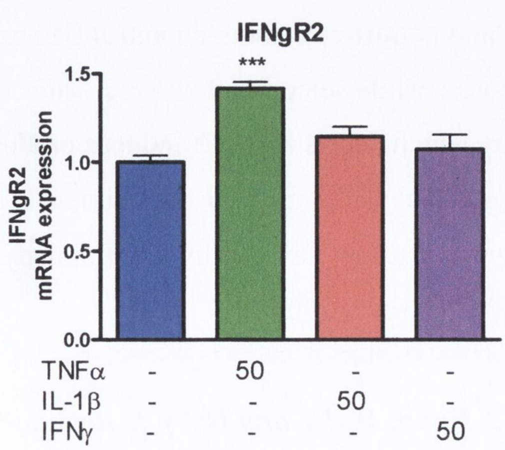
(d) TNFR2 mRNA



(e) IFNgR1 mRNA



(f) IFNgR2 mRNA



4.8 Discussion

4.8.1 β_2-AR activation induces an anti-inflammatory profile of neuronal cytokine receptor expression but does not alter neuronal viability

The β-AR agonist salbutamol, the β_2-AR agonist salmeterol, the β_1-AR agonist xamoterol, and the cAMP analogue db-cAMP, did not significantly alter neuronal viability or LDH release. This is consistent with other *in vitro* studies which have shown that low concentrations of NA have no effect on neuronal viability (Fu et al., 1998). Only very high doses of NA (50-200 μM) which are much higher than the equivalent doses of AR agonists used in these studies, can induce progressive neurotoxicity, and the ED50 for NA is 150 μM (Fu et al., 1998). NA can also be protective as it can promote long-term survival and function of dopaminergic neurons in mesencephalic cultures (Troadec et al., 2001), and can prevent degeneration of cholinergic neurons subjected to low-level oxidative stress (Traver et al., 2005). However these effects occur independently of AR activation (Troadec et al., 2001) and neither β_1- nor β_2-AR agonists (xamoterol and procaterol, respectively) were able to mimic the effects of NA (Traver et al., 2005). Interestingly, db-cAMP and other cAMP enhancing agents significantly increased the survival of postnatal mesencephalic DA neurons *in vitro* (Lara et al., 2003).

Salbutamol, salmeterol, and db-cAMP, but not xamoterol, increased the anti-inflammatory receptor IL-1R2 and the anti-apoptotic receptor TNFR2. Db-CAMP also decreased the pro-inflammatory receptor IL-1R1. Salbutamol and db-cAMP also increased IFNgR1. The most dramatic of these effects is the increase in IL-1R2 which displays a 15-fold increase in response to salbutamol and salmeterol, and a 40-fold increase in response to db-cAMP. Overall these results indicate that activators of the β_2-AR and pathway promote an anti-inflammatory, anti-apoptotic environment in neuronal cultures, while selective β_1-AR activation does not. This induction is consistent with a study which shows that NA induces production of IL-1R2 but not IL-1R1 from primary mixed glial cells, and this is coupled with a prevention of neurotoxicity in cortical primary neurons induced by conditioned medium from IL-1β treated mixed glial cells (McNamee et al., 2010).

4.8.2 TNFα, IL-1β, and IFNγ do not alter neuronal viability

The pro-inflammatory cytokines TNFα, IL-1β, and IFNγ do not alter cell viability. This is not consistent with studies showing that TNFα induces *in vitro* cell death in neurons (Williams et al., 2005) and differentiated PC12 cells (Koski et al., 2004), or that IL-1β treatment can cause retinal neuronal death (Abcouwer et al., 2008), partially increase death of neuroblastoma cells at high doses (Bielarczyk et al., 2005), and increase apoptosis of NPCs (Wang et al., 2007). However much of the literature states that cytokines alone are usually not capable of inducing cell death in pure neuronal cultures and that glial activation is required. Indeed many of the studies which show cytokine-induced neuronal death are typically either *in vivo* studies or have used mixed glial and neuron cultures. For example, studies have shown that TNFα, via neuronal TNFR1 and caspase-3 activation, is only neurotoxic in the presence of microglia or microglial-CM (Taylor et al., 2005), and in primary cultures of rat mesencephalon TNFα induced toxicity which was selective for DA neurons and did not affect glial viability (McGuire et al., 2001). Similarly, IL-1β increased NMDA- and glutamate-induced hippocampal neuron death in co-cultures of mixed neurons and microglia in a process that did not require that activated glia be in cell-to-cell contact with the neurons and was dependent on IL-1R1 (Ma et al., 2002). IFNγ has been shown to induce neuronal death, although again the presence of glia is required. For example, *in vitro* studies on mixed microglia and midbrain neuron cultures showed that IFNγ resulted in dopaminergic cell loss (Mount et al., 2007). Interestingly, IFNγ can also occasionally have a beneficial effect as pre-treatment of hippocampal cell cultures with IFNγ protects neurons against glutamate-induced death by increasing recovery of intracellular Ca^{2+} concentrations (Lee et al., 2006).

TNFα, IL-1β, and IFNγ in combination also did not alter neuronal viability. Other studies have shown that combining a number of cytokines together can induce cell death, although again glial activation is required. For example, co-treatment of neuron-astrocyte cultures with IL-1β and IFNγ induced expression of TNFα and resulted in neuronal death (Downen et al., 1999), and TNFα and IL-1β from Aβ-activated microglia increased cell death in a neuroblastoma cell line (Chiarini et al., 2006). Also IFNγ induced TNFα secretion in microglia co-cultured with astrocytes, and this IFNγ and TNFα activation of microglia inhibited Aβ degradation (Yamamoto et al., 2007). The combination of three major pro-inflammatory cytokines IL-1β, TNFα and IFNγ, caused astrocytes to express iNOS and make dangerously large amounts of NO via MAPKs (Chiarini et al., 2006), which is known to induce neuronal death.

4.8.3 Cytokines induce neuronal cytokine receptor expression

The pro-inflammatory cytokines TNFα and IFNγ induced expression of neuronal cytokine receptors. In particular, TNFα induced expression of the IFNγ receptors IFNgR1 and IFNgR2, and IFNγ induced expression of the TNFα receptors TNFR1 and TNFR2. This shows that the cytokines are capable of inducing the expression of receptors of other cytokines, implying cross-talk between the signalling pathways. This is consistent with a study on two human neuroblastoma cell lines showing that IFNγ increased TNFR expression to a different extent in both cell lines; IFNγ induced TNFR1 in the GI-LI-N line but it induced TNFR2 in the LAN-5 line. In contrast to this TNFα had no effect on IFNgR expression in either cell line (Montaldo et al., 1994). TNFα and IFNγ also up-regulated transcription of TNFR1 in the human oligodendroglial cell lines HOG and MO3.13 (Buntinx et al., 2004b).

However, unlike the following studies, the results in this section did not find any alteration caused by IL-1β or on IL-1Rs. IL-1β and TNFα induce expression of IL-1R1 in astrocytes and neurons *in vitro* (Friedman, 2001). In mouse pulmonary epithelial cells IL-1β increased soluble and surface TNFR2. This effect was dependent on TNFR1 expression and did not occur until 24 hours after IL-1β treatment, indicating effects were post-transcriptional (Saperstein et al., 2009). In a mouse organ culture assay model of mouse airways, IL-1β increased the mRNA and protein expression of IL-1R1 in the epithelial cells and TNFR1 and TNFR2 in the epithelial cells and smooth muscle cells, suggesting a transcriptional mechanism. Also, TNFR2 expression was increased after 1 day, while TNFR1 expression only increased after 4 days (Cardell et al., 2008). As the studies conducted in this section measured mRNA expression at 6 hours, this could explain why no IL-1β induced up-regulation of cytokine receptor expression was found. Future studies could examine later time points to determine whether or not an alteration in cytokine receptor expression is seen at 24 or 48 hours.

4.8.4 Conclusion

The primary finding of these studies was that β_2-AR agonists and db-cAMP are capable of increasing and decreasing the expression of neuronal cytokine receptors in a manner that promotes an anti-inflammatory and anti-apoptotic environment. This is an important finding as it indicates that β_2-AR agonists and db-cAMP could help protect neuronal cultures from pro-inflammatory or neurotoxic agents. These studies also showed that cytokines are capable of inducing the expression of the receptors for other cytokines, emphasising the existence of complex signalling pathways between the cytokines during an inflammatory response. However the expression of these cytokine receptors was only examined at the mRNA level, therefore future studies could examine the effect of cytokines, β_2-AR agonists, and db-cAMP on the levels of protein expression of these cytokine receptors to ensure that the transcriptional effects seen here are maintained to the translation level. These studies showed that cytokines failed to induce neuronal death in primary neuronal cultures. This is not unusual as many other studies show that glial activation is necessary to induce neuronal death. The next step is to investigate the direct effect of thrombin and Aβ on neurons and the role of glia in inducing neuronal death.

5. The effect of thrombin and Aβ on neurons

5.1 Introduction

The studies described in Chapter 3 of this thesis showed that thrombin, and to a lesser extent Aβ, are capable of directly activating glial cells to induce the production of numerous inflammatory molecules including the cytokines IL-1β and TNFα, and that β-AR activation is capable of dramatically reducing this inflammatory response. However the studies described in Chapter 4 of this thesis showed that IL-1β, TNFα, and IFNγ, either alone or in combination, were not sufficient to alter neuronal viability. It was hypothesised that other soluble factors might be responsible as studies have shown that soluble factors produced by activated microglia can be cytotoxic to neurons (Flavin et al., 1997a, Flavin et al., 1997b). CM transfer systems are routinely used to examine the effect of soluble factors released from glial cells on neuronal function. This experimental approach ensures that soluble factors are transferred but no cell-cell contact is possible. Neuronal cultures exposed to CM from stimulated macrophages or microglial cultures induced an increase in the number of apoptotic neuronal cells, while CM from fibroblast or astrocyte cultures did not (Flavin et al., 1997a), showing that microglial and macrophage derived soluble factors induce apoptosis in neuronal cultures. Dialysis of the CM, with a 3500 or a 50000 molecular weight cut-off, provided a retentate which produced the same apoptotic effect as non-dialysed CM, and trypsin digestion prevented this indicating that the soluble factors are proteins between 3500 and 50000 molecular weight and that they are susceptible to trypsin. In addition, even after storing for up to two weeks at -20°C, CM continued to exert effects, demonstrating that the candidate factors are both potent and stable. Potentially cytotoxic molecules secreted by macrophages include ROS, TNFα, glutamate, quinolinate, thromboxane A2, proteases and lipases (Flavin et al., 1997a). Treating macrophages with dexamethasone, a potent anti-inflammatory glucocorticoid, prevented the toxic effect induced by macrophage CM in neurons, while indomethacin, a nonsteroidal anti-inflammatory agent, and the anti-inflammatory cytokine TGFβ had a similar but less profound protective effect (Flavin et al., 1997b), indicating that the toxic mediators are inflammatory in nature.

The studies in this chapter were carried out to assess the effect of thrombin and Aβ on neuronal viability, either by direct treatment of the neurons or by the addition of media from thrombin- or Aβ-activated glial cells, and to investigate the effect of β-AR activation on thrombin- or Aβ-induced changes in neuronal viability. Thrombin and Aβ have been reported to be directly toxic to neurons and a wide range of studies have shown that neuronal apoptosis can be induced by thrombin (Rao et al., 2007, Park and Jin, 2008, Sanchez et al., 2009, Thirumangalakudi et al., 2009), and Aβ (Moore and O'Banion, 2002, Walsh et al., 2002). Glial-mediated neurotoxicity is also an important driver of neurological damage in AD, PD, and stroke. In AD, neurotoxicity is dependent on glial activation, which results in the induction of iNOS and inflammatory cytokines such as IL-1β and TNFα (Akama and Van Eldik, 2000). Also, the potency of Aβ-induced neurotoxicity on cultured neurons was increased dramatically in the presence of microglia, with this increase being primarily attributed to microglial activation (Liu and Hong, 2003). In studies using thrombin as an inflammatory stimulus, microglial activation preceded neuronal loss (Huang et al., 2008b), and neuronal loss was reduced by inhibition of microglial activation (Fujimoto et al., 2007). Also reducing the number of microglia present, using a macrophage depletion method, protected the neurons from cytotoxicity (Katsuki et al., 2006). This indicates that microglia play an important role in thrombin- and Aβ-induced neuronal cell death. The experiments in this chapter used a CM transfer system to investigate the effect that soluble factors released from glia in response to thrombin and Aβ treatment have on neuronal viability. Many CM studies have shown that glial inflammation is toxic to neurons. For instance, treatment of microglia with LPS induced cell death in neurons (Munch et al., 2003) and in PC12 cells, a model for dopamine neurons (Wang et al., 2005). However, one CM study showed that LPS-induced inflammation did not affect neuronal viability (Gibbons and Dragunow, 2006), while another showed that CM from glia treated with LPS can have neurotoxic and neuroprotective actions on neurons, depending on dosage (Li et al., 2007). It also appears that neurotoxicity is mediated by microglial activation, while astrocytes have a suppressive role in this process, as the addition of microglia but not astrocytes to neuronal cultures dramatically increases LPS-induced neurotoxicity (Wang et al., 2005).

In these studies it was expected that CM from glial cells treated with thrombin or Aβ would decrease neuronal viability. However it was shown that CM from glial cells treated with Aβ did not alter neuronal viability, while CM from glial cells treated with thrombin actually increased neuronal viability. It was hypothesised that this increase may occur as a result of the production of neurotrophic factors or anti-inflammatory cytokines induced by thrombin specifically. CM studies have shown that the release of the neurotrophic factors GDNF and NGF, and the anti-inflammatory cytokines IL-10 and TGFβ, from glia, is protective to neurons (Hashimoto et al., 2005, Nakajima et al., 2007, Cacci et al., 2008). GDNF is one of the most potent neurotrophic factors and is a survival factor for midbrain dopaminergic neurons and motoneurons in the CNS (Lin et al., 1993, Verity et al., 1999, Sariola and Saarma, 2003), while NGF is essential for the proliferation, differentiation, and survival of neuronal and non-neuronal cells (Dechant and Neumann, 2002, Chao et al., 2006). IL-10 is a potent anti-inflammatory cytokine which inhibits cytokine-induced production of TNFα, IL-1β, and IFNγ, chemokines, and MHC-class-II expression, thus negatively regulating inflammation (Bogdan et al., 1992, Sheng et al., 1995, Denys et al., 2002, Ledeboer et al., 2002, Mosser and Zhang, 2008). It also has direct trophic actions on neurons (Zhou et al., 2009). TGFβ is an anti-inflammatory cytokine known to regulate macrophage activity (Flavin et al., 1997b), and it inhibits CD40-induced IFNγ expression (Nguyen et al., 1998), LPS-induced TNFα production (Bogdan et al., 1992), and endotoxin-induced NO production from glia (Vincent et al., 1997). TGFβ also promotes neuronal survival and, although it is most likely not neurotrophic by itself, it is required to promote the survival of neuron populations that is induced by neurotrophins such as GDNF (Mehler and Kessler, 1997, Krieglstein et al., 2002).

It was also hypothesised that the thrombin-induced increase in neuronal viability may occur as a result of modulation of neuronal cytokine receptor expression induced by thrombin specifically. This hypothesis was based on studies which have shown that the pro-inflammatory molecule LPS can alter cytokine receptor expression. For example, LPS increases IL-1R1 expression in the hippocampus, and this increase is blocked by IL-4 (Barry et al., 2005). However, the studies on the effect of LPS on TNFRs are contradictory, as some studies indicate that LPS induces TNFR1, but not TNFR2, in neurons (Wen et al., 2006) and in the olfactory bulb (Mori et al., 2005), while another states that LPS induces TNFR2 but not TNFR1 in the mouse brain (Bette et al., 2003). Another study states that TNFR1 is induced in virtually all neurons and in non-neuronal cells, while TNFR2 is only expressed and increased in non-neuronal cells (Li et al., 2004),. If thrombin and Aβ alter cytokine receptor expression on neurons it could make the neurons more, or less, susceptible to the actions of cytokines and the associated inflammatory response.

β_2-AR agonists, by increasing intracellular cAMP concentrations, have anti-inflammatory actions (Feinstein et al., 2002). Studies from Chapters 3 and 4 of this thesis showed that the β-AR agonist, salbutamol, the β_2-AR agonist, salmeterol, and the cAMP analogue, db-cAMP, promoted an anti-inflammatory environment by decreasing thrombin- or Aβ-induced expression of pro-inflammatory cytokines, chemokines and CAMs in glial cultures, and increasing expression of the anti-inflammatory IL-1R2 and the anti-apoptotic TNFR2 in neuronal cultures. The second objective of this section was to investigate if β_2-AR agonists were capable of altering the increase in neuronal viability that was induced by thrombin treated glial cells. This hypothesis was based on studies which have shown that NA protects against glial-induced neuronal cell death: For example, co-treatment of microglia with NA significantly reduced neuronal cell death in response to treatment with LPS CM by blocking IL-1β production from glia, and dramatically decreasing the amount of NO induced in neurons by LPS CM (Madrigal et al., 2005). A follow up study by the same group (Madrigal et al., 2006) showed that NA reduced LPS-CM-induced neuronal cell death, whether it was added to the microglia or the neurons. However neuronal iNOS expression in response to LPS-CM was reduced by 70% when NA was added to the microglia, but it was only reduced by 35% when NA was added to the neurons, indicating that NA is more effective when used on microglia than when used directly on neurons (Madrigal et al., 2006).

The objective of this section was to investigate whether direct treatment with thrombin or Aβ, or CM from mixed glial cells treated with thrombin or Aβ, could induce neurotoxicity in primary neuronal cultures, and to examine neuroprotective effects of β_2-AR agonists. However, once it was observed that thrombin CM increased neuronal viability, thrombin or Aβ treated glial cultures were examined to determine whether there were neurotrophic factors or anti-inflammatory cytokines present. It was also investigated whether it was microglia or astrocytes which were the producers of these molecules, and whether thrombin or Aβ could alter neuronal cytokine receptor expression. The next objective was to investigate the effect of β-AR agonists and db-cAMP on neuronal viability in response to CM treatment. Both pre-treatment of the glia and the neurons with these agents was examined to determine whether any beneficial effects were mediated via modulating glial cell function or via direct actions on the neurons. The release of neurotrophic factors or anti-inflammatory cytokines from glial cells treated with β-AR agonists or db-cAMP was also examined.

5.2 Thrombin and Aβ do not alter neuronal viability

The following studies were carried out to assess the ability of thrombin and Aβ to alter neuronal viability. Vehicle, thrombin (20 U/ml), or Aβ (2.5 μM) was administered to primary neuronal cultures for 24 hours. An Alamar Blue cell viability assay and an LDH assay were carried out after 24 hours. Complete-NBM was used as a control treatment for unstimulated cells.

Thrombin and Aβ do not alter neuronal viability: When an Alamar blue assay or an LDH assay was conducted thrombin and Aβ did not significantly alter cell viability or LDH release (Figure 5.2.1).

Figure 5.2.1:

(a) Alamar Blue Assay (b) LDH Assay

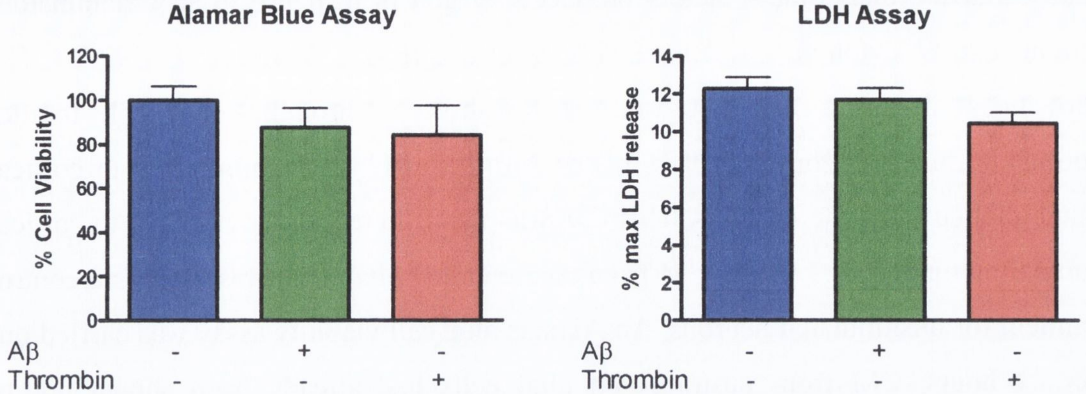


Figure 5.2.1: Thrombin and Aβ do not alter neuronal viability

Thrombin (20 U/ml) or Aβ (2.5 μM) was administered to primary neuronal cultures and (a) an Alamar Blue cell viability assay or (b) an LDH assay was carried out after 24 hours. A one-way ANOVA demonstrated that none of the treatments significantly altered (a) cell viability or (b) LDH release.

Data expressed as means ± SEM (n=7-8).

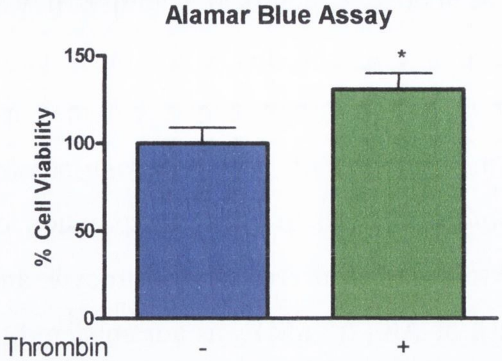
5.3 Conditioned media (CM) from glial cells treated with thrombin, but not Aβ, increases neuronal viability

Studies indicate that soluble factors produced by glia in response to an inflammatory stimulus can be cytotoxic to neurons (Flavin et al., 1997a, Flavin et al., 1997b). The following studies were carried out to assess the ability of thrombin- or Aβ-CM to alter neuronal viability. Thrombin (20 U/ml) or Aβ (2.5 μM) was administered to cortical mixed glial cultures for 24 hours. This CM was then filtered and transferred to cortical neuronal cultures for 24 hours. CM from unstimulated glial cells was used as a control treatment for unstimulated neurons. An Alamar blue cell viability assay was carried out after 24 hours. CM from unstimulated glial cells had already been shown not to significantly alter neuronal viability (Glial-CM 100 ± 8.88 vs. non-CM 116 ± 9.41; data expressed as Mean ± SEM, n=8).

Thrombin-, but not Aβ-, CM increases neuronal viability: When an Alamar blue assay was conducted, CM from glial cells treated with thrombin, but not Aβ, significantly increased neuronal viability (Figure 5.3.1). For this reason the remaining experiments were conducted using thrombin-CM.

Figure 5.3.1:

(a) Thrombin-CM



(b) Aβ-CM

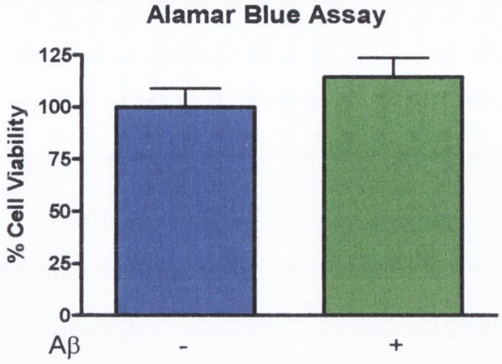


Figure 5.3.1: Thrombin-, but not Aβ-, CM increases neuronal viability

CM from glial cells treated with thrombin (20 U/ml) or Aβ (2.5 μM) was administered to primary neuronal cultures for 24 hours, after which an Alamar blue cell viability assay was carried out. A student's t-test demonstrated that (a) thrombin-CM increases neuronal viability ($P=0.031$) with an Alamar blue cell viability assay. (b) Aβ CM did not alter neuronal viability.

Data expressed as means ± SEM (n=7-8). *$P<0.05$ vs. control (Student's t-test).

5.4 Thrombin, but not Aβ, induces neurotrophic factors and anti-inflammatory cytokines in mixed glial, astrocyte, and microglial cultures

The previous studies showed that thrombin-CM increased neuronal viability. It was hypothesised that this increase in neuronal viability could be due to the presence of neurotrophic factors that promote neuronal survival or anti-inflammatory cytokines that combat the actions of pro-inflammatory cytokines in the CM. The following studies were carried out to assess the ability of thrombin and Aβ to induce expression of neurotrophic factors and anti-inflammatory cytokines in mixed glial, astrocyte and microglial cultures. Vehicle, thrombin (20 U/ml), or Aβ (2.5 μM) was administered to mixed glial cultures for 6 hours. Vehicle or thrombin (20 U/ml) was also administered to enriched astrocyte and microglial cultures for 6 hours. Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically, the mRNA expression of the neurotrophic factors, GDNF and NGFβ, and the anti-inflammatory cytokines, IL-10 and TGFβ, were examined.

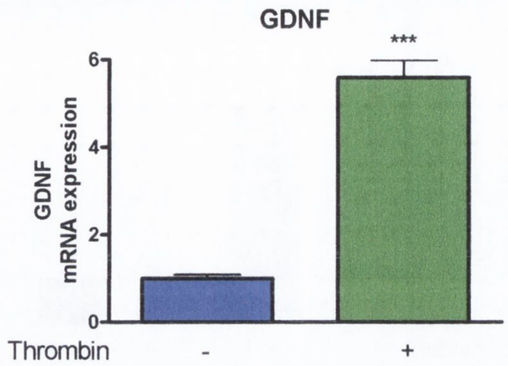
Thrombin induces neurotrophic factors and anti-inflammatory cytokines in mixed glial cultures: Thrombin significantly increased GDNF, NGFβ, IL-10, and TGFβ mRNA expression in mixed glial cultures (Figure 5.4.1).

Thrombin induces neurotrophic factors and anti-inflammatory cytokines in astrocytes and microglia: Thrombin significantly increased GDNF, NGFβ, IL-10 and TGFβ mRNA expression in astrocyte cultures. Thrombin significantly increased IL-10 and NGFβ mRNA expression, but not GDNF or TGFβ, in microglial cultures (Figure 5.4.2). The thrombin-induced increase in microglial cultures was to a much lesser extent than the thrombin-induced increase in mixed glial or astrocyte cultures.

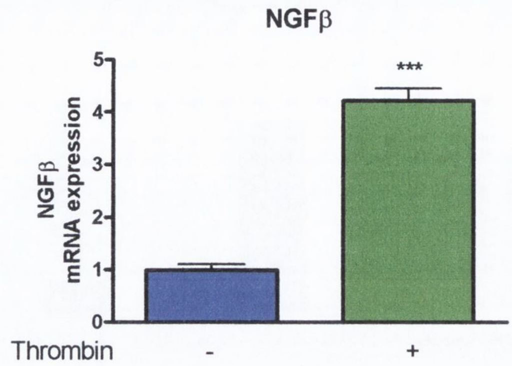
Aβ induces some anti-inflammatory cytokines, but not neurotrophic factors, in mixed glial cultures: Aβ significantly increased TGFβ mRNA expression, but not GDNF, NGFβ, or IL-10 mRNA, in mixed glial cultures (Figure 5.4.3).

Figure 5.4.1:

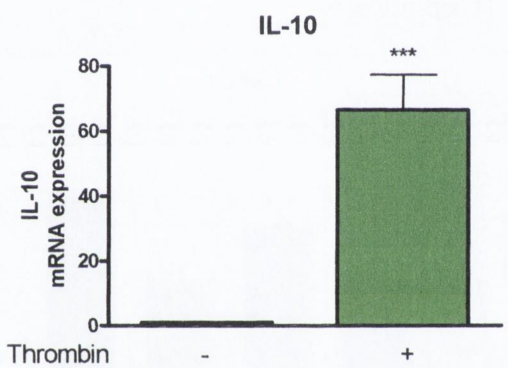
(a) GDNF mRNA



(b) NGFβ mRNA



(c) IL-10 mRNA



(d) TGFβ mRNA

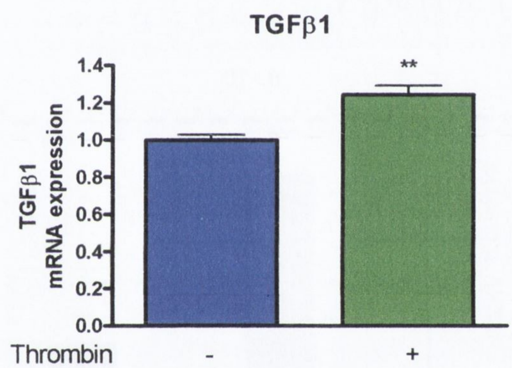


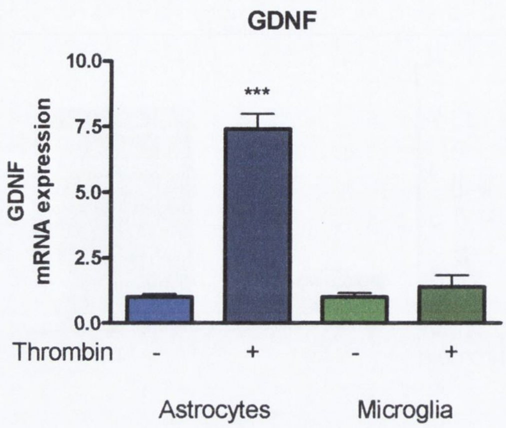
Figure 5.4.1: Thrombin induces neurotrophic factors and anti-inflammatory cytokines in mixed glial cells

Thrombin (20 U/ml) was administered to mixed glial cells for 6 hours. A student t-test revealed a significant effect of thrombin treatment in (a) GDNF ($P<0.0001$), (b) NGFβ ($P<0.0001$), (c) IL-10 ($P=0.0001$), and (d) TGFβ ($P=0.0011$) mRNA expression. Thrombin significantly increased (a) GDNF, (b) NGFβ, (c) IL-10, and (d) TGFβ mRNA expression.

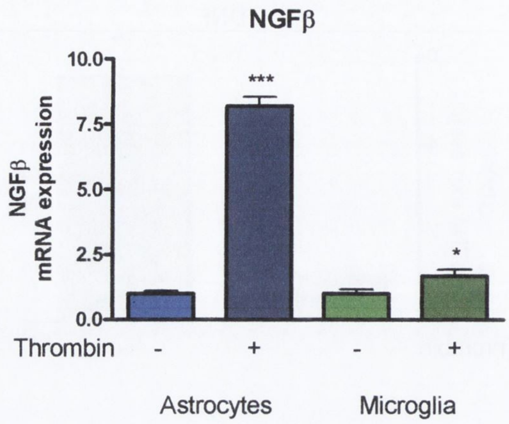
Data expressed as means ± SEM (n=5-6), **$P<0.01$, ***$P<0.001$ vs. control (Student t-test).

Figure 5.4.2:

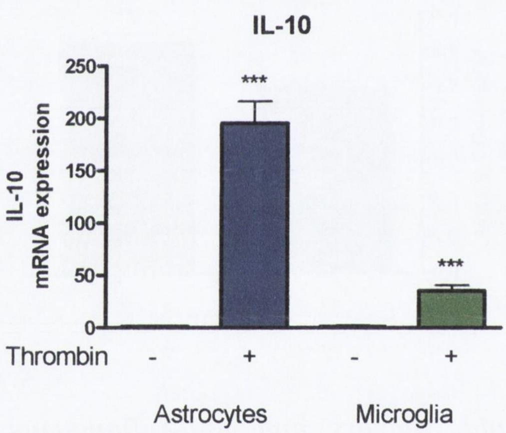
(a) GDNF mRNA



(b) NGFβ mRNA



(c) IL-10 mRNA



(d) TGFβ mRNA

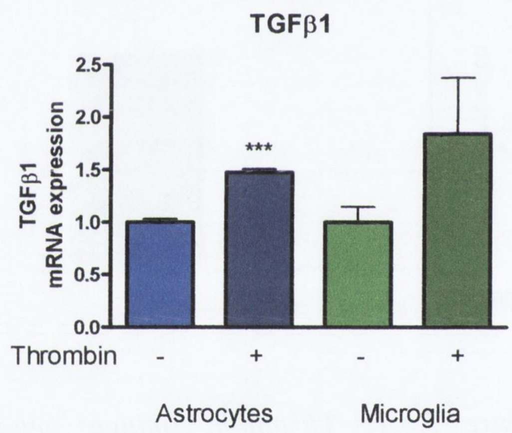


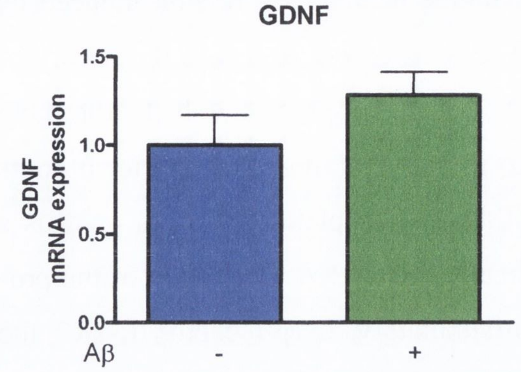
Figure 5.4.2: Thrombin induces neurotrophic factors and anti-inflammatory cytokines in astrocytes and microglia

Thrombin (20 U/ml) was administered to astrocyte or microglial cultures for 6 hours. In astrocytes, a student t-test revealed a significant effect of thrombin treatment in (a) GDNF ($P<0.0001$), (b) NGFβ ($P<0.0001$), (c) IL-10 ($P<0.0001$), and (d) TGFβ ($P<0.0001$) mRNA expression. In microglia, a student t-test revealed a significant effect of thrombin treatment in (c) IL-10 ($P=0.0002$) and (b) NGFβ ($P=0.049$) mRNA expression, but not (a) GDNF or (d) TGFβ.

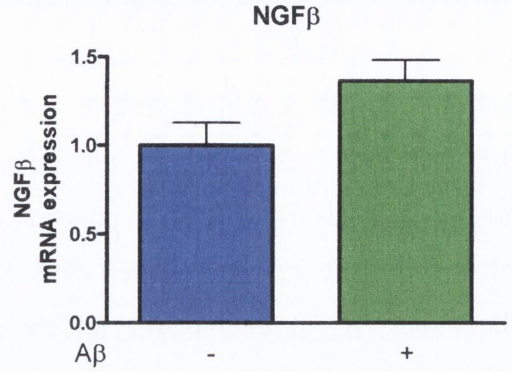
Data expressed as means ± SEM (n=5-6), *$P<0.05$, ***$P<0.001$ vs. control (Student t-test).

Figure 5.4.3:

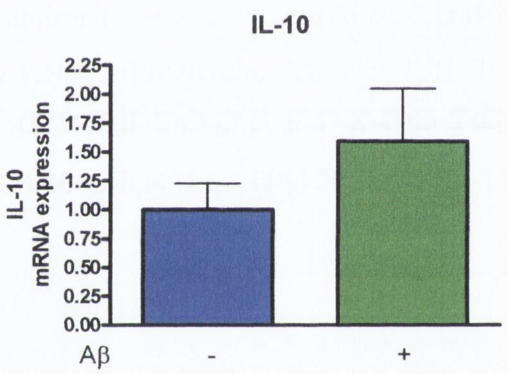
(a) GDNF mRNA



(b) NGFβ mRNA



(c) IL-10 mRNA



(d) TGFβ mRNA

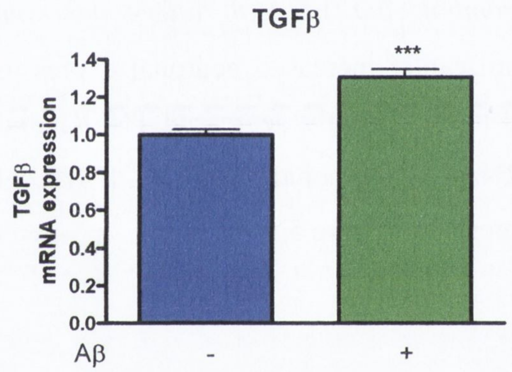


Figure 5.4.3: Aβ induces anti-inflammatory cytokines, but not neurotrophic factors, in mixed glial cells

Aβ (2.5 μM) was administered to mixed glial cells for 6 hours. A student t-test revealed a significant effect of Aβ treatment in (d) TGFβ (P=0.0001) mRNA expression, but not (a) GDNF, (b) NGFβ, (c) IL-10 mRNA expression.

Data expressed as means ± SEM (n=5-6), ***P<0.001 vs. control (Student t-test).

5.5 Thrombin, but not Aβ, alters neuronal cytokine receptor expression

It was hypothesised that the thrombin-induced increase in neuronal viability may also occur as a result of modulation of neuronal cytokine receptor expression induced by thrombin specifically. The following studies were carried out to assess the ability of thrombin and Aβ to induce cytokine receptor expression in neurons. Vehicle, thrombin (20 U/ml), or Aβ (2.5 μM) was administered to primary neuronal cultures for 6 hours prior to the analysis of gene expression by RT-PCR. Complete-NBM was used as a control treatment for unstimulated cells. Specifically the mRNA expression of the pro-inflammatory IL-1β receptor IL-1R1, the anti-inflammatory IL-1β receptor IL-1R2, the pro-inflammatory TNFα receptor TNFR1, the anti-inflammatory TNFα receptor TNFR2, and the IFNγ receptors IFNgR1 and IFNgR2, were examined.

Thrombin, but not Aβ, alters neuronal cytokine receptor expression: Thrombin significantly increased neuronal expression of IL-1R2, TNFR2, and IFNgR2 mRNA, and it significantly decreased TNFR1 and IFNgR1 mRNA, but failed to alter IL-1R1 mRNA. Aβ did not alter IL-1R1, IL-1R2, TNFR1, TNFR2, IFNgR1, or IFNgR2 mRNA expression (Figure 5.5.1).

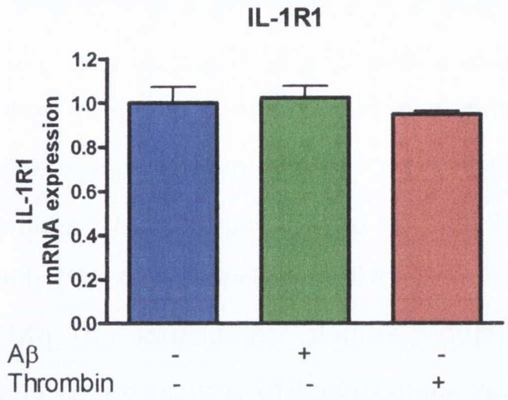
Figure 5.5.1: Thrombin, but not Aβ, alters neuronal cytokine receptor expression

Thrombin (20 U/ml) or Aβ (2.5 μM) was administered to primary neuronal cultures for 6 hours. A one-way ANOVA demonstrated a significant effect of treatment on (b) IL-1R2 [$F_{(2,15)}$=9.15, P=0.0025], (c) TNFR1 [$F_{(2,15)}$=21.76, P<0.0001], (d) TNFR2 [$F_{(2,15)}$=192.50, P<0.0001], (e) IFNgR1 [$F_{(2,15)}$=25.20, P<0.0001], and (f) IFNgR2 [$F_{(2,15)}$=11.48, P=0.0009] mRNA expression. There was no effect on (a) IL-1R1 mRNA. Thrombin significantly increased (b) IL-1R2, (d) TNFR2, and (f) IFNgR2 mRNA, and it significantly decreased (c) TNFR1 and (e) IFNgR1 mRNA expression. Aβ had no effect.

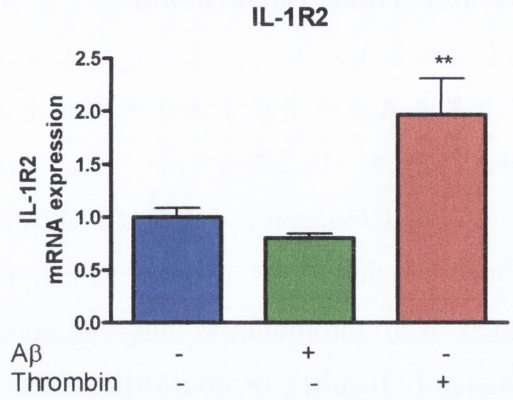
Data expressed as means ± SEM (n=6), **P<0.01, ***P<0.001 vs. control (Newman-Keuls post-hoc test).

Figure 5.5.1:

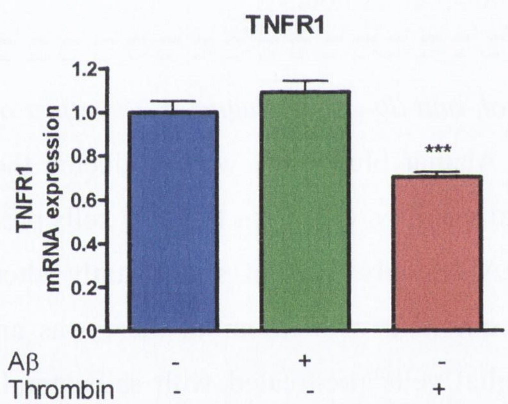
(a) IL-1R1 mRNA



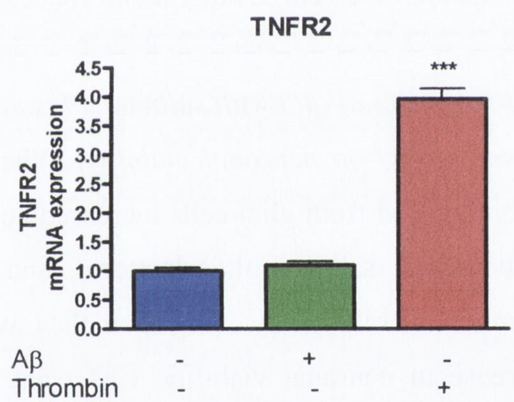
(b) IL-1R2 mRNA



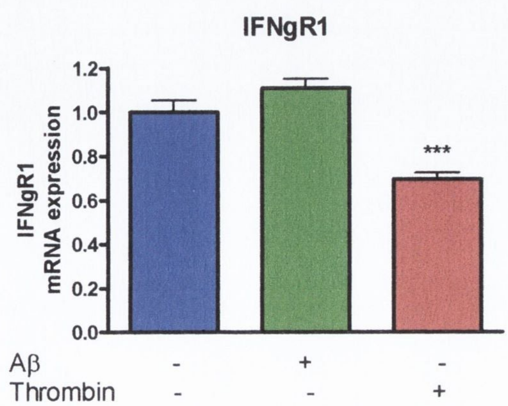
(c) TNFR1 mRNA



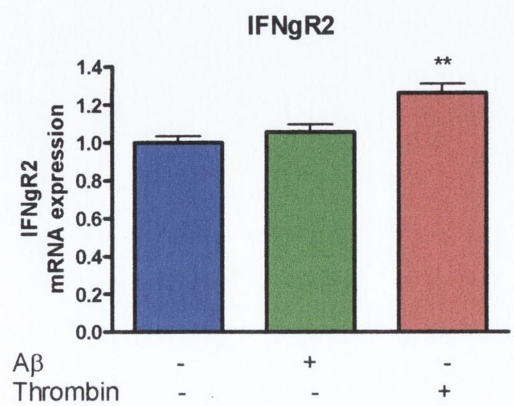
(d) TNFR2 mRNA



(e) IFNgR1 mRNA



(f) IFNgR2 mRNA



5.6 The effect of CM from glial cells pre-treated with the β-AR agonists, salbutamol and salmeterol, or the cAMP analogue db-cAMP, then treated with thrombin, on neuronal viability

Studies indicate that β_2-ARs, by increasing intracellular cAMP concentrations, have anti-inflammatory and neurotrophic actions (Furukawa et al., 1989, Verity et al., 1999, Eijkelkamp et al., 2004, Frieri and Capetandes, 2008). The following studies were carried out to assess the ability of CM from glial cells pre-treated with the β-AR agonist salbutamol, the β_2-AR agonist salmeterol, and the cAMP analogue db-cAMP, then treated with thrombin, to alter neuronal viability. Vehicle, salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to cortical mixed glial cultures for 2 hours prior to administration of thrombin (20 U/ml) for 24 hours. This CM was then filtered and transferred to cortical neuronal cultures for 24 hours. CM from unstimulated glial cells was used as a control treatment for unstimulated neurons. An Alamar blue cell viability assay was carried out after 24 hours.

Pre-treatment of glia with salbutamol, salmeterol, and db-cAMP augments the effect of thrombin-CM on neuronal viability: When an Alamar blue assay was conducted the thrombin-CM from glial cells increased neuronal viability. CM from the glial cells pre-treated with salbutamol, salmeterol, and db-cAMP only did not significantly alter neuronal viability, however when they were combined with thrombin there was an increase in neuronal viability. CM from the glial cells pre-treated with salbutamol, salmeterol, and db-cAMP, then treated with thrombin, significantly augmented the thrombin-induced increase in neuronal viability (Figure 5.6.1).

Figure 5.6.1:

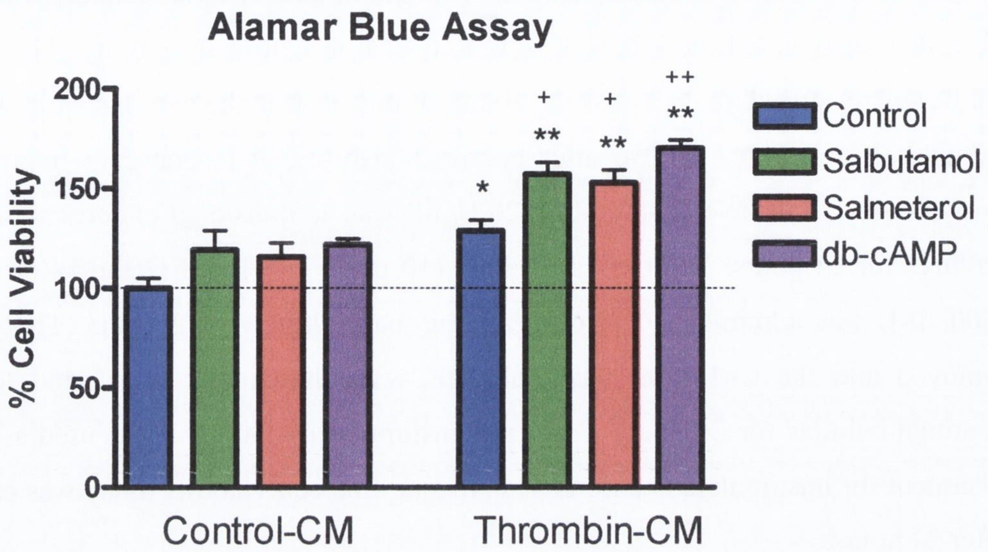


Figure 5.6.1: Pre-treatment of glia with salbutamol, salmeterol, and db-cAMP augments the effect of thrombin CM on neuronal viability

CM from glial cells pre-treated with salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) for 2 hours, and then treated with thrombin (20 U/ml) for 24 hours, was administered to primary neuronal cultures. An Alamar blue cell viability assay was carried out after 24 hours. A two-way ANOVA demonstrated a significant effect of thrombin treatment ($F_{(1,40)}$=44.99, P<0.0001) and a significant effect of pre-treatment ($F_{(3,40)}$=4.86, P=0.0056). CM from glia treated with thrombin, with or without salbutamol, salmeterol or db-cAMP, increased neuronal viability. Salbutamol, salmeterol, and db-cAMP significantly augmented this increase.

Data expressed as means ± SEM (n=6). *P<0.05, **P<0.01 vs. control, +P<0.05, ++P<0.01 vs. thrombin (Fisher's LSD post-hoc test).

5.7 The effect of CM from glial cells treated with thrombin on neuronal viability when the neurons were pre-treated with salbutamol, salmeterol, or db-cAMP

The previous study showed that the pre-treatment of glia with salbutamol, salmeterol, or db-cAMP augmented the effect of thrombin-CM on neuronal viability. The following studies were carried out to assess the ability of pre-treating the neurons with salbutamol, salmeterol, and db-cAMP, to alter neuronal viability in response to treatment with thrombin- CM. Vehicle or thrombin (20 U/ml) was administered to cortical mixed glial cultures for 24 hours. Vehicle, salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to cortical neuronal cultures for 2 hours. This was then removed and the CM from the glial cells was filtered and transferred to cortical neuronal cultures for 24 hours. CM from unstimulated glial cells was used as a control treatment for unstimulated neurons. An Alamar blue cell viability assay was carried out after 24 hours.

Pre-treatment of neurons with salbutamol, salmeterol, or db-cAMP does not alter the increase in neuronal viability induced by thrombin-CM: When an Alamar blue assay was conducted the thrombin-CM from glial cells increased neuronal viability. Pre-treatment of the neurons with salbutamol, salmeterol, or db-cAMP did not significantly alter neuronal viability (Figure 5.7.1).

Figure 5.7.1:

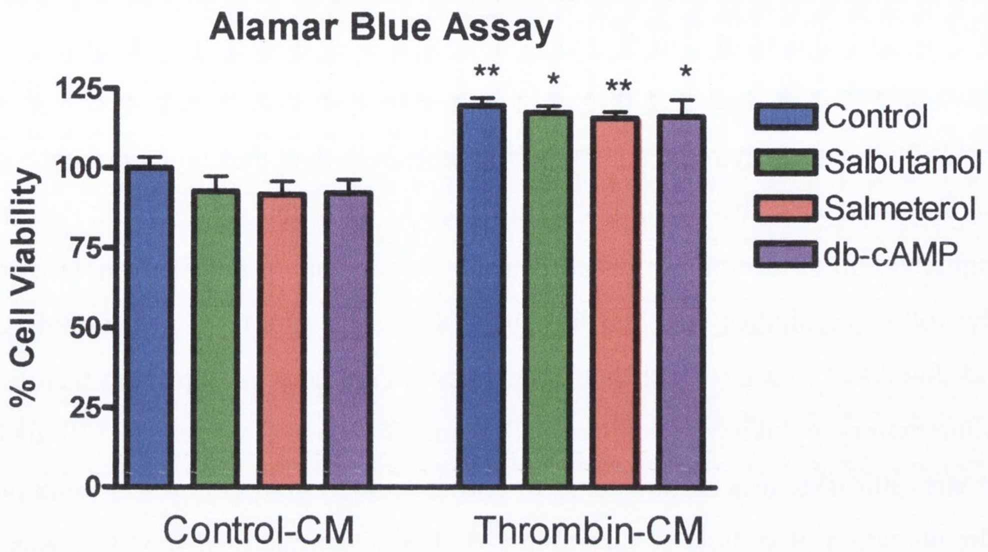


Figure 5.7.1: Pre-treatment of neurons with salbutamol, salmeterol, or db-cAMP does not alter the increase in neuronal viability induced by thrombin-CM

CM from glial cells treated with thrombin (20 U/ml) for 24 hours, was administered to primary neuronal cultures which had been pre-treated with salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) for 2 hours. An Alamar blue cell viability assay was carried out after 24 hours. A two-way ANOVA demonstrated a significant effect of thrombin treatment ($F_{(1,40)}$=73.30, P<0.0001). Thrombin CM, regardless of neuronal pre-treatment, significantly increased neuronal viability.

Data expressed as means ± SEM (n=6). *P<0.05, **P<0.01 vs. control (Fisher's LSD post-hoc test).

5.8 The effect of salbutamol, salmeterol, and db-cAMP on the thrombin-induced increase in neurotrophic factors and anti-inflammatory cytokines

The previous studies showed that the pre-treatment of glia, but not neurons, with salbutamol, salmeterol, and db-cAMP augmented the thrombin-CM-induced increase in neuronal viability, and that thrombin induced expression of neurotrophic factors and anti-inflammatory cytokines. It was hypothesised that the augmentation in neuronal viability induced by salbutamol, salmeterol, and db-cAMP could be due to an augmentation of thrombin-induced neurotrophic factors or anti-inflammatory cytokines. The following studies were carried out to assess the ability of salbutamol, salmeterol, and db-cAMP to alter the thrombin-induced increase in neurotrophic factors and anti-inflammatory cytokines. Vehicle, salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to cortical mixed glial cultures 2 hours prior to the administration of vehicle or thrombin (20 U/ml). Complete-DMEM:F12 was used as a control treatment for unstimulated cells. Cells were harvested after 6 hours for the analysis of gene expression by RT-PCR. Specifically, the mRNA expression of the neurotrophic factors, GDNF and NGFβ, and the anti-inflammatory cytokines, IL-10 and TGFβ, were examined.

Salbutamol and db-cAMP significantly augment the thrombin-induced increase in GDNF and NGFβ, but salmeterol only augments the thrombin-induced increase in GDNF in mixed glial cells: Salbutamol, salmeterol, and db-cAMP significantly increased GDNF and NGFβ mRNA expression. Salbutamol and db-cAMP significantly augmented the thrombin-induced increase in GDNF and NGFβ mRNA expression, but salmeterol only augmented the thrombin-induced increase in GDNF mRNA expression (Figure 5.8.1).

Salbutamol and db-cAMP, but not salmeterol, augment the thrombin-induced increase in the anti-inflammatory cytokines, IL-10 and TGFβ, in mixed glial cells: Salbutamol, salmeterol, and db-cAMP did not alter IL-10 mRNA expression. Salbutamol and db-cAMP but not salmeterol significantly increased TGFβ mRNA expression. Salbutamol and db-cAMP, but not salmeterol, significantly augmented the thrombin-induced increase in IL-10 and TGFβ mRNA expression (Figure 5.8.2).

Figure 5.8.1: Salbutamol, salmeterol, and db-cAMP augment the thrombin-induced increase in GDNF and NGFβ in mixed glial cells

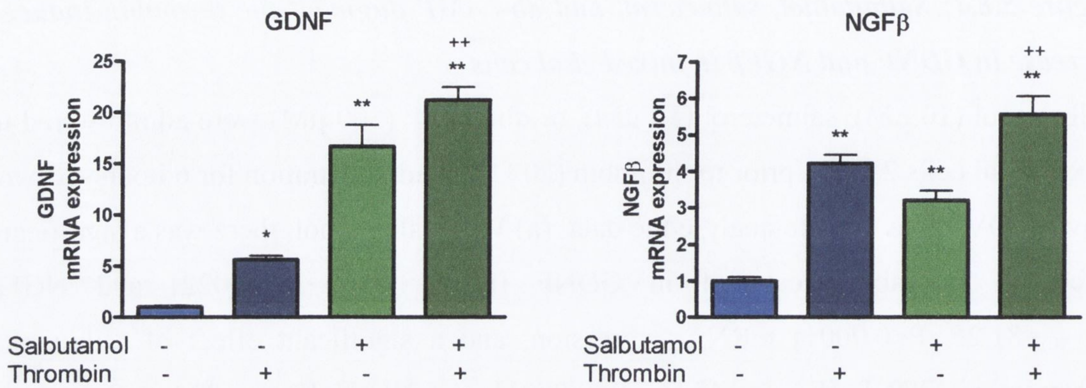
Salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) were administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA was used to analyse the data. (a) With salbutamol, there was a significant effect of thrombin treatment in GDNF [$F_{(1,19)}$=12.56, P=0.0022] and NGFβ [$F_{(1,20)}$=81.25, P<0.0001] mRNA expression, and a significant effect of salbutamol treatment in GDNF [$F_{(1,19)}$=147.56, P<0.0001] and NGFβ [$F_{(1,20)}$=33.00, P<0.0001] mRNA expression. (b) With salmeterol, there was a significant interaction effect between salmeterol pre-treatment and thrombin treatment in GDNF [$F_{(1,17)}$=36.47, P<0.0001] and NGFβ [$F_{(1,20)}$=5.51, P=0.029] mRNA expression. (c) With db-cAMP, there was a significant effect of thrombin treatment in GDNF [$F_{(1,19)}$=26.63, P<0.0001] and NGFβ [$F_{(1,20)}$=210.80, P<0.0001] mRNA expression, and a significant effect of db-cAMP treatment in GDNF [$F_{(1,19)}$=133.67, P<0.0001] and NGFβ [$F_{(1,20)}$=103.64, P<0.0001] mRNA expression.

Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

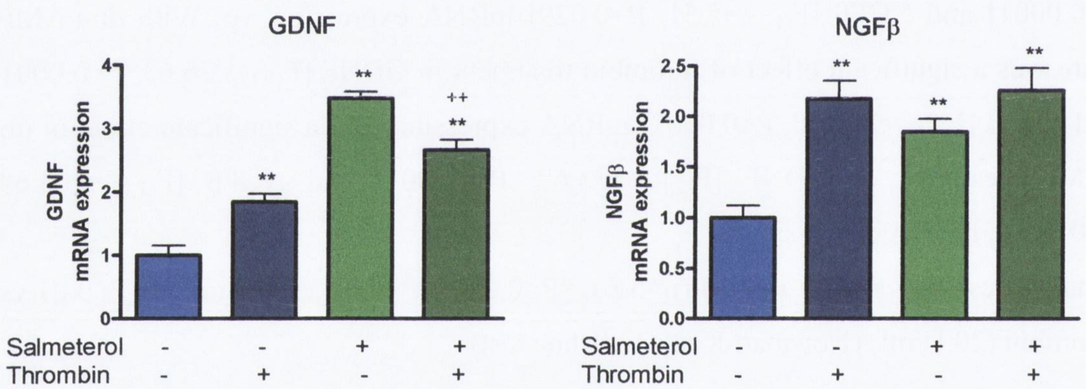
Figure 5.8.1:

GDNF mRNA **NGFβ mRNA**

(a) Salbutamol treatment



(b) Salmeterol treatment



(c) db-cAMP treatment

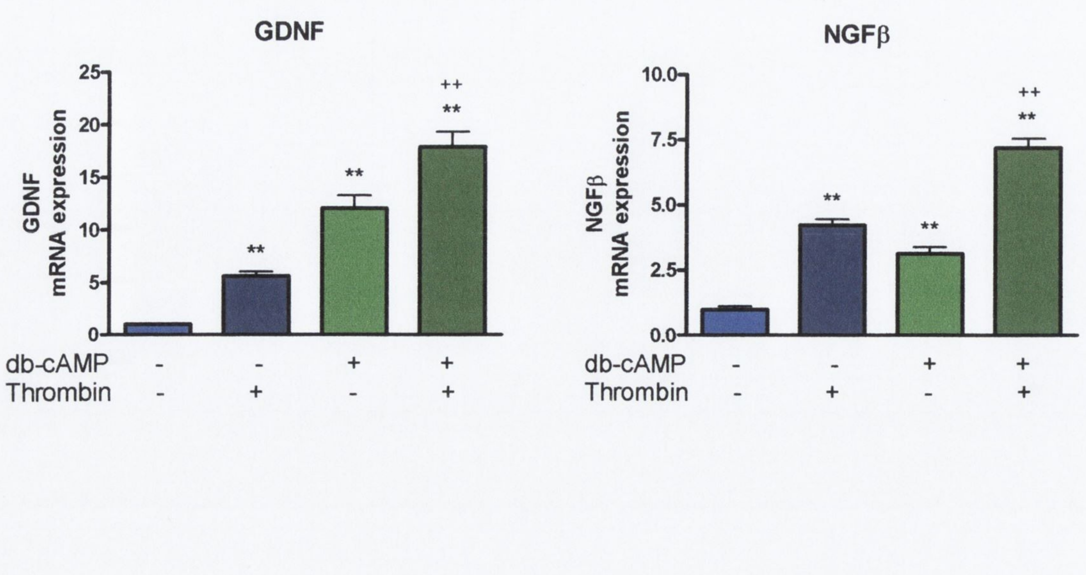


Figure 5.8.2: Salbutamol and db-cAMP, but not salmeterol, augment the thrombin-induced increase in IL-10 and TGFβ in mixed glial cells

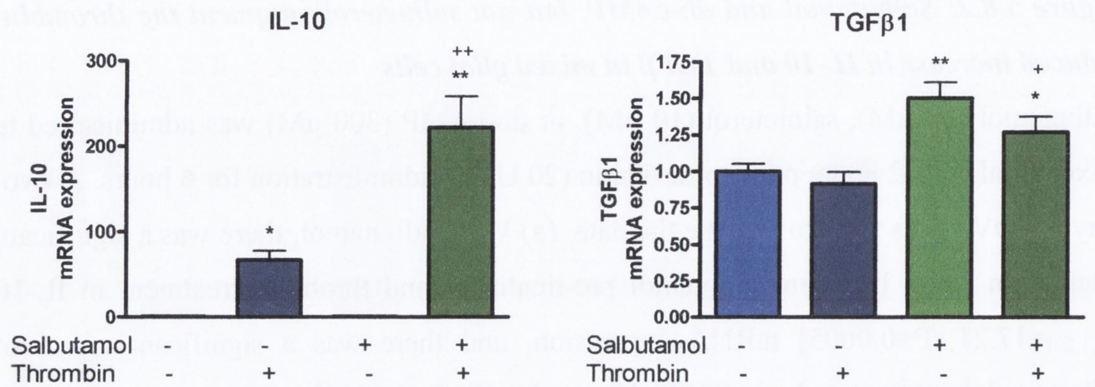
Salbutamol (10 μM), salmeterol (10 μM), or db-cAMP (300 μM) was administered to mixed glial cells 2 hours prior to thrombin (20 U/ml) administration for 6 hours. A two-way ANOVA was used to analyse the data. (a) With salbutamol, there was a significant interaction effect between salbutamol pre-treatment and thrombin treatment in IL-10 [$F_{(1,20)}$=17.21, P=0.0005] mRNA expression, and there was a significant effect of salbutamol treatment only in TGFβ [$F_{(1,20)}$=23.47, P<0.0001] mRNA expression. (b) With salmeterol, there was a significant effect of thrombin treatment only in IL-10 [$F_{(1,17)}$=124.30, P<0.0001] and TGFβ [$F_{(1,20)}$=6.71, P=0.018] mRNA expression. (c) With db-cAMP, there was a significant interaction effect between db-cAMP pre-treatment and thrombin treatment in IL-10 [$F_{(1,19)}$=42.56, P<0.0001] mRNA expression, and a significant effect of db-cAMP treatment only in TGFβ [$F_{(1,20)}$=41.22, P<0.0001] mRNA expression.

Data expressed as means ± SEM (n=5-6), *P<0.05, **P<0.01 vs. control, +P<0.05, ++P<0.01 vs. thrombin (20 U/ml) (Newman-Keuls post-hoc test).

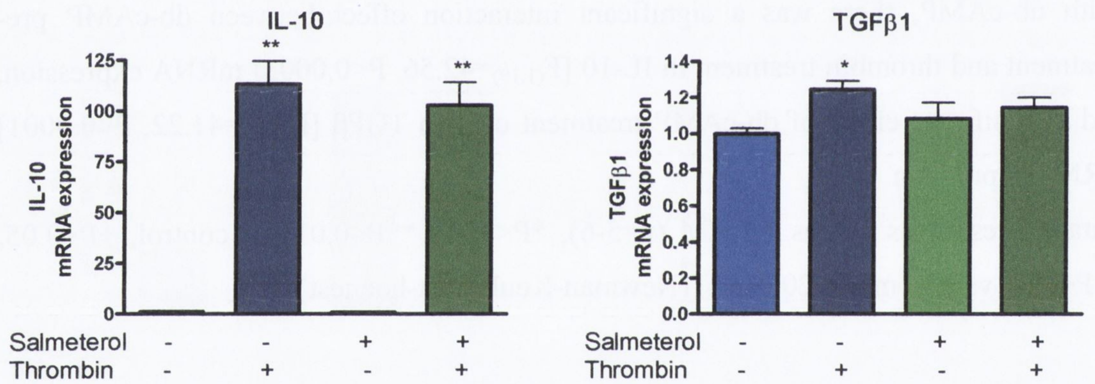
Figure 5.8.2:

IL-10 mRNA **TGFβ mRNA**

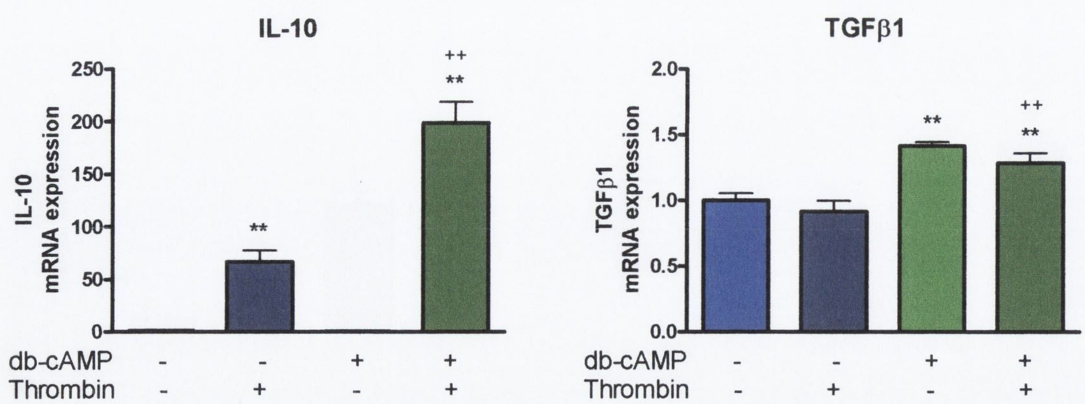
(a) Salbutamol treatment



(b) Salmeterol treatment



(c) db-cAMP treatment



5.9 Discussion

5.9.1 Thrombin and Aβ did not directly alter neuronal viability

Thrombin and Aβ did not alter neuronal viability or LDH release. This is in contradiction to a wide range of *in vitro* studies that have shown that thrombin induces neuronal apoptosis and results in neuronal cell death (Rao et al., 2007, Park and Jin, 2008, Sanchez et al., 2009, Thirumangalakudi et al., 2009), and that Aβ can directly kill neurons (Moore and O'Banion, 2002, Walsh et al., 2002). However many other studies have suggested that thrombin or Aβ alone are not sufficient to induce neuronal death and that it is necessary to use co-culture systems, growing the neurons in the presence of astrocytes or microglia. For example, treatment of co-cultures of neurons and microglia with thrombin results in the cell death of neurons (Lee et al., 2005, Lee da et al., 2006, Huang et al., 2008a). Also mixed neuron and astrocyte cultures showed that low doses of thrombin (1 and 2 U/ml) did not induce cell death, while doses greater than 5 U/ml resulted in a dose-dependent release of LDH, indicating rising levels of cell death (Hua et al., 2007). Other studies state that Aβ-induced neurotoxicity is dependent on glial activation and the resulting induction of iNOS and inflammatory cytokines (Akama and Van Eldik, 2000), and that neuronal death increases dramatically in the presence of microglia, with this increase being primarily attributed to microglial activation (Liu and Hong, 2003). Due to this, the following experiments used CM transfer systems.

5.9.2 CM from glial cells treated with thrombin, but not Aβ, increases neuronal viability

In these studies it was expected that CM from glial cells treated with thrombin or Aβ would decrease neuronal viability. This hypothesis was based on the findings in Chapter 3 of this thesis that showed that thrombin and Aβ induced a significant inflammatory response in cultured glial cells, on a study which showed that CM from microglial cultures treated with thrombin induced degeneration of dopaminergic neurons, with this cell death being prevented by inhibiting microglial activation (Lee et al., 2005), and on studies which showed that CM from microglia cultures which had been treated with Aβ caused apoptosis in cortical neurons in a process that was dependent on iNOS and TNFα (Combs et al., 1999, Combs et al., 2001). However in this study CM from glial cells treated with Aβ did not alter neuronal viability, while CM from glial cells treated with thrombin actually increased neuronal viability. This is consistent with other CM studies which have shown that CM from astrocyte cultures treated with thrombin promoted the survival of human hybrid neurons (Ishida et al., 2006), and that CM obtained from PAR-activated astrocytes similarly protected cortical neurons (Wang et al., 2007). This is also consistent with studies which showed that CM from mixed glial cultures which had been treated with Aβ did not cause apoptosis, while CM from microglia only cultures which had been treated with Aβ caused apoptosis in hippocampal neurons, indicating that astrocytes could play a role in preventing apoptosis (von Bernhardi and Eugenin, 2004). Together these studies indicate that microglia are responsible for inducing neurotoxic effects in neurons, while astrocytes can suppress this neurotoxic effect, possibly having a protective role in the prevention of microglial-induced neuronal apoptosis. However, in order to determine which cell type mediates the thrombin-induced increase in neuronal viability, further studies would be necessary to investigate the effects of CM from thrombin-activated cultures of enriched astrocytes or microglia on neuronal viability. The fact that the studies in this section used mixed glial cultures (which best represent the *in vivo* environment in the brain) instead of microglial only or astrocyte only cultures means that the astrocytic effects appear to be predominant and could explain the finding that there was an increase in neuronal viability in response to thrombin, and no effect in response to Aβ.

5.9.3 A role for neurotrophic factors, anti-inflammatory cytokines, and neuronal cytokine receptor expression in the thrombin-induced increase in neuronal viability?

Considering the ability of thrombin to increase neuronal viability, it was hypothesised that this increase may occur as a result of the production of neurotrophic factors or anti-inflammatory cytokines induced by thrombin specifically. It was shown that thrombin increased mRNA expression of the neurotrophic factors, GDNF and NGFβ, and the anti-inflammatory cytokines, IL-10 and TGFβ, in mixed glial cultures and in astrocyte cultures. Thrombin also increased NGFβ and IL-10 in microglial cultures, but to a much lesser extent than in astrocyte cultures. The most dramatic increase was in IL-10 mRNA expression which was increased 60-fold in mixed glial cultures and 200-fold in astrocyte cultures, while GDNF and NGFβ mRNA expression were increased approximately 5-fold in mixed glial cultures and 7.5-fold in astrocyte cultures. To the best of the author's knowledge, this is the first study to report that thrombin increases GDNF mRNA expression. The increase in NGFβ is consistent with other studies that showed that thrombin induces NGF secretion from different cell types including astrocytes (Neveu et al., 1993, Debeir et al., 1996) and vascular, but not bladder, smooth muscle cells (Sherer et al., 1997, Sherer et al., 2000). The proteolytic activity of thrombin is required to enhance the production of NGF as the thrombin inhibitor hirudin results in a marked decrease of the thrombin-induced increase in NGF expression. Also the effects appear to be specific to thrombin as another serine protease, trypsin, did not increase NGF secretion (Neveu et al., 1993). The thrombin induced increase in IL-10 is consistent with studies which show that thrombin induces IL-10 expression in cultured rat microglia, where it acts as a negative feedback regulation for TNFα (Kim et al., 2002) and that astrocytes can produce IL-10 to a much greater extent than microglia (Ledeboer et al., 2002). The increase in TGFβ1 is also consistent with a study which showed that human fibroblasts release TGFβ1 in response to thrombin stimulation (Huang and Yang, 2003), and that cultures of mixed glia produce TGFβ (Vincent et al., 1997).

Aβ increased TGFβ1 mRNA expression in mixed glial cultures. This is consistent with a study which states that Aβ induces TGFβ1 from cultured microglial cells (Meda et al., 1999), and a study using transgenic Tg2576 mice which showed that Aβ plaque development was correlated with an increase in TGFβ1 in surrounding astrocytes, indicating that Aβ mediated up-regulation of TGFβ1 (Apelt and Schliebs, 2001). However another study states that Aβ does not induce TGFβ1 from microglia (Franciosi et al., 2005), and levels of TGFβ1 are not increased in the CSF of AD patients (Blasko et al., 2006). Aβ did not alter expression of IL-10, GDNF or NGFβ mRNA expression in mixed glial cultures. This is consistent with a study which states that Aβ does not induce IL-10 from cultured microglial cells (Franciosi et al., 2005), however numerous other studies state that Aβ does induce IL-10 production from cultured microglial cells (Meda et al., 1999, Nagai et al., 2001, Lee et al., 2002, Lue and Walker, 2002), and transgenic Tg2576 mice also showed increased IL-10 in astrocytes, indicating that Aβ mediated up-regulation of IL-10 (Apelt and Schliebs, 2001). This appears to be the first study investigating whether Aβ can induce GDNF production from glial cells, however one study did show that levels of GDNF are not altered in the CSF of AD patients (Blasko et al., 2006), which would appear to correlate with the finding here that Aβ does not induce GDNF expression. The finding that Aβ does not alter NGFβ is not in keeping with studies showing that astrocytes, but not microglia, treated with $A\beta_{42}$ increased the production of NGF (Heese et al., 1998, Saez et al., 2006, Schulte-Herbruggen et al., 2007), and that levels of NGF are increased in the CSF of AD patients (Blasko et al., 2006).

While these studies showed quite dramatic results in the level of mRNA expression of GDNF, NGF, TGFβ, and IL-10, future studies would be required to examine the effect of thrombin and Aβ on the levels of protein expression to ensure that the transcriptional effects are being maintained at a protein level. Also, although the studies conducted here indicate that there may be a link between the up-regulation of neurotrophic factors and anti-inflammatory cytokines and an increase in neuronal viability induced by thrombin but not Aβ, a causal relationship remains to be determined. The hypothesis of a causal relationship is supported by many CM studies which have demonstrated the role of neurotrophic factors and anti-inflammatory cytokines in promoting survival of numerous cell types including neurons. For example, astrocytes under conditions of *in vitro* ischemia release GDNF, TGFβ1, and NT-3, and CM from these astrocytes increases the number of surviving ischemic microglia, astrocytes, and neurons, with the microglia being protected by TGFβ1 and NT-3, the astrocytes being protected by GDNF, and the neurons being protected by NT-3 (Lin et al., 2006). Microglia treated with CM from neurons produced GDNF, NGF, IL-10, and TGFβ1, and CM from these microglia, when transferred back onto neurons, increased the survival and maturation of GABAergic, cholinergic, and catecholaminergic neurons (Nakajima et al., 2007). Chronic exposure of microglia to LPS resulted in an increase in the production of IL-10, and CM from these chronically activated microglia increased neuronal viability and differentiation, indicating a protective role for IL-10 (Cacci et al., 2008). In addition to this, IL-10 also has direct trophic actions on neurons (Zhou et al., 2009), and TGFβ is required to promote the survival of neuron populations that is induced by neurotrophins such as GDNF (Mehler and Kessler, 1997, Krieglstein et al., 2002). In order to determine a causal relationship it would be necessary to conduct experiments using neutralising antibodies against GDNF, NGFβ, IL-10 and TGFβ1. For example, IL-10 neutralising antibodies increased thrombin-induced TNFα and IFNγ release, indicating that IL-10 can function as a negative feedback regulator for the thrombin-induced inflammatory response (Kim et al., 2002, Naldini et al., 2006). In order to determine if GDNF, NGFβ, IL-10, or TGFβ1 are responsible for the thrombin-induced increase in neuronal viability it would be necessary to conduct experiments using neutralising antibodies against these molecules or using cultures prepared from KO mice.

It was also hypothesised that the thrombin-induced increase in neuronal viability may occur as a result of modulation of neuronal cytokine receptor expression induced by thrombin. Thrombin, but not Aβ, altered expression of cytokine receptors in neurons. Thrombin increased the anti-inflammatory IL-1R2 and the anti-apoptotic TNFR2, and decreased the pro-apoptotic TNFR1. It also decreased IFNgR1 and increased IFNgR2. The most significant of these increases were in IL-1R2 and TNFR2. Overall this indicates that thrombin promotes an anti-inflammatory and anti-apoptotic environment in neurons. Despite the wealth of literature discussing the role of thrombin in cytokine production, there is apparently no literature examining the role of thrombin in cytokine receptor expression. The only other study which directly measured Aβ induced cytokine receptor expression showed that $A\beta_{1-42}$ dramatically increased IFNgR expression in human aortic endothelial cell line, while only $A\beta_{1-40}$ increased IFNgR expression in human aortic smooth muscle cell line, suggesting that Aβ can up-regulate cytokine receptor expression but that different forms differentially activate different cell types (Suo et al., 1998). However this study used a 10 μM dose of Aβ which was four times higher than the dose that was used in the experiments in this section. Another point is that these molecules were only examined at the mRNA level, therefore future studies could examine the effect of thrombin and Aβ on the levels of protein expression of the cytokine receptors.

5.9.4 Pre-treatment of glia with β-AR agonists or db-cAMP augments the increase in neuronal viability induced by thrombin-CM: A role for neurotrophic factors and anti-inflammatory cytokines?

Pre-treatment of glia with the β-AR agonist salbutamol, the β_2-AR agonist salmeterol, and the cAMP analogue db-cAMP augmented the increase in neuronal viability induced by thrombin-CM. However, pre-treatment of neurons with salbutamol, salmeterol, or db-cAMP did not alter the increase in neuronal viability induced by thrombin-CM. This indicates that β_2-AR agonists increase neuronal viability via the actions of glial-derived factors as opposed to through a direct effect on neurons. To the best of the author's knowledge, this is the first paper to report such a finding. While other papers have examined NA in CM systems, these papers have induced neuronal death by using LPS as a glial stimulus, and demonstrated that NA protected against LPS-CM induced neuronal cell death (Madrigal et al., 2005, Madrigal et al., 2006).

As the β-AR agonist-induced augmentation of the thrombin-induced increase in neuronal viability was mediated via the effect of β-AR agonists on glia, it was hypothesised that this increase may occur as a result of an augmentation of the neurotrophic factors and anti-inflammatory cytokines induced by thrombin. Salbutamol, salmeterol, and db-cAMP increased the neurotrophic factors, GDNF and NGFβ. Salbutamol and db-cAMP also increased the anti-inflammatory cytokine TGFβ, but not IL-10. In addition to this, salbutamol and db-cAMP augmented all the neurotrophic factors induced by thrombin, while salmeterol only augmented GDNF. These findings are in concurrence with a number of other studies: For example, db-cAMP and forskolin, an adenylate cyclase activator, increased GDNF release in neuroblastoma and glioblastoma cell lines (Verity et al., 1999). Although there is currently no information on NA-induced GDNF production, studies have shown that treatment of cultured mouse astrocytes with DA receptor agonists, or a novel catecholaminergic and serotoninergic activity enhancer, increased the secretion of GDNF and NGF (Ohta et al., 2002, Ohta et al., 2003), indicating that neurotransmitters like NA could play a role in GDNF modulation. NA, but not salbutamol, increased NGF mRNA in astroglial cells (Furukawa et al., 1989), and many other studies have shown that β-AR agonists such as isoproterenol and β_2-AR agonists such as clenbuterol increase NGF expression in cultured astrocytes and *in vivo* (Mocchetti et al., 1989, Schwartz and Mishler, 1990, Semkova and Krieglstein, 1999, Rami et al., 2003). Db-cAMP and other cAMP activators also increased NGF *in vitro* (Quarless and Heinrich, 1987, Schwartz and Mishler, 1990).

The effects of β-AR agonists on IL-10 induction appear to be tissue specific (Eijkelkamp et al., 2004). β-AR agonists (including salbutamol and salmeterol) have been shown to increase IL-10 production in peripheral blood CD4+ T cells (Peek et al., 2005), in monocytic cell line U937 cells cultured with LPS or IL-1β (Kavelaars et al., 1997), in rats with experimental autoimmune myocarditis (EAM) (Nishii et al., 2006), and in the peritoneal cavity (Eijkelkamp et al., 2004). However they decreased levels in the lung and the terminal ileum (Eijkelkamp et al., 2004) and in PBMCs, but had no effect in monocytes (Takahashi et al., 2004), or in dendritic cells (Machida et al., 2005). Db-cAMP increased IL-10 mRNA and protein in a stimulated human Jurkat T-cell line (Benbernou et al., 1997). These studies support the findings of this study which showed that the β-AR agonists and db-cAMP were not sufficient to induce IL-10 expression alone, but did augment IL-10 when combined with thrombin treatment. With regard to

TGFβ1, β_2-AR agonists have been shown to modulate airway epithelial cell and smooth muscle release of TGFβ (Frieri and Capetandes, 2008), and salbutamol has tissue-specific effects as it did not alter LPS-induced TGFβ levels in the terminal ileum, but increased levels in liver and peritoneal cavity (Eijkelkamp et al., 2004). NA also increases TGFβ1 expression *in vivo* (Tsai et al., 2001, Briest et al., 2004) and *in vitro* in rat cardiac fibroblasts (Fisher and Absher, 1995, Lai et al., 2009).

Again, these molecules were only examined at the mRNA level therefore future studies could examine the effect of β-AR agonists and db-cAMP on the levels of GDNF, NGFβ, IL-10 and TGFβ1protein expression.

5.9.5 Conclusion

In summary, these experiments showed that CM from mixed glial cells treated with thrombin increased neuronal viability, and that thrombin greatly increased the mRNA expression of the neurotrophic factors, GDNF and NGFβ, and the anti-inflammatory cytokines, IL-10 and TGFβ1, in mixed glial and astrocyte cultures, but only slightly increased NGFβ and IL-10 in microglial cultures. Thrombin also promotes an anti-inflammatory and anti-apoptotic environment in neurons by increasing mRNA expression of the anti-inflammatory IL-1R2 and the anti-apoptotic TNFR2, and decreasing expression of the pro-apoptotic TNFR1. Meanwhile CM from mixed glial cells treated with Aβ did not alter neuronal viability, Aβ only slightly increased TGFβ1 mRNA expression in mixed glial cultures, and Aβ did not alter mRNA expression of cytokine receptors in neurons. These experiments also showed that pre-treating mixed glial cells with salbutamol, salmeterol, and db-cAMP prior to thrombin augmented the increase in neuronal viability induced by thrombin-CM, however pre-treating the neurons had no effect. Salbutamol and db-cAMP augmented the thrombin-induced increase in GDNF, NGFβ, IL-10, and TGFβ1, while salmeterol only augmented GDNF. A causal relationship between up-regulation of neurotrophic factors and anti-inflammatory cytokines and an increase in neuronal viability remains to be determined, and this could be achieved with the use of neutralising antibodies or cultures prepared from KO mice.

6. Concluding remarks and future directions

6.1 Thrombin has pro-inflammatory, anti-inflammatory, and neurotrophic actions

The principle finding of this thesis is that thrombin induces the expression of pro-inflammatory molecules from mixed glial, microglial, and astrocyte cultures, but it also induces neurotrophic factors and anti-inflammatory cytokines from astrocyte and mixed glial cultures. Thrombin also induces an anti-inflammatory profile of cytokine receptor expression on neurons, and media from thrombin-activated glial cells increases neuronal viability (Figure 6.1).

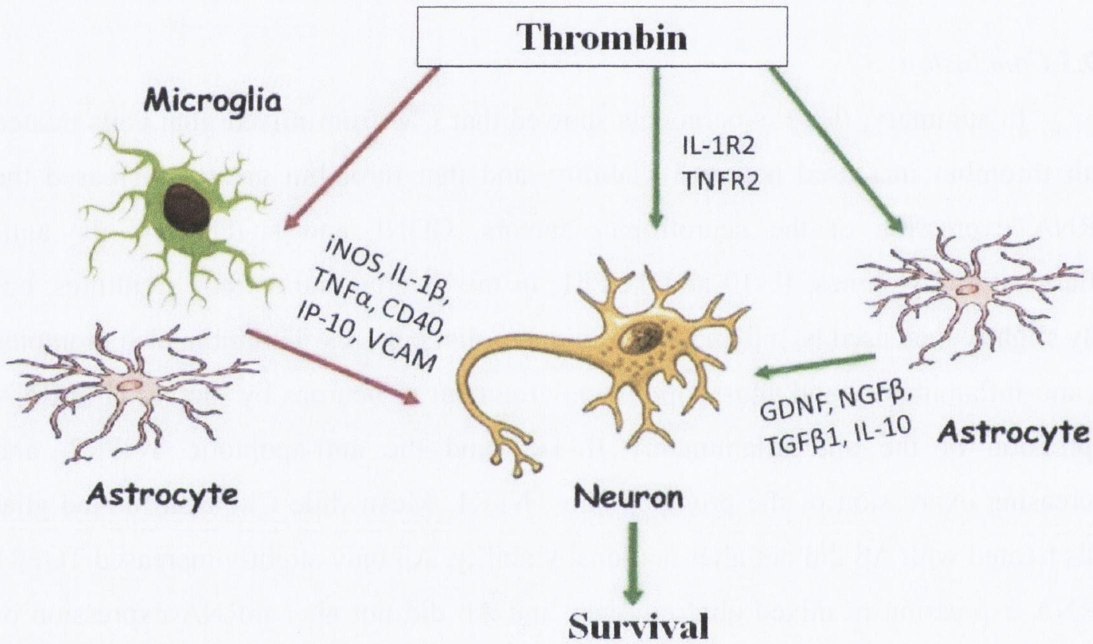


Figure 6.1: The pro-inflammatory, anti-inflammatory, and neurotrophic actions of thrombin

The red arrows indicate thrombin's pro-inflammatory effects and the green arrows indicate thrombin's anti-inflammatory and neurotrophic effects. Thrombin induces expression of the pro-inflammatory molecules iNOS, IL-1β, TNFα, CD40, IP-10 and VCAM-1 from mixed glial, astrocyte, and microglial cultures, however it also induces expression of the neurotrophic factors GDNF and NGFβ, and the anti-inflammatory cytokines IL-10 and TGFβ1 from mixed glial and astrocyte cultures. In neuronal cultures, thrombin induces the expression of the anti-inflammatory IL-1β receptor (IL-1R2) and the anti-apoptotic TNFα receptor (TNFR2). The overall effect of this is that media from thrombin-activated glial cells increases neuronal viability.

These findings that thrombin has pro-inflammatory, anti-inflammatory, and neurotrophic effects are consistent with a recent paper by Luo and colleagues (2007) which investigated both the neurotoxic and neurotrophic actions of thrombin. They found that thrombin induced pro-inflammatory factors such as NO, IL-1β, and TNFα, and toxic factors such as glutamate from astrocytes and microglia which contributed to neurodegeneration. However they also found that thrombin induced astrocytic release of chemokines, glutathione peroxidase and polyunsaturated fatty acids which are known to promote neuronal survival (Luo et al., 2007). Thrombin also mediated either neuronal death or survival depending on the time of thrombin administration: For example, in the 6-OHDA model of PD, administering thrombin with 6-OHDA significantly enhanced behavioural deficits, while administering thrombin 1 or 7 days after 6-OHDA prevented 6-OHDA-induced neurochemical and behavioural deficits (Cannon et al., 2007). Another study by Turgeon and colleagues (1999) proposed a hypothesis, based on the interaction of cell signalling pathways, to explain the toxic and trophic effects of thrombin. Thrombin mediates its toxic effects by binding to PAR-1, which induces signal transduction, stimulation of PLC and translocation of MAPK, leading to gene expression. For example, PAR-1 activation stimulates caspase pathways which induce neuronal death. Meanwhile, activation of neurotrophic receptors (such as receptors for NGF, GDNF, or BDNF) which are coupled to tyrosine kinases also leads to stimulation of PLC and MAPK, leading to gene expression. Turgeon hypothesises that due to the similar post-activation pathways potential overlaps occur either in the cytoplasm, in the nucleus, or at the translational level (Turgeon and Houenou, 1999). Both of these studies propose interesting theories for the actions of thrombin and future studies should investigate these processes.

The findings in this thesis showed that thrombin induced expression of pro-inflammatory molecules in both microglia and astrocytes, but that it was the astrocytes that predominantly expressed anti-inflammatory molecules such as TGFβ and IL-10 in response to thrombin (Figure 6.1). This indicates that the anti-inflammatory actions of thrombin are largely mediated by astrocytes, although further studies are required to confirm this. In concurrence with this, an interesting study by Schubert and colleagues (2000) investigated the anti-inflammatory actions of astrocytes on microglia and showed that astrocytes, depending on their activation state, exert negative feedback regulation on activated microglial cell functions and inhibits LPS- and IFNγ-induced microglial NO release (Figure 6.2). For example, resting astrocytes release TGFβ1 and are differentiated, confluent, and have a low proliferative rate, and CM from these astrocytes effectively inhibits microglial NO release. However activated astrocytes are non-differentiated and highly proliferative and CM from these astrocytes did not inhibit microglial NO release. There is also significant interplay of cytokines that are released from microglia and astrocytes. For example, TNFα acts as a positive feedback signal reinforcing microglial activation and inducing IL-1β production thereby promoting activation of astrocytes. However IL-6 exerts a negative feedback on TNFα signalling thus inhibiting the escalating microglial activation that leads to the critical secondary activation of astrocytes (Schubert et al., 2000).

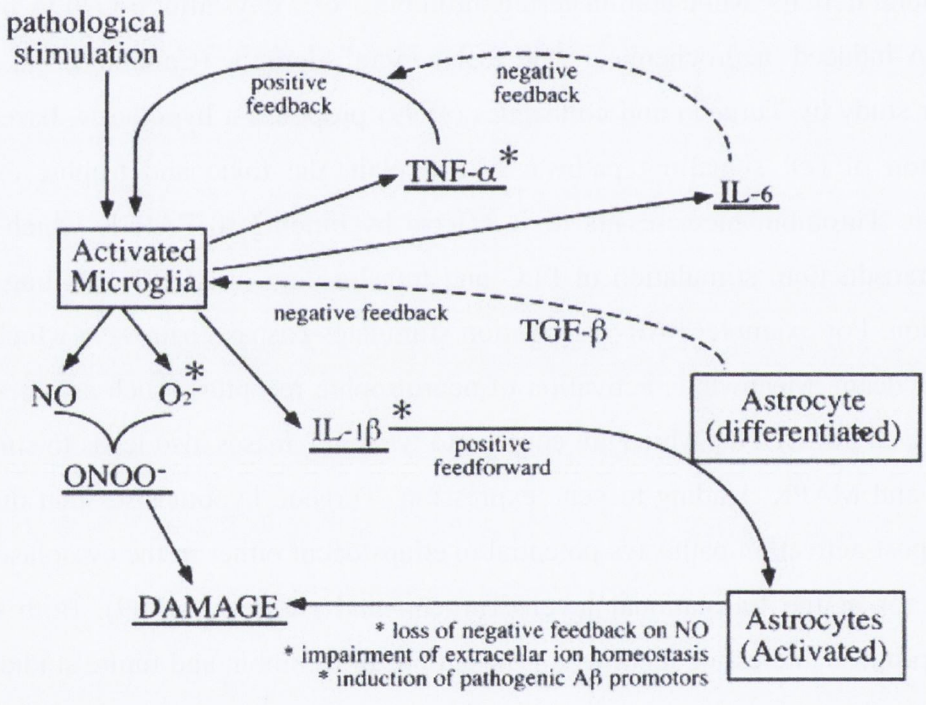


Figure 6.2: Astrocyte and cytokine modulation of microglial activation (adapted from Schubert et al., 2000)

Differentiated (resting), but not activated, astrocytes inhibit microglial activation by the release of TGFβ. TNFα has a positive feedback mechanism to promoter IL-1β release which has a positive feed-forward mechanism to activate astrocytes. This results in loss of negative feedback on NO, impairment of extracellular ion homeostasis and induction of pathogenic Aβ promoters. IL-6 has a negative feedback mechanism to inhibit TNFα.

6.2 Aβ has pro-inflammatory actions

Aβ induces the expression of pro-inflammatory molecules from mixed glial, microglial, and astrocyte cultures, however it induces a restricted inflammatory profile relative to thrombin (Aβ only induces IL-1β and TNFα), and the magnitude of expression of these inflammatory molecules is much less than that induced by thrombin. Aβ also induces expression of the anti-inflammatory cytokine TGFβ1 in mixed glial cultures, but unlike thrombin it does not induce neurotrophic factors or the anti-inflammatory cytokine IL-10, which has a neurotrophic effect itself (Zhou et al., 2009). It has no effect on neuronal cytokine receptor expression and media from Aβ-activated glial cells does not neuronal viability (Figure 6.3).

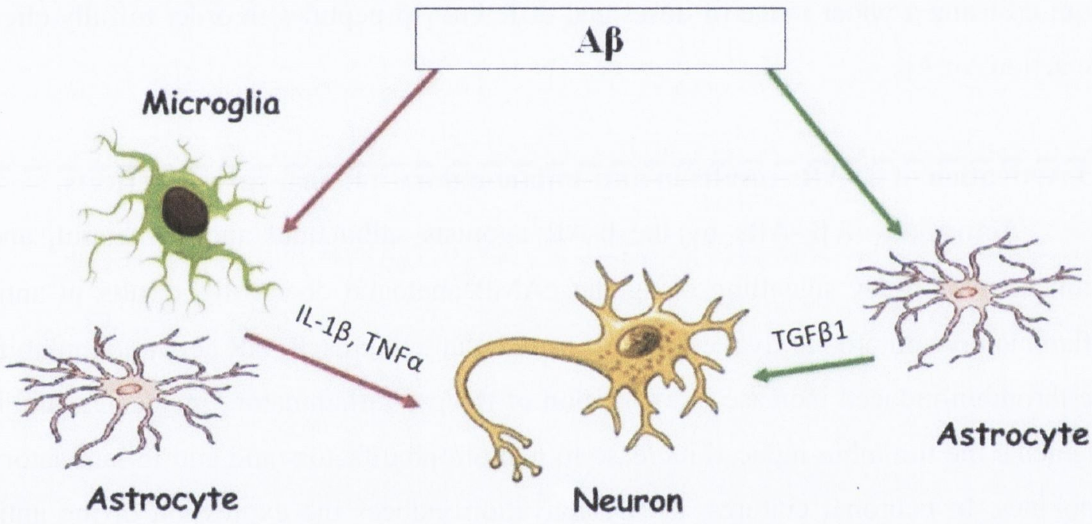


Figure 6.3: The pro-inflammatory actions of Aβ

The red arrows indicate Aβ's pro-inflammatory effects and the green arrows indicate Aβ's anti-inflammatory effects. Aβ induces expression of the pro-inflammatory molecules IL-1β and TNFα from mixed glial, astrocyte, and microglial cultures, however it also induces modest expression of the anti-inflammatory cytokine TGFβ1 from mixed glial and astrocyte cultures. Aβ does not alter expression of cytokine receptors in neurons and CM from mixed glial cells treated with Aβ does not alter neuronal viability.

Aβ induces an inflammatory response in mixed glial cultures, but this response is insufficient to induce apoptosis in neuronal cultures. Although other studies have shown that CM from microglial cultures treated with Aβ caused apoptosis in cortical neurons, these studies used considerably higher doses of Aβ than were used in the current experiments (2.5 μM), occasionally as high as 60 μM (Combs et al., 1999, Combs et al., 2001), however other studies reported similar results using 2 μM Aβ (von Bernhardi and Eugenin, 2004), indicating that the dose of Aβ required to elicit a response can be extremely variable. As well as dose, the peptide species of Aβ used appears to be important: For example, $A\beta_{25\text{-}35}$ was shown to induce NGF expression while $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ failed to alter NGF synthesis (Heese et al., 1998, Saez et al., 2006). As Aβ only increases TGFβ1 it is consistent that Aβ would not have neurotrophic effects on neurons as TGFβ is most likely not neurotrophic by itself, but is required to promote the survival of neuron populations that is induced by neurotrophins such as GDNF (Mehler and Kessler, 1997, Krieglstein et al., 2002). Further studies are required using a wider range of doses and different Aβ peptides in order to fully elicit the actions of Aβ.

6.3 Activation of β_2-ARs results in anti-inflammatory and pro-survival effects

Activation of β_2-ARs by the β-AR agonists salbutamol and salmeterol, and mimicking of β-AR signalling using the cAMP analogue db-cAMP, results in anti-inflammatory and pro-survival effects. In mixed glial cultures, β_2-AR activation inhibits the thrombin-induced increase in expression of the pro-inflammatory molecules, and it augments the thrombin-induced increase in neurotrophic factors and anti-inflammatory cytokines. In neuronal cultures, β_2-AR activation induces the expression of the anti-inflammatory IL-1β receptor (IL-1R2) and the anti-apoptotic TNFα receptor (TNFR2). The overall effect of this is that β_2-AR activation augments the increase in neuronal viability induced by media from thrombin-activated glial cells (Figure 6.4). It has been shown that thrombin results in a decrease in cAMP (Bogatcheva et al., 2002) so it is logical that increasing cAMP inhibits the pro-inflammatory effects of thrombin. However the mechanism of action for the β-AR-induced augmentation of the anti-inflammatory actions of thrombin has yet to be elucidated.

Although the studies in this thesis showed that activation of the β-AR pathway by agonists and activators of cAMP, PKA and EPAC mimicked the anti-inflammatory actions of NA, β-AR antagonists, and inhibitors of cAMP and PKA were unable to prevent the ability of NA to combat thrombin-induced inflammation. Other studies have obtained similar results and suggest that PKA-independent pathways, such as the EPAC or ERK pathways, may also play a role in mediating the NA-induced inhibition of glial activation (Dello Russo et al., 2004, Qian et al., 2009).

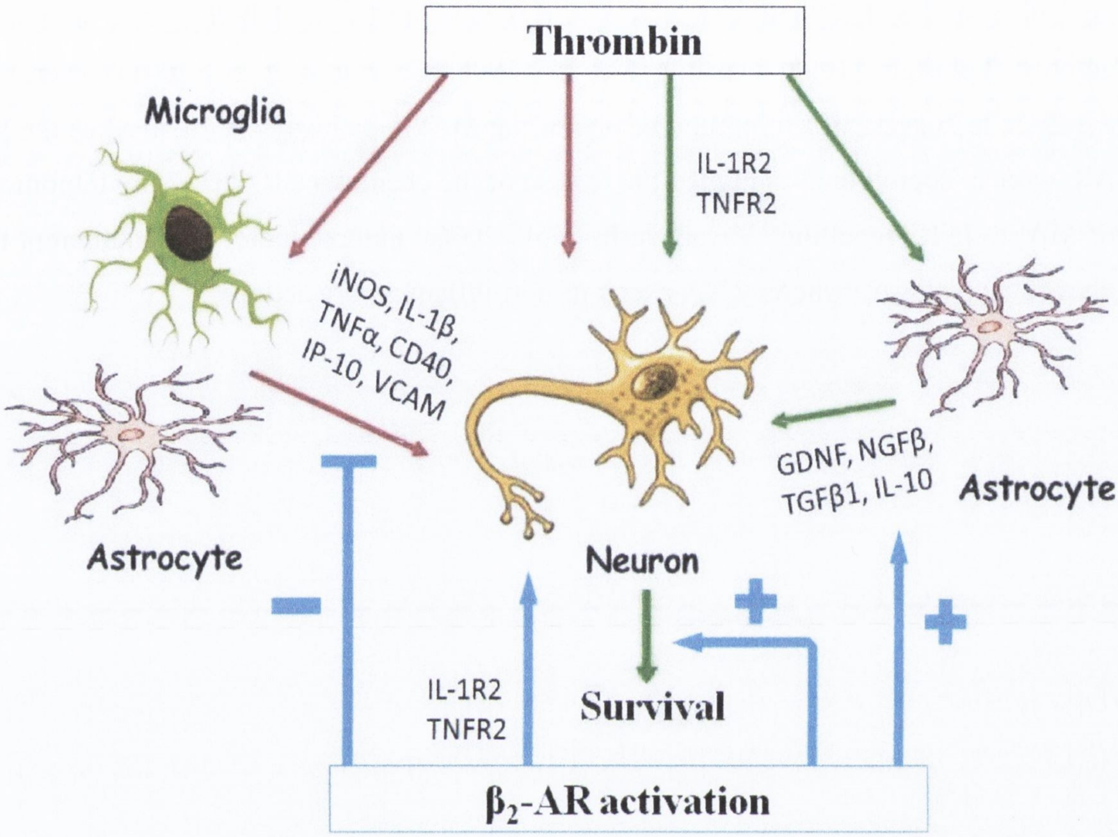


Figure 6.4: Activation of β_2-ARs results in anti-inflammatory and pro-survival effects
The red arrows indicate thrombin's pro-inflammatory effects, the green arrows indicate thrombin's anti-inflammatory and neurotrophic effects, and the blue arrows indicate the effects of β_2-AR activation. In mixed glial cultures, β_2-AR activation inhibits the thrombin-induced expression of the pro-inflammatory molecules TNFα, CD40, IP-10 and VCAM-1, and it augments the thrombin-induced expression of the neurotrophic factors GDNF and NGFβ, and the anti-inflammatory cytokines IL-10 and TGFβ1. In neuronal cultures, β_2-AR activation induces the expression of the anti-inflammatory IL-1β receptor (IL-1R2) and the anti-apoptotic TNFα receptor (TNFR2). The overall effect of this is that β_2-AR activation augments the increase in neuronal viability induced by media from thrombin-activated glial cells.

Further research is required in order to elucidate other pathways which may be involved in mediating the anti-inflammatory actions of NA. For example, some studies have shown that the anti-inflammatory actions of NA are mediated in a cell- and gene-specific manner through a number of transcription factors, with particular emphasis on NFκB and IκB (Feinstein et al., 2002, Gavrilyuk et al., 2002, Dello Russo et al., 2004), and other studies have indicated that NA can function by modulating gene promoter activity (Feinstein, 1998, Nakamura et al., 1998, Gavrilyuk et al., 2001). There is evidence to suggest that inhibition of one of the MAPK pathways is involved as the β-AR agonist isoproterenol inhibited the release of the chemokine RANTES by inhibition of MAPK-JNK signalling (Miyabayashi et al., 2006). Future studies should attempt to dissect the pathways whereby NA exerts its anti-inflammatory actions.

6.4 Future directions

The research presented in this thesis has yielded a number of important leads for future research as outlined below.

1) NA and salmeterol inhibited the thrombin-induced increase in TNFα and IL-1β mRNA and protein, indicating that the effects occurred at a transcriptional level and that they were maintained at the level of protein expression. However the other molecules were only examined at the mRNA level, therefore future studies could examine the effect of thrombin and β-AR activation on the levels of protein expression of iNOS, CD40, IP-10, and VCAM-1.
2) While activation of the β-AR pathway with β-AR agonists and cAMP and PKA activators mimicked the effect of NA, β-AR antagonists and inhibitors of cAMP and PKA were unable to block the actions of NA. Further research is required in order to elucidate other pathways which may be involved in mediating the anti-inflammatory actions of NA. One such target for research could be the ERK pathway (Qian et al., 2009).
3) Media from thrombin-activated mixed glial cells increased neuronal viability. Other studies have indicated that the protective effects of thrombin are mediated by astrocytes while the neurotoxic effects are mediated by microglia (Lee et al., 2005, Ishida et al., 2006, Wang et al., 2007). In order to determine which cell type mediates the thrombin-induced increase in neuronal viability, it would be necessary to investigate the effects of media from thrombin-activated cultures of enriched astrocytes or microglia on neuronal viability.
4) Thrombin induced the expression of GDNF, NGFβ, IL-10, and TGFβ1 mRNA from astrocytes. Other CM studies have shown that the release of these molecules from glia is protective to neurons (Hashimoto et al., 2005, Nakajima et al., 2007, Cacci et al., 2008). In order to determine if these molecules are responsible for the thrombin-induced increase in neuronal viability it would be necessary to conduct experiments using neutralising antibodies against these molecules or using cultures prepared from KO mice.
5) Determine if thrombin- and β-AR agonist-induced GDNF and NGFβ production can protect against glutamate-induced neurotoxicity.

6) All of the studies in this thesis were conducted *in vitro* therefore future studies should investigate the effect of β-AR activation on acute thrombin- and Aβ-induced inflammation *in vivo*.

Investigation into these areas would provide clear insights into the functioning of thrombin and the mechanisms of action of β-AR activation both *in vitro* and *in vivo*. This could lead to useful therapeutic interventions that may be used in the treatment of stroke, PD, AD, and other neurodegenerative diseases.

7. Appendices

7.1 List of Figures and Tables

Chapter 4:

Chapter 5:

Chapter 6:

7.2 Raw Data

Chapter 3

Raw PCR data from Section 3.2:

	Control	LPS		Control	Thrombin 10 U/ml	Thrombin 20 U/ml
iNOS	0.10	185.61	***iNOS***	0.10	471.81	588.63
	0.68	141.25		0.68	423.03	561.25
	1.65	202.73		1.65	462.84	464.29
	0.91	232.65		0.91	677.43	432.04
	1.43	121.69		1.43	520.86	426.63
	1.24	193.83		1.24	748.11	788.68
Mean	1.00	179.60	Mean	1.00	550.70	543.60
SEM	0.23	16.75	SEM	0.23	53.59	56.22
IL-1β	1.28	18.05	***IL-1β***	1.28	45.93	44.44
	1.32	15.90		1.32	41.62	41.12
	1.05	20.14		1.05	43.51	29.55
	0.76	38.16		0.76	53.87	40.56
	0.88	13.23		0.88	32.99	28.20
	0.71	17.35		0.71	49.89	38.87
Mean	1.00	20.47	Mean	1.00	44.63	37.12
SEM	0.11	3.66	SEM	0.11	2.95	2.72
TNFα	0.59	10.76	***TNFα***	0.59	21.68	23.97
	1.08	11.51		1.08	18.58	22.39
	1.02	11.77		1.02	17.55	26.67
	0.95	15.77		0.95	31.02	21.62
	1.11	9.78		1.11	24.80	20.04
	1.25	14.19		1.25	16.68	19.69
Mean	1.00	12.29	Mean	1.00	21.72	22.40
SEM	0.09	0.92	SEM	0.09	2.23	1.07
CD40	1.27	37.86	***CD40***	1.27	44.58	40.89
	1.43	24.06		1.43	36.18	36.60

	0.93	20.94
	0.78	8.43
	0.89	15.09
	0.71	22.64
Mean	1.00	21.50
SEM	0.12	4.03
VCAM-1	0.80	10.25
	1.13	9.07
	0.93	9.60
	0.87	5.76
	1.07	8.42
	1.21	12.12
Mean	1.00	9.20
SEM	0.07	0.86
IP-10	0.34	83.14
	0.87	66.20
	0.87	78.21
	1.35	58.93
	1.25	59.47
	1.32	70.10
Mean	1.00	69.34
SEM	0.16	4.02

	0.93	47.18	24.80
	0.78	44.17	25.86
	0.89	46.29	23.90
	0.71		32.97
Mean	1.00	43.68	30.83
SEM	0.12	1.95	2.88
VCAM-1	0.80	18.26	21.22
	1.13	12.67	12.49
	0.93	12.88	14.53
	0.87	11.35	16.29
	1.07	10.06	11.56
	1.21	19.07	21.96
Mean	1.00	14.05	16.34
SEM	0.07	1.52	1.79
IP-10	0.34	92.28	130.51
	0.87	91.65	81.29
	0.87	91.25	90.97
	1.35	79.78	121.79
	1.25	112.73	93.00
	1.32	113.52	126.58
Mean	1.00	96.87	107.40
SEM	0.16	5.48	8.70

	Control	**Aβ 2.5 μM**	**Aβ 5 μM**	**Aβ 10 μM**
iNOS	0.10	0.84	1.38	2.16
	0.68	1.04	1.29	1.86
	1.65	0.68	2.43	5.46
	0.91	0.24	6.81	3.70
	1.43	3.47	4.47	12.35
	1.24	3.09		
Mean	1.00	1.56	3.28	5.10
SEM	0.23	0.56	1.05	1.92

IL-1β	1.28	5.87	6.09	7.53
	1.32	3.90	5.83	6.45
	1.05	4.64	6.74	9.15
	0.76	6.05	10.87	10.89
	0.88	7.36	7.94	7.90
	0.71			11.87
Mean	1.00	5.56	7.49	8.96
SEM	0.11	0.60	0.92	0.85
TNFα	0.59	3.84	5.12	5.95
	1.08	4.00	5.77	4.71
	1.02	4.31	6.19	6.54
	0.95	5.09	7.47	7.48
	1.11	6.46	5.96	5.74
	1.25			9.61
Mean	1.00	4.74	6.10	6.67
SEM	0.09	0.48	0.39	0.70
CD40	1.27	1.66	1.36	2.93
	1.43	1.62	1.46	2.31
	0.93	1.09	1.28	1.90
	0.78	1.17	1.30	1.83
	0.89	1.58	1.31	3.47
	0.71			
Mean	1.00	1.42	1.34	2.49
SEM	0.12	0.12	0.03	0.31
VCAM-1	0.80	1.38	1.55	1.28
	1.13	1.16	1.64	1.20
	0.93	1.37	1.89	1.53
	0.87	0.11	0.19	3.38
	1.07	2.03	2.60	1.35
	1.21	2.78	3.01	2.68
Mean	1.00	1.47	1.81	1.90

SEM	0.07	0.37	0.40	0.37
IP-10	0.34	0.63	0.87	0.67
	0.87	0.72	0.87	0.64
	0.87	1.06	1.15	1.35
	1.35	0.10	0.14	0.99
	1.25	5.28	3.51	4.19
	1.32	4.30	3.95	
Mean	1.00	2.01	1.75	1.57
SEM	0.16	0.90	0.65	0.67

Raw PCR data from Section 3.3:

	Microglia				***Astrocytes***			
	Con	**LPS**	**Throm**	**Aβ**	**Con**	**LPS**	**Throm**	**Aβ**
iNOS	0.78	199.89	220.96	16.78	1.16	294.60	229.94	36.70
	0.49	261.62	145.86	10.85	0.46	253.32	233.68	54.07
	1.26	98.81	100.42	5.62	0.98	184.44	136.02	28.68
	1.18	216.39	97.21	76.90	1.27	346.73	222.97	162.21
	1.29	144.29	73.51	18.72	1.13	289.22	154.29	74.75
		196.82	111.33	28.76		390.43	209.69	109.70
Mean	1.00	186.30	124.90	26.27	1.00	293.10	197.80	77.69
SEM	0.16	23.30	21.49	10.62	0.14	29.30	17.13	20.67
IL-1β	0.41	49.90	40.97	12.24	1.68	30.94	41.73	21.67
	0.73	58.80	70.93	19.15	1.72	26.27	45.42	24.81
	0.94	23.15	48.28	8.27	0.54	25.50	28.05	17.40
	1.62	30.02	21.12	30.54	0.74	29.44	36.56	36.01
	1.54	24.93	56.74	21.67	0.66	31.37	31.07	29.28
	0.76	26.27	27.38	25.39	0.66	41.37	41.16	32.00
Mean	1.00	35.51	44.24	19.54	1.00	30.82	37.33	26.86
SEM	0.20	6.14	7.56	3.37	0.22	2.33	2.74	2.81
TNFα	0.46	26.82	14.25	5.52	1.55	26.27	17.30	17.67
	0.75	41.28	17.16	7.60	0.62	21.33	22.09	22.03

	1.12	22.11	25.29	4.63	0.98	14.60	12.08	16.78
	1.32	19.57	8.41	8.98	0.98	20.61	11.52	29.96
	1.56	29.99	14.58	12.30	0.88	26.67	13.72	24.42
	0.79	20.81	10.10	7.62		25.14	13.76	25.07
Mean	1.00	26.76	14.96	7.78	1.00	22.44	15.08	22.66
SEM	0.17	3.32	2.44	1.11	0.15	1.88	1.63	2.02
CD40	1.19	116.54	87.02	9.50	1.35	62.86	44.99	8.32
	0.96	75.43	77.24	12.78	0.61	49.13	51.80	11.21
	0.69	57.81	109.66	7.01	1.16	45.37	23.26	4.80
	0.62	38.08	37.60	20.61	1.04	50.56	34.86	24.82
	1.45	66.68	58.56	18.72	0.84	58.37	27.21	13.19
	1.08	62.45	71.22	17.79		80.31	34.31	15.85
Mean	1.00	69.50	73.55	14.40	1.00	57.77	36.07	13.03
SEM	0.13	10.69	10.04	2.24	0.13	5.21	4.38	2.83
IP-10	0.44	127.83	88.18	10.63	0.96	160.95	50.78	6.86
	0.81	144.22	89.15	12.86	1.13	140.34	44.43	9.63
	1.52	99.19	70.46	2.81	1.20	175.76	45.10	12.72
	0.72	81.49	44.03	15.67	0.86	103.45	54.96	40.38
	1.38	122.52	67.56	16.90	0.86	140.24	43.52	29.22
	1.12	109.58	69.74			166.31	45.53	26.40
Mean	1.00	114.10	71.52	11.77	1.00	147.80	47.39	20.87
SEM	0.17	9.08	6.74	2.49	0.07	10.61	1.84	5.39

Raw PCR data from Section 3.4:

	Control	**Thrombin**	**NA 1µM**	**NA 1µM +Throm**	**NA 10µM**	**NA 10µM +Throm**
iNOS	1.16	618.38	3.86	886.63	3.39	264.03
	1.11	553.57	3.74	1164.64	7.88	399.13
	0.44	657.77	3.17	846.50	5.43	145.71
	0.80	529.33	2.67	194.45	0.87	67.47
	1.38	314.10	4.71	287.95	5.72	174.92
	1.11	600.71	1.70	612.20	2.61	229.64

Mean	1.00	545.60	3.31	665.40	4.32	213.50
SEM	0.14	49.94	0.43	152.50	1.03	46.43
IL-1β	0.86	25.32	0.84	23.86	0.73	16.30
	1.14	23.67	0.98	30.40	1.12	21.09
	0.50	29.54	0.70	22.71	1.05	13.97
	0.93	24.18	0.80	17.54	0.19	8.92
	1.46	25.03	1.12	16.29	0.90	15.42
	1.12	31.89	0.83	27.24	0.79	17.50
Mean	1.00	26.60	0.88	23.01	0.80	15.53
SEM	0.13	1.36	0.06	2.22	0.14	1.65
TNFα	0.82	6.23	0.34	4.93	0.24	3.56
	1.02	10.03	0.30	5.87	0.32	3.51
	0.57	11.66	0.22	6.25	0.37	4.05
	1.04	9.61	0.22	3.51	0.06	2.25
	1.34	14.51	0.55	3.47	0.45	2.97
	1.20	17.56	0.21	5.05	0.28	4.02
Mean	1.00	11.60	0.31	4.85	0.29	3.39
SEM	0.11	1.63	0.05	0.47	0.05	0.28
CD40	0.81	24.10	0.53	26.45	0.46	19.05
	1.10	31.47	0.59	31.96	0.63	23.92
	0.65	34.25	0.42	29.56	0.59	11.88
	0.87	32.68	0.33	15.72	0.14	7.22
	1.51	36.23	0.71	16.57	0.65	12.45
	1.07	55.96	0.39	28.72	0.44	18.67
Mean	1.00	35.78	0.49	24.83	0.49	15.53
SEM	0.12	4.38	0.06	2.84	0.08	2.48
VCAM-1	0.89	5.16	0.54	2.72	0.41	2.17
	1.08	8.72	0.61	2.17	0.62	1.77
	0.66	7.23	0.42	2.75	0.48	1.48
	0.96	5.96	0.53	2.68	0.24	0.82

	1.31	8.10	0.69	3.37	0.57	2.17
	1.09	8.89	0.35	3.36	0.56	3.34
Mean	1.00	7.35	0.52	2.84	0.48	1.96
SEM	0.09	0.62	0.05	0.19	0.06	0.34
IP-10	1.03	35.50	0.92	28.16	0.69	5.93
	1.10	47.70	0.73	25.77	0.76	9.08
	0.82	44.18	0.96	28.48	0.72	3.55
	0.79	38.05	0.32	5.66	0.20	0.69
	1.30	49.26	0.94	19.25	0.82	6.93
	0.96	51.24	0.71	29.04	0.88	13.22
Mean	1.00	44.32	0.76	22.73	0.68	6.57
SEM	0.08	2.59	0.10	3.72	0.10	1.78

	Control	**Aβ**	**NA 1μM**	**NA 1 +Aβ**	**NA 10μM**	**NA 10 +Aβ**
IL-1β	0.74	2.36	0.99	2.37	0.41	2.25
	0.46	2.77	0.64	2.66	0.48	2.11
	0.64	1.40	0.69	2.54	0.41	3.03
	1.26	2.96	0.74	3.22	1.10	3.45
	1.18	2.11	0.91	2.23	0.78	2.60
	1.71	3.57		0.96	1.01	
Mean	1.00	2.53	0.79	2.33	0.70	2.69
SEM	0.19	0.31	0.07	0.31	0.13	0.25
TNFα	0.50	6.43	0.36	1.08	0.09	0.49
	0.70	8.22	0.25	0.55	0.04	0.44
	0.32	1.91	0.10	0.98	0.01	1.01
	1.90	10.72	0.55	1.19	0.28	0.12
	0.84	3.27	0.12	0.45	0.30	1.08
	1.75	8.46	0.31	1.54	0.19	
Mean	1.00	6.50	0.28	0.96	0.15	0.63
SEM	0.27	1.37	0.07	0.17	0.05	0.18

CD40	0.63	1.52	0.50	0.96	1.00	0.87
	1.24	2.07	0.76	1.14	0.34	0.85
	0.68	3.15	0.64	1.37	0.64	1.27
	1.38	1.34	1.04	0.93	0.48	0.93
	1.29	1.59	1.24	0.76	0.47	0.95
	0.78		0.99	0.83	0.81	
Mean	1.00	1.94	0.86	1.00	0.62	0.97
SEM	0.14	0.33	0.11	0.09	0.10	0.08

Raw PCR data from Section 3.5:

Microglia	**Control**	**Thrombin**	**NA 1μM**	**NA1+Thr**	**NA 10μM**	**NA10+Thr**
iNOS	1.22	600.76	1.14	514.86	1.95	474.89
	1.17	591.84	1.11	455.55	1.91	581.31
	1.32	482.75	0.97	493.78	1.40	459.23
	0.85	475.08	1.32	524.61	0.93	397.33
	0.45	390.43	1.11	400.78	0.97	390.18
Mean	1.00	508.20	1.13	477.90	1.43	460.60
SEM	0.16	39.48	0.06	22.64	0.22	34.45
IL-1β	0.79	110.68	1.02	93.65	0.95	93.17
	0.91	115.72	0.99	84.37	0.88	
	0.85	89.43	0.98	80.56	1.00	79.06
	1.26	86.31	0.80	87.26	0.55	104.42
	1.19	84.46	0.79	76.76	0.46	77.76
Mean	1.00	97.32	0.92	84.52	0.77	88.60
SEM	0.09	6.58	0.05	2.89	0.11	6.32
TNFα	0.84	17.34	0.84	20.15	0.72	13.88
	1.05	26.40	0.72	27.69	0.57	16.92
	0.94	21.38	0.92	21.82	0.76	13.95
	0.93	25.21	0.67	19.99	0.60	24.00
	1.23	28.25	0.53	22.77	0.50	22.31
Mean	1.00	23.72	0.74	22.48	0.63	18.21
SEM	0.07	1.95	0.07	1.40	0.05	2.11

CD40	1.13	114.00	0.68	72.30	0.74	66.59
	1.05	112.24	0.84	75.26	0.67	86.71
	1.13	76.39	0.69	75.44	0.54	63.12
	0.89	78.88	0.67	78.91	0.44	51.61
	0.81	55.26	0.64	78.55	0.48	59.63
Mean	1.00	87.36	0.70	76.09	0.57	65.53
SEM	0.06	11.29	0.04	1.21	0.06	5.85

Astrocytes	**Control**	**Thrombin**	**NA 1 μM**	**NA 1 μM +Throm**	**NA 10 μM**	**NA 10 μM +Throm**
iNOS	0.70	281.49	2.70	274.55	2.18	198.36
	1.35	285.95	4.18	279.35	6.14	215.52
	1.02	255.68	3.33	302.09	4.57	234.55
	0.79	212.14	3.25	216.58	2.98	172.55
	1.14	230.53	4.45	155.32	3.81	134.06
Mean	1.00	253.20	3.58	245.60	3.94	191.00
SEM	0.12	14.28	0.32	26.61	0.68	17.51
IL-1β	0.81	37.22	0.93	32.72	0.94	29.50
	1.29	41.41	1.14	35.20	1.31	33.30
	0.81	43.31	1.07	41.38	1.26	24.64
	1.02	37.30	1.09	35.45	0.63	32.76
	1.06	46.64	1.47	28.20	0.96	26.67
Mean	1.00	41.18	1.14	34.59	1.02	29.37
SEM	0.09	1.80	0.09	2.14	0.12	1.68
TNFα	0.93	11.03	0.64	11.41	0.40	13.25
	1.22	10.66	0.60	10.11	0.72	8.05
	0.80	10.46	0.76	10.64	0.96	10.96
	1.16	16.52	1.04	14.81	0.33	10.57
	0.88	14.64	0.82	14.95	0.50	9.53
Mean	1.00	12.66	0.77	12.39	0.58	10.47
SEM	0.08	1.23	0.08	1.04	0.11	0.86

CD40	1	53.15	0.84	44.41	0.72	31.33
	1.28	52.33	1.02	58.73	0.86	35.66
	1.01	53.4	0.94	59.64	0.92	32.32
	0.84	33.41	0.71	30.19	0.48	31.15
	0.86	41.3	0.79	20.46	0.61	25.87
Mean	1	46.72	0.86	42.69	0.72	31.27
SEM	0.08	4.03	0.05	7.74	0.08	1.57

Microglia	**Control**	**Aβ**	**NA 1 μM**	**NA 1μM +Aβ**	**NA 10 μM**	**NA 10μM +Aβ**
IL-1β	1.06	3.81	1.26	3.01	0.85	1.64
	1.27	3.75	1.26	3.28	0.76	1.49
	0.88	3.49	1.08	2.94	0.86	1.96
	0.80		0.89		0.70	1.81
	0.99		1.07		0.76	
Mean	1.00	3.68	1.11	3.08	0.79	1.72
SEM	0.08	0.10	0.07	0.10	0.03	0.10
TNFα	1.05	2.86	1.11	2.31	1.18	1.49
	1.10	2.89	1.11	2.76	0.78	2.08
	0.95	2.77	1.04	2.63	0.99	1.87
	0.95		1.01		0.71	1.65
	0.95		1.34		1.03	
Mean	1.00	2.84	1.12	2.57	0.94	1.77
SEM	0.03	0.04	0.06	0.13	0.09	0.13
CD40	0.96	1.67	0.83	1.52	1.00	1.42
	1.12	1.70	0.90	1.33	1.09	1.04
	1.07	1.24	1.00	1.16	1.05	1.34
	0.95	1.14	0.94	0.94	0.61	1.22
	0.89	0.96	0.80	0.97	0.83	0.83
Mean	1.00	1.34	0.90	1.18	0.92	1.17
SEM	0.04	0.15	0.04	0.11	0.09	0.11

Astrocytes	Control	Aβ	NA 1 µM	NA 1µM +Aβ	NA 10 µM	NA 10µM +Aβ
IL-1β	0.99	6.08	2.47	6.51	1.90	2.56
	1.46	7.45	2.85	6.54	2.19	2.91
	0.98	5.33	3.52	6.32	3.16	2.09
	0.88		1.44		0.80	1.98
	0.69		1.70		1.40	
Mean	1.00	6.29	2.40	6.46	1.89	2.39
SEM	0.13	0.62	0.38	0.07	0.40	0.21
TNFα	1.11	5.50	1.57	6.62	1.00	2.68
	0.95	7.19	1.84	4.09	1.22	3.29
	0.88	5.96	1.74	4.65	1.03	2.80
	0.76		0.65		0.50	2.96
	1.30		1.06		0.95	
Mean	1.00	6.22	1.37	5.12	0.94	2.93
SEM	0.09	0.50	0.23	0.76	0.12	0.13
CD40	0.81	1.23	0.88	1.29	0.86	2.00
	1.07	1.38	1.00	1.43	1.00	1.94
	0.78	1.30	0.94	1.14	1.22	1.92
	0.97	0.92	0.74	0.84	0.93	1.48
	1.37	0.96	0.92	1.07	1.13	2.77
Mean	1.00	1.16	0.90	1.15	1.03	2.02
SEM	0.11	0.09	0.04	0.10	0.07	0.21

Microglia	Con	LPS	NA 0.1µM	NA 0.1 +LPS	NA 1µM	NA 1 +LPS	NA 10µM	NA 10 +LPS
iNOS	1.34	28.63	1.37	43.49	2.17	54.50	2.12	36.27
	0.65	33.16	0.90	33.98	1.66	40.24	1.37	29.33
	0.80	28.18	1.34	45.02	1.38	44.10	1.36	32.42
	0.98	25.52	1.66	31.91	1.44	31.67	1.32	31.07
	1.22	27.94	1.91	33.44	2.25	36.43	1.75	27.60
		20.18	0.82	34.40	1.29	40.87	1.39	19.55

Mean	1.00	27.27	1.33	37.04	1.70	41.30	1.55	29.37
SEM	0.13	1.74	0.17	2.32	0.17	3.16	0.13	2.31
IL-1β	1.87	23.39	2.02	25.33	0.76	28.97	1.05	23.71
	0.99	28.53	1.11	20.98	0.63	21.51	0.72	19.72
	0.46	14.59	1.14	14.77	1.10	17.45	0.63	14.19
	0.66	13.97	1.76	16.31	1.03	17.72	0.79	16.58
	1.27	14.77	2.06	15.45	0.94	13.38	0.54	13.24
	0.76	15.76	1.21	17.43		15.88	0.70	14.61
Mean	1.00	18.50	1.55	18.38	0.89	19.15	0.74	17.01
SEM	0.21	2.46	0.18	1.65	0.09	2.24	0.07	1.64
TNFα	0.95	10.93	1.07	15.90	0.86	12.54	0.94	10.29
	0.96	14.55	1.10	14.79	1.07	22.77	1.20	20.46
	1.01	12.06	1.44	15.65	0.99	18.82	0.77	10.92
	0.88	13.16	1.53	15.77	1.10	16.36	0.70	14.67
	1.07	16.61	1.20	18.66	1.51	16.11	0.82	15.51
	1.13	13.74	0.97	17.48	0.78	13.14	0.55	11.30
Mean	1.00	13.51	1.22	16.37	1.05	16.62	0.83	13.86
SEM	0.04	0.81	0.09	0.58	0.10	1.55	0.09	1.58
CD40	1.24	19.09	1.17	30.82	0.91	25.01	1.07	22.16
	0.96	27.55	1.09	25.51	1.14	23.25	1.14	18.03
	0.95	17.42	1.16	20.05	0.75	19.22	0.79	14.23
	0.87	21.42	1.25	24.55	1.07	20.71	1.11	20.87
	1.07	20.79	1.17	26.31	1.14	12.55	0.87	12.35
	0.91	17.79	0.80	17.17	1.04	18.69	1.04	14.47
Mean	1.00	20.68	1.11	24.07	1.01	19.90	1.00	17.02
SEM	0.05	1.52	0.06	1.97	0.06	1.77	0.06	1.62

Astrocytes	**Con**	**LPS**	**NA 0.1µM**	**NA 0.1 +LPS**	**NA 1µM**	**NA 1 +LPS**	**NA 10µM**	**NA 10 +LPS**
iNOS	1.52	95.24	0.57	119.14	1.43	128.76	1.33	108.76
	0.76	63.05	0.19	102.38	1.05	136.10	3.81	93.24

	1.62	90.00	1.14	120.48	3.90	130.38	4.48	85.14
	0.95	84.86	1.52	100.76	3.81	117.52	2.19	94.86
	0.48	45.14		123.71	1.71	190.48	1.43	176.48
	0.67	86.48		162.86	2.10	169.81	0.29	95.43
Mean	1.00	77.46	0.86	121.60	2.33	145.50	2.25	109.00
SEM	0.19	7.88	0.30	9.16	0.50	11.54	0.65	13.85
IL-1β	1.12	24.90	1.77	25.79	2.02	24.05	1.22	24.90
	0.62	16.78	0.70	21.66	1.47	33.39	0.92	18.42
	0.75	17.73	1.10	26.17	1.84	30.65	1.89	21.06
	0.97	20.76	1.79	28.53	3.04	23.60	1.32	17.45
	1.52	14.22		32.34	1.39	28.71	1.47	25.89
	1.02	20.56		28.76	1.89	24.95	0.90	21.93
Mean	1.00	19.16	1.34	27.21	1.94	27.56	1.29	21.61
SEM	0.13	1.52	0.27	1.47	0.24	1.63	0.15	1.38
TNFα	1.51	35.09	1.30	44.42	1.02	36.95	1.47	29.40
	1.19	30.46	0.70	34.04	1.47	46.07	1.23	28.74
	0.74	35.09	1.40	46.81	1.82	41.05	1.23	28.32
	0.63	34.39	2.00	50.60	2.11	44.74	0.49	30.95
	1.02	18.67		50.18	1.23	66.74	1.82	47.72
	0.91	38.28		59.19	1.37	52.14	1.09	30.74
Mean	1.00	31.99	1.35	47.54	1.50	47.95	1.22	32.64
SEM	0.13	2.85	0.27	3.39	0.16	4.29	0.18	3.05
CD40	0.74	27.52	0.69	24.39	0.52	24.03	1.24	20.20
	0.99	20.04	0.63	20.83	0.99	25.84	2.01	19.49
	1.32	22.68	0.85	26.53	1.07	23.81	1.54	19.60
	0.99	19.43	0.00	21.96	1.27	23.75	1.32	24.88
	0.74	9.66	1.13	27.25	0.80	25.43	1.40	30.52
	1.21	25.38		28.10	1.05	26.81	0.91	24.41
Mean	1.00	20.78	0.66	24.84	0.95	24.94	1.40	23.18
SEM	0.10	2.56	0.19	1.21	0.11	0.52	0.15	1.77

Raw PCR data from Section 3.7:

	β2-AR			***β-actin***		
	Mixed glia	**Astrocytes**	**Microglia**	**Mixed glia**	**Astrocytes**	**Microglia**
	2.43	1.00	0.30	15.91	16.44	15.66
	3.26	0.80	0.21	16.60	16.10	17.37
	1.93	1.18	0.27	16.41	16.96	15.92
	2.54	1.04	0.32	16.11	17.54	15.73
	4.01	0.94	0.42	16.54	16.39	15.65
	2.68			16.58		
Mean	2.81	0.99	0.30	16.36	16.68	16.07
SEM	0.30	0.06	0.03	0.12	0.25	0.33

Raw data from Section 3.8: Alamar Blue Cell Viability Assay

Mixed Glia	**Control**	**LPS**	**Thrombin**	**Aβ**
	120.98	109.84	108.07	110.94
	88.94	131.10	98.98	102.81
	91.33	117.72	101.85	109.98
	86.07	114.57	84.16	81.77
	98.98	82.39	86.07	80.34
	111.90	90.15	88.94	95.64
	111.90	99.84	87.99	113.81
	89.90	83.68	58.82	97.07
Mean	100.00	103.70	89.36	99.04
SEM	4.66	6.21	5.30	4.55

Microglia	**Control**	**LPS**	**Thrombin**	**Aβ**
	101.26	87.33	87.38	98.53
	101.77	102.97	98.85	100.41
	100.32	100.58	94.52	97.50
	96.65	99.21	94.10	96.90
Mean	100.00	97.52	93.71	98.33
SEM	1.16	3.49	2.37	0.77

Astrocytes	Control	LPS	Thrombin	Aβ
	100.22	99.26	98.78	96.99
	101.66	100.70	100.54	99.06
	101.58	101.02	101.18	99.22
	96.54	98.54	99.74	96.28
Mean	100.00	99.88	100.10	97.89
SEM	1.20	0.59	0.52	0.74

Mixed Glia	Control	NA 0.1 µM	NA 1 µM	NA 10 µM
	97.70	111.84	107.24	107.58
	104.51	113.71	112.86	104.51
	97.36	89.87	88.16	74.88
	94.64	96.68	104.34	100.60
	100.43	102.30	107.58	104.68
	105.37	107.92	98.38	67.39
Mean	100.00	103.70	103.10	93.27
SEM	1.74	3.77	3.56	7.13

Mixed Glia	Control	Salbutamol	Salmeterol	db-cAMP
	72.05	51.58	119.29	111.42
	69.69	41.34	144.49	125.59
	109.84	46.85	135.04	137.40
	65.75	73.62	123.23	113.78
	141.34	126.38	112.21	109.06
	141.34	118.50	129.53	76.77
Mean	100.00	76.38	127.30	112.30
SEM	14.59	15.27	4.72	8.33

Raw PCR data from Section 3.9:

	Control	Thrombin	Salbutamol	Thrombin+ Salbutamol
iNOS	0.40	1067.28	0.75	597.44
	0.28	1111.15	5.73	759.69

	1.36	1444.07	13.90	2325.33
	1.68	2211.08	10.02	3015.09
	0.72	1201.20	17.36	2073.23
	1.58	657.18	4.90	1832.16
Mean	1.00	1282.00	8.78	1767.00
SEM	0.25	213.00	2.52	380.80
IL-1β	0.79	61.93	1.25	60.71
	0.90	69.41	0.90	60.98
	0.78	79.59	0.70	98.38
	1.32	89.26	1.07	113.64
	1.04	72.55	1.12	131.30
	1.17	35.47	0.79	126.42
Mean	1.00	68.03	0.97	98.57
SEM	0.09	7.54	0.09	12.81
TNFα	0.55	12.34	0.29	6.53
	0.86	17.22	0.30	4.38
	0.90	15.01	0.47	4.55
	1.14	6.51	0.59	4.74
	0.88	14.83	0.49	8.26
	1.68	25.35	0.41	6.25
Mean	1.00	15.21	0.43	5.79
SEM	0.16	2.52	0.05	0.62
CD40	0.82	61.65	1.02	44.05
	1.11	70.30	0.61	37.85
	0.83	82.15	0.75	60.68
	0.94	57.91	0.62	57.53
	0.92	72.06	1.12	93.75
	1.38	58.65	0.62	85.87
Mean	1.00	67.12	0.79	63.29
SEM	0.09	3.86	0.09	9.12

VCAM-1	0.68	9.27	0.64	1.95
	0.67	16.65	0.71	3.56
	1.00	17.02	1.16	4.66
	1.31	10.11	0.95	7.12
	0.98	16.31	0.95	4.98
	1.35	9.20	0.91	5.78
Mean	1.00	13.09	0.89	4.68
SEM	0.12	1.60	0.08	0.73
IP-10	0.78	99.94	0.68	16.52
	1.46	128.42	0.72	33.34
	0.86	147.96	0.95	91.23
	0.83	124.99	0.69	129.68
	1.06	154.89	1.40	138.81
		187.82	0.91	88.75
Mean	1.00	140.70	0.89	83.06
SEM	0.13	12.29	0.11	20.23

	Aβ	**Salbutamol**		**Aβ**	**Salbutamol**
IL-1β	6.38	40.08	***TNFα***	4.42	9.95
	7.01	30.73		5.58	3.32
	11.64	36.58		5.14	10.71
	7.84	54.34		4.13	10.55
	1.79			4.69	
	6.49				
Mean	6.86	40.43	Mean	4.79	8.63
SEM	1.29	5.02	SEM	0.26	1.78

Raw PCR data from Section 3.10:

	Throm	**Salm 0.1 +Throm**	**Salm 1 +Throm**	**Salm 10 +Throm**	**Xam 0.1 +Throm**	**Xam 1 +Throm**	**Xam 10 +Throm**
iNOS	0.59	0.82	1.03	0.45	2.04	1.73	0.65
	0.83	1.22	1.11	0.63	2.24	2.04	0.98

	0.92	1.43	1.18	0.68	2.29	2.11	1.08
	1.09	1.61	1.61	0.75	2.30	2.27	1.26
	1.10	1.73	1.62	0.86	2.40	2.30	1.68
	1.48	1.97	1.85	0.86	2.42	2.43	1.68
Mean	1.00	1.46	1.40	0.71	2.28	2.15	1.22
SEM	0.12	0.17	0.14	0.06	0.06	0.10	0.17
IL-1β	0.88	0.79	1.00	0.93	1.31	1.35	1.23
	1.00	0.67	1.00	0.95	1.44	1.21	1.52
	1.30	1.31	1.14	1.14	1.56	1.45	1.68
	0.88	0.98	0.68	0.79	1.25	1.37	1.55
	1.21	0.99	1.08	0.96	1.51	1.61	1.95
	0.73	1.28	1.47	1.05	1.47	1.37	1.03
Mean	1.00	1.00	1.06	0.97	1.42	1.40	1.49
SEM	0.09	0.10	0.10	0.05	0.05	0.05	0.13
TNFα	1.27	0.37	0.34	0.30	0.91	0.87	0.89
	1.12	0.26	0.30	0.30	1.05	0.75	1.47
	1.20	0.31	0.47	0.38	0.63	0.74	1.16
	0.80	0.42	0.32	0.24	0.76	0.77	1.13
	0.96	0.38	0.51	0.33	0.83	1.09	1.28
	0.66	0.44	0.35	0.36	0.66	0.66	0.96
Mean	1.00	0.36	0.38	0.32	0.81	0.82	1.15
SEM	0.10	0.03	0.03	0.02	0.06	0.06	0.09
CD40	1.00	0.31	0.41	0.38	1.06	0.94	0.85
	1.30	0.36	0.43	0.49	1.47	1.22	1.80
	1.63	0.38	0.40	0.59	0.93	1.04	1.60
	0.47	0.48	0.23	0.34	0.69	0.84	0.93
	0.94	0.24	0.21	0.19	0.79	0.95	0.99
	0.66	0.42	0.30	0.39	0.63	0.68	0.39
Mean	1.00	0.37	0.33	0.40	0.93	0.95	1.09
SEM	0.17	0.03	0.04	0.05	0.12	0.07	0.21

VCAM-1	1.10	0.36	0.65	0.33	1.16	1.13	0.82
	1.41	0.70	0.85	0.37	1.59	1.43	0.85
	1.38	1.20	0.54	0.36	1.81	1.55	0.76
	0.73	0.57	0.17	0.17	0.98	1.28	0.93
	0.85	0.56	0.30	0.08	1.05	0.75	0.84
	0.54	0.87	1.01	0.38	1.46	1.40	0.17
Mean	1.00	0.71	0.59	0.28	1.34	1.26	0.73
SEM	0.14	0.12	0.13	0.05	0.13	0.12	0.11
IP-10	1.24	0.76	0.91	0.23	1.88	1.59	0.89
	1.26	0.62	0.81	0.14	2.45	2.04	1.07
	1.29	1.01	0.91	0.11	2.17	1.85	0.94
	0.89	1.01	0.56	0.13	1.26	1.66	1.02
	0.88	0.73	0.62	0.06	1.62	1.47	0.92
	0.44	0.72	0.60	0.19	1.76	1.85	0.38
Mean	1.00	0.81	0.73	0.14	1.86	1.74	0.87
SEM	0.14	0.07	0.07	0.02	0.17	0.09	0.10

	Aβ	**Salm 0.1 +Aβ**	**Salm 1 +Aβ**	**Salm 10 +Aβ**	**Xam 0.1 +Aβ**	**Xam 1 +Aβ**	**Xam 10 +Aβ**
IL-1β	5.09	59.78	83.70	2.99	26.96	110.80	15.29
	6.91	41.58	32.59	6.22	74.96	150.65	3.91
	7.77	44.34	35.12	6.12	95.56	43.71	1.62
	2.31	10.13	44.05	5.45	61.80	67.81	41.27
	4.84	53.30	47.73		37.88	48.92	6.10
			50.47		47.20	55.76	30.19
Mean	5.38	41.83	48.94	5.20	57.39	79.61	16.40
SEM	0.95	8.56	7.52	0.76	10.32	17.28	6.56
TNFα	1.21	9.45	6.81	2.31	1.94	3.10	2.31
	1.06	4.61	1.76	4.65	6.42	2.32	0.89
	1.62	8.62	7.10	0.51	10.73	3.63	1.11
	3.13	3.61	2.35	1.46	3.78	4.82	8.32
	1.63	8.28	3.34	1.97	18.72		2.95

	3.52		7.63				
Mean	2.03	6.92	4.83	2.18	8.32	3.47	3.12
SEM	0.42	1.17	1.08	0.69	2.99	0.52	1.36

	Con	Salm 0.1	Salm 1	Salm 10	Xam 0.1	Xam 1	Xam 10
iNOS	0.23	0.43	0.42	1.65	0.76	1.55	2.46
	0.37	0.57	0.99	1.37	0.63	2.15	3.39
	1.39	1.39	0.70	2.01	0.91	1.40	5.21
	1.46	2.76	1.05	1.88	1.74	2.75	3.06
	1.55	0.51	1.60	1.26	0.55	1.17	4.23
		0.54	0.57	1.86	1.02	0.99	
Mean	1.00	1.03	0.89	1.67	0.94	1.67	3.67
SEM	0.29	0.37	0.17	0.12	0.18	0.27	0.48
IL-1β	0.67	1.02	1.00	0.89	1.13	1.36	1.14
	1.04	0.92	1.26	1.10	1.62	1.92	1.37
	1.11	1.32	1.05	0.96	1.39	1.20	2.87
	1.27	1.27	1.04	0.60	1.45	1.62	1.46
	1.12	1.18	0.90	0.82	1.32	1.26	3.72
	0.80	1.13	1.41	1.22	2.26	2.25	1.02
Mean	1.00	1.14	1.11	0.93	1.53	1.60	1.93
SEM	0.09	0.06	0.08	0.09	0.16	0.17	0.45
TNFα	0.42	0.32	0.40	0.33	1.15	1.30	1.85
	0.99	0.44	0.56	0.56	1.86	1.90	1.13
	1.15	0.97	1.08	0.82	1.62	1.45	1.40
	1.36	0.96	0.71	0.32	2.02	2.38	3.91
	1.56	0.57	0.68	0.31	1.41	1.51	
	0.53	0.57	0.70	0.51	1.35	2.18	
Mean	1.00	0.64	0.69	0.47	1.57	1.79	2.07
SEM	0.19	0.11	0.09	0.08	0.13	0.18	0.63
CD40	0.79	0.34	0.30	0.86	0.85	0.89	1.47
	1.09	0.28	0.33	0.79	0.66	1.08	1.63

	1.13	0.33	0.39	0.59	0.71	0.59	2.18
	0.98	0.31	0.27	0.47	0.99	0.91	1.34
	0.99	0.30	0.28	0.49	0.67	0.59	1.96
	1.01	0.36	0.39	0.67	0.60	0.62	0.48
Mean	1.00	0.32	0.33	0.65	0.75	0.78	1.51
SEM	0.05	0.01	0.02	0.06	0.06	0.08	0.24
VCAM-1	0.67	0.85	0.77	0.71	0.67	1.12	0.98
	0.81	0.95	0.90	0.77	1.25	1.21	1.17
	0.92	0.96	0.92	0.77	1.30	1.21	1.20
	1.07	0.97	1.02	0.80	1.36	1.40	1.59
	1.26	1.10	1.13	0.87	1.39	1.57	1.61
	1.27	1.32	1.25	1.09	1.51	1.64	2.43
Mean	1.00	1.02	1.00	0.84	1.25	1.36	1.50
SEM	0.10	0.07	0.07	0.06	0.12	0.09	0.21
IP-10	0.47	0.46	0.59	0.80	1.04	0.77	1.60
	0.87	0.53	0.46	1.16	0.84	1.20	1.70
	1.30	0.60	0.43	0.66	0.81	0.85	4.31
	1.46	0.45	0.52	0.61	1.00	1.03	1.85
	1.49	0.39	0.48	0.97	0.61	0.92	3.69
	0.41	0.46	0.63	0.62	0.57	0.55	1.18
Mean	1.00	0.48	0.52	0.80	0.81	0.89	2.39
SEM	0.20	0.03	0.03	0.09	0.08	0.09	0.52

Raw PCR data from Section 3.11:

	Control	Thrombin	dbcAMP 100 µM	Throm+ dbcAMP 100 µM	dbcAMP 500 µM	Throm+ dbcAMP 500 µM
IL-1β	0.79	61.93	1.54	61.34	2.54	64.69
	0.90	69.41	1.16	60.96	1.66	73.27
	0.78	79.59	1.36	63.88	1.86	74.47
	1.32	89.26	2.14	114.76	4.12	122.45
	1.04	72.55	1.64	82.09	2.00	78.41

	1.17	35.47	1.32	62.24	1.73	78.08
Mean	1.00	68.03	1.53	74.21	2.32	81.89
SEM	0.09	7.54	0.14	8.75	0.38	8.36
TNFα	0.55	12.34	0.13	1.95	0.15	1.24
	0.86	17.22	0.11	1.63	0.11	1.65
	0.90	15.01	0.17	1.39	0.17	1.34
	1.14	6.51	0.24	2.63	0.24	1.96
	0.88	14.83	0.17	2.54	0.11	1.89
	1.68	25.35	0.12	1.36	0.15	1.44
Mean	1.00	15.21	0.16	1.92	0.16	1.59
SEM	0.16	2.52	0.02	0.23	0.02	0.12
CD40	0.82	61.65	0.39	39.58	0.73	36.13
	1.11	70.30	0.53	39.70	0.44	35.77
	0.83	82.15	0.50	36.44	0.87	40.09
	0.94	57.91	0.67	57.16	0.85	38.65
	0.92	72.06	0.54	33.41	0.66	49.96
	1.38	58.65	0.52		0.52	
Mean	1.00	67.12	0.53	41.26	0.68	40.12
SEM	0.09	3.86	0.04	4.14	0.07	2.59
VCAM-1	0.68	9.27	0.57	7.40	0.63	5.38
	0.67	16.65	0.58	6.40	0.48	5.15
	1.00	17.02	0.77	7.18	0.72	5.41
	1.31	10.11	0.94	6.47	0.51	6.23
	0.98	16.31	1.05	7.58	0.85	6.27
	1.35	9.20	0.67	8.30	0.62	5.15
Mean	1.00	13.09	0.76	7.22	0.63	5.60
SEM	0.12	1.60	0.08	0.29	0.06	0.21
IP-10	0.78	99.94	1.52	89.58	1.42	67.07
	1.46	128.42	1.54	97.59	0.63	86.84
	0.86	147.96	1.40	98.07	1.69	109.78

	0.83	124.99	2.36	175.77	1.08	162.95
	1.06	154.89	1.02	134.20	1.70	110.01
		187.82	1.79	117.61	1.53	108.28
Mean	1.00	140.70	1.60	118.80	1.34	107.50
SEM	0.13	12.29	0.18	13.19	0.17	13.10

	Thrombin	**db-cAMP +Throm**	**BNZ +Throm**	**pCPT +Throm**
IL-1β	1.04	1.15	1.60	0.19
	1.23	0.86	0.99	0.19
	0.94	0.84	1.04	0.11
	0.73	1.53	1.08	0.71
	1.30	1.34	1.14	0.49
	0.77	1.19	1.10	0.26
Mean	1.00	1.15	1.16	0.32
SEM	0.10	0.11	0.09	0.09
TNFα	0.88	0.14	0.22	0.18
	1.58	0.09	0.15	0.18
	0.89	0.07	0.15	0.14
	0.94	0.13	0.15	0.53
	1.11	0.11	0.16	0.41
	0.60	0.11	0.16	0.19
Mean	1.00	0.11	0.16	0.27
SEM	0.13	0.01	0.01	0.07
CD40	1.03	0.62	0.90	0.27
	1.12	0.35	0.52	0.37
	0.96	0.49	0.54	0.32
	0.88	0.57	0.75	0.77
	1.12	0.51	0.72	0.58
	0.89	0.50	0.69	0.43
Mean	1.00	0.50	0.69	0.46
SEM	0.04	0.04	0.06	0.08

VCAM-1	0.84	0.25	0.40	0.07
	1.15	0.28	0.27	0.06
	0.89	0.36	0.16	0.09
	1.01	0.20	0.13	0.13
	1.43	0.33	0.44	0.18
	0.67	0.15	0.25	0.05
Mean	1.00	0.26	0.28	0.10
SEM	0.11	0.03	0.05	0.02
IP-10	1.04	0.37	0.71	0.01
	1.01	0.15	0.43	0.04
	1.00	0.41	0.16	0.05
	0.58	0.13	0.23	0.14
	1.47	0.32	0.73	0.12
	0.90	0.12	0.32	0.03
Mean	1.00	0.25	0.43	0.06
SEM	0.12	0.05	0.10	0.02

	Aβ	**dbcAMP +Aβ**	**BNZ +Aβ**	**pCPT +Aβ**
IL-1β	6.38	31.01	19.53	14.09
	7.01	23.64	24.96	5.00
	11.64	54.39	30.36	22.18
	7.84	28.52	10.99	34.24
	1.79	25.00	28.43	6.36
	6.49			7.14
Mean	6.86	32.51	22.85	14.83
SEM	1.29	5.62	3.49	4.69
TNFα	4.42	0.92	0.37	5.70
	5.58	2.94	1.07	1.69
	5.14	0.75	1.35	2.93
	4.13	0.84	2.27	8.54
	1.19	1.33	0.71	3.87

	4.69	0.25	2.09	
Mean	4.19	1.17	1.31	4.54
SEM	0.64	0.38	0.31	1.19

Raw PCR data from Section 3.12:

	Thrombin	**Salm +Throm**	**SQ22536 +Throm**	**SQ22536 +Salm +Throm**	**KT5720 +Throm**	**KT5720 +Salm +Throm**
TNFα	1.01	0.30	0.90	0.27	1.36	0.46
	1.25	0.44	1.37	0.28	1.50	0.34
	0.99	0.37	0.85	0.24	1.30	0.39
	1.02	0.36	0.86	0.27	1.22	0.52
	0.95	0.30	1.02	0.31	1.22	0.40
	0.77		0.66	0.23	0.75	0.45
Mean	1.00	0.35	0.94	0.27	1.22	0.43
SEM	0.06	0.02	0.10	0.01	0.10	0.03
CD40	1.20	0.54	1.38	0.77	1.57	0.61
	1.19	0.93	1.35	0.81	1.41	0.53
	1.16	0.55	1.00	0.45	1.35	0.44
	0.93	0.56	0.91	0.52	1.00	0.44
	0.73	0.48	0.96	0.55	0.61	0.43
	0.79		0.82	0.50	0.88	0.62
Mean	1.00	0.61	1.07	0.60	1.14	0.51
SEM	0.09	0.08	0.10	0.06	0.15	0.04
VCAM-1	1.16	0.93	1.45	0.65	1.05	0.20
	1.00	0.71	1.24	0.53	1.08	0.17
	1.02	0.58	1.10	0.55	1.01	0.13
	0.68	0.44	1.02	0.45	0.61	0.16
	0.83	0.89	1.08	0.48	1.14	0.18
	1.31		1.43	0.82	1.32	0.25
Mean	1.00	0.71	1.22	0.58	1.04	0.18
SEM	0.09	0.09	0.08	0.06	0.10	0.02

IP-10	1.35	0.83	1.48	0.92	1.29	0.04
	1.11	0.75	1.26	0.39	0.76	0.03
	0.96	0.42	1.26	0.37	1.03	0.02
	0.82	0.50	0.98	0.41	0.69	0.04
	0.69	0.64	0.79	0.41	0.63	0.03
	1.07		1.55	0.82	1.30	0.09
Mean	1.00	0.63	1.22	0.55	0.95	0.04
SEM	0.10	0.08	0.12	0.10	0.12	0.01

Raw PCR data from Section 3.13:

	Control	Thrombin	NA+ Throm	Prop	Prop+ Throm	Prop+NA +Throm
TNFα	1.00	16.35	12.08	1.67	24.73	14.35
	1.05	17.85	8.53	2.95	28.55	10.34
	1.34	16.84	10.85	2.14	21.62	17.48
	0.87	27.63	12.18	1.13	22.44	14.76
	0.57	16.97	11.33	0.73	25.73	15.70
	1.17	19.06	10.07	1.02	16.93	12.79
Mean	1.00	19.11	10.84	1.61	23.33	14.24
SEM	0.11	1.75	0.56	0.34	1.63	1.00
CD40	1.08	63.79	61.55	1.54	77.89	45.37
	1.46	63.89	49.42	1.62	96.38	36.06
	1.06	95.09	75.42	1.44	93.55	49.63
	0.86	78.03	46.49	0.65	71.28	37.11
	0.55	42.23	44.11	0.67	73.23	43.53
	0.99	55.54	37.64	0.72	49.62	32.38
Mean	1.00	66.43	52.44	1.10	76.99	40.68
SEM	0.12	7.47	5.61	0.19	6.95	2.67
VCAM-1	1.25	19.49	10.95	2.76	19.60	10.46
	1.43	21.28	13.34	2.51	20.55	9.01
	1.23	16.52	15.23	2.07	16.68	3.01
	0.64	18.10	12.28	0.49	16.36	5.72

	0.58	8.77	10.50	0.68	14.17	6.69
	0.86	12.69	5.72	0.90	11.86	4.24
Mean	1.00	16.14	11.34	1.57	16.54	6.52
SEM	0.14	1.90	1.32	0.41	1.33	1.15
IP-10	1.49	57.46	58.87	2.02	59.84	26.36
	1.70	57.66	55.07	3.57	75.60	37.10
	0.99	62.02	70.45	2.40	65.38	21.67
	0.60	62.02	50.82	0.35	60.70	25.12
	0.49	52.54	41.06	0.39	55.41	19.70
	0.73	60.21	43.05	0.63	42.81	15.94
Mean	1.00	58.65	53.22	1.56	59.96	24.32
SEM	0.20	1.47	4.43	0.54	4.43	2.98

	Aβ	**NA +Aβ**	**Prop +Aβ**	**Prop+ NA+Aβ**
IL-1β	1.11	0.58	1.66	0.87
	0.84	0.84	0.45	1.57
	0.76	0.47	0.80	1.82
	1.52	0.88	0.99	2.09
	0.57	0.37	0.96	0.97
	1.20	1.65	0.42	0.94
Mean	1.00	0.80	0.88	1.38
SEM	0.14	0.19	0.19	0.21
TNFα	1.09	0.17	0.56	0.36
	0.62	0.40	0.20	0.28
	0.85	0.14	0.63	0.51
	1.64	0.22	0.91	1.45
	0.51	0.24	0.34	0.10
	1.30	0.34	0.20	0.22
Mean	1.00	0.25	0.47	0.29
SEM	0.17	0.04	0.11	0.07

Raw PCR data from Section 3.14:

	Thrombin	NA +Throm	Met +Throm	Met+NA +Throm	But +Throm	But+NA +Throm
TNFα	1.03	0.06	0.83	0.23	0.32	0.13
	0.84	0.08	1.07	0.19	0.83	0.12
	0.82	0.09	0.85	0.22	0.76	0.19
	0.92	0.07	0.79	0.23	0.48	0.16
	1.40	0.04	1.34	0.25	0.57	0.15
			0.94	0.25	0.62	0.16
Mean	1.00	0.07	0.97	0.23	0.60	0.15
SEM	0.11	0.01	0.08	0.01	0.07	0.01
CD40	1.05	0.46	1.26	0.67	0.71	0.53
	0.75	0.44	1.33	0.68	1.23	0.54
	1.14	0.63	1.31	0.55	1.14	0.56
	0.75	0.45	0.93	0.60	0.75	0.54
	1.31	0.21	1.11	0.68	0.27	0.48
			1.56	0.66	0.98	0.61
Mean	1.00	0.44	1.25	0.64	0.85	0.54
SEM	0.11	0.07	0.09	0.02	0.14	0.02
VCAM-1	1.00	0.32	2.83	0.91	1.67	0.73
	0.88	0.27	2.92	1.14	2.33	0.43
	1.53	0.34	2.95	1.24	2.59	0.49
	0.82	0.30	2.09	0.88	1.32	0.42
	0.42	0.19	2.38	0.91	2.52	0.39
	1.36	0.45	2.89	1.33	2.01	0.55
Mean	1.00	0.31	2.68	1.07	2.07	0.50
SEM	0.16	0.03	0.15	0.08	0.20	0.05
IP-10	1.06	0.04	3.40	1.17	2.21	0.82
	0.53	0.04	3.44	0.92	3.42	0.44
	1.27	0.06	3.43	0.92	3.53	0.63
	0.77	0.03	2.81	0.85	1.56	0.34

	1.37	0.01	2.90	0.80	2.47	0.39
		0.08	3.96	1.11	3.47	0.64
Mean	1.00	0.04	3.32	0.96	2.78	0.54
SEM	0.16	0.01	0.17	0.06	0.33	0.08

Chapter 4

Raw PCR data from Section 4.4:

	Control	Salbutamol		Control	dbcAMP
IL-1R1	1.77	0.86	***IL-1R1***	1.12	0.77
	1.06	0.67		1.08	0.59
	0.97	0.95		1.22	0.70
	0.57	0.44		0.81	0.52
	0.63	0.62		0.76	0.57
		0.77		1.01	0.52
Mean	1.00	0.72	Mean	1.00	0.61
SEM	0.21	0.08	SEM	0.07	0.04
IL-1R2	1.73	35.23	***IL-1R2***	1.50	71.88
	0.19	18.02		1.04	52.06
	1.58	8.04		0.76	36.78
	0.50	56.50		0.71	28.30
		20.76			24.48
		30.55			
Mean	1.00	28.18	Mean	1.00	42.70
SEM	0.39	6.88	SEM	0.18	8.70
TNFR1	1.61	1.12	***TNFR1***	1.28	1.36
	0.53	1.18		1.09	1.27
	1.11	0.83		1.21	1.08
	0.88	1.09		0.88	1.16
	0.86	0.75		0.72	0.77
		0.66		0.82	0.84
Mean	1.00	0.94	Mean	1.00	1.08
SEM	0.18	0.09	SEM	0.09	0.10

TNFR2	1.15	3.58	***TNFR2***	1.19	2.41
	0.65	3.26		1.14	3.42
	1.52	1.20		1.61	1.71
	0.98	5.02		1.16	1.71
	0.70	1.60		0.39	1.28
		2.80		0.50	0.90
Mean	1.00	2.91	Mean	1.00	1.91
SEM	0.16	0.57	SEM	0.19	0.37
IFNgR1	1.43	1.03	***IFNgR1***	1.24	0.91
	0.94	1.06		1.02	0.84
	0.99	0.65		1.43	0.93
	0.74	1.11		0.96	0.98
	0.90	1.10		0.70	0.58
		0.88		0.65	0.68
Mean	1.00	0.97	Mean	1.00	0.82
SEM	0.12	0.07	SEM	0.12	0.06
IFNgR2	1.17	1.89	***IFNgR2***	0.95	1.97
	0.87	1.33		1.14	3.24
	1.39	1.01		1.53	1.83
	0.51	2.66		1.00	2.35
	1.07	1.30		0.71	1.14
		1.98		0.68	1.03
Mean	1.00	1.69	Mean	1.00	1.93
SEM	0.15	0.25	SEM	0.13	0.33

	Control	**Salmeterol 1 µM**	**Salmeterol 10 µM**	**Xamoterol 1 µM**	**Xamoterol 10 µM**
IL-1R1	1.77	0.89	0.61	1.15	1.57
	1.06	0.66	1.21	0.89	1.07
	0.97	0.83	1.70	1.26	0.43
	0.57	0.73	0.56	0.59	0.47
	0.63	1.01	0.73	1.06	1.04

		0.72	0.78		
Mean	1.00	0.81	0.93	0.99	0.92
SEM	0.21	0.05	0.18	0.12	0.21
IL-1R2	1.73	20.55	85.21	19.77	15.19
	0.19	31.18	14.25	0.97	1.27
	1.58	50.77	19.19	15.64	7.34
	0.50	24.79	21.03	2.64	8.49
		10.39	3.94		
		29.31	35.92		
Mean	1.00	27.83	29.92	9.76	8.07
SEM	0.39	5.50	11.84	4.68	2.85
TNFR1	1.61	1.10	1.14	1.72	1.64
	0.53	0.96	1.59	1.19	1.11
	1.11	1.75	1.22	0.48	0.83
	0.88	0.71	0.64	1.26	0.93
	0.86	1.39	0.36	0.87	1.61
		0.69	0.50		
Mean	1.00	1.10	0.91	1.10	1.22
SEM	0.18	0.17	0.20	0.21	0.17
TNFR2	1.15	4.69	2.54	6.69	4.23
	0.65	2.18	3.34	1.39	1.32
	1.52	1.91	1.88	1.35	1.33
	0.98	3.32	1.21	3.09	2.58
	0.70	1.81	2.08	3.05	5.14
				2.49	2.55
Mean	1.00	2.78	2.21	3.01	2.86
SEM	0.16	0.55	0.35	0.80	0.63
IFNgR1	1.43	1.17	0.98	0.92	1.36
	0.94	1.07	0.81	0.83	1.17
	0.99	0.69	0.82	0.88	1.07

	0.74	0.81	0.71	0.94	0.59
	0.90	0.85	0.63	0.49	1.35
		0.68		1.11	1.32
Mean	1.00	0.88	0.79	0.86	1.15
SEM	0.12	0.08	0.06	0.08	0.12
IFNgR2	1.17	1.64	1.10	0.91	1.02
	0.87	1.35	1.07	0.80	0.79
	1.39	1.35	1.09	1.71	0.64
	0.51	0.71	0.71	0.42	0.82
	1.07	1.30	0.74	0.38	1.22
Mean	1.00	1.27	0.94	0.84	0.90
SEM	0.15	0.15	0.09	0.24	0.10

Raw PCR data from Section 4.7:

	Control	**IL-1β**	**TNFα**	**IFNγ**
IL-1R1	1.10	1.40	1.47	1.20
	1.12	2.09	1.51	0.75
	0.85	0.89	1.77	0.68
	0.78	1.15	1.42	0.69
	0.86	1.32	1.32	1.65
	1.29			0.57
Mean	1.00	1.37	1.50	0.92
SEM	0.08	0.20	0.08	0.17
IL-1R2	1.14	0.02	0.67	0.80
	0.85	1.51	1.02	0.04
	0.81	0.12	1.67	0.38
	0.28	0.50	0.37	
	1.92	0.52	0.98	
Mean	1.00	0.53	0.94	0.41
SEM	0.27	0.26	0.22	0.22
TNFR1	1.34	1.03	0.98	1.98

	1.03	4.45	0.77	2.37
	0.80	1.07	1.09	1.66
	0.91	2.15	0.81	1.62
	0.87	0.68	0.73	2.22
	1.05	1.05	2.15	2.15
Mean	1.00	1.74	0.88	2.00
SEM	0.08	0.58	0.07	0.13
TNFR2	1.27	1.23	1.08	1.50
	1.06	1.01	1.71	2.40
	0.96	2.14	0.94	1.55
	0.91	1.53	1.12	1.38
	0.91	1.16	1.11	2.31
	0.90			1.03
Mean	1.00	1.41	1.19	1.70
SEM	0.06	0.20	0.13	0.22
IFNgR1	0.96	1.33	1.40	0.97
	1.19	1.14	1.33	1.19
	0.96	1.17	1.44	0.94
	0.97	1.09	1.55	1.42
	0.91	1.01	1.36	1.04
	1.02			0.88
Mean	1.00	1.15	1.42	1.07
SEM	0.04	0.05	0.04	0.08
IFNgR2	0.41	1.66	2.08	1.18
	1.14	1.09	0.81	1.10
	1.04	0.99	1.45	0.93
	0.94	1.15	1.47	0.52
	1.12	1.03	1.95	1.00
	1.35			0.89
Mean	1.00	1.18	1.55	0.94
SEM	0.13	0.12	0.22	0.09

Chapter 5

Raw PCR data from Section 5.4:

	Mixed Glia		Astrocytes		Microglia	
	Control	**Thrombin**	**Control**	**Thrombin**	**Control**	**Thrombin**
GDNF	0.85	4.63	1.17	8.65	0.72	0.70
	1.17	5.00	1.46	8.13	0.80	3.04
	0.89	7.27	0.67	8.67	1.22	1.40
	1.22	5.59	0.75	7.65	0.79	1.18
	0.86	5.08	1.09	6.11	1.70	0.00
	0.00	6.08	0.86	5.28	0.78	0.62
Mean	1.00	5.61	1.00	7.42	1.00	1.39
SEM	0.08	0.39	0.12	0.58	0.16	0.44
NGFβ	0.92	4.06	1.47	8.74	1.15	1.85
	0.88	3.36	1.22	7.80	0.70	2.39
	0.99	4.51	0.73	9.50	1.70	2.37
	0.88	4.13	0.81	8.26	0.99	1.29
	0.81	4.11	0.86	6.96	0.70	0.85
	1.52	5.13	0.90	7.89	0.76	1.31
Mean	1.00	4.22	1.00	8.19	1.00	1.68
SEM	0.11	0.24	0.12	0.36	0.16	0.26
IL-10	1.30	62.67	3.18	199.94	0.05	34.86
	0.93	72.20	0.80	267.26	1.06	40.00
	1.14	95.39	0.33	114.13	0.17	21.49
	1.23	90.36	0.09	217.75	0.14	24.52
	0.90	55.56	0.60	205.12	3.57	56.33
	0.50	23.47		167.48		33.74
Mean	1.00	66.61	1.00	195.30	1.00	35.16
SEM	0.12	10.68	0.56	20.95	0.67	5.08
TGFβ1	0.91	1.12	1.10	1.49	0.89	1.31
	1.07	1.22	1.05	1.56	0.64	3.11
	0.97	1.19	0.92	1.50	1.15	1.18

	1.08	1.24	0.99	1.44	1.48	0.82
	0.96	1.46	1.03	1.36	0.83	3.85
	1.02	1.24	0.91	1.50		0.76
Mean	1.00	1.25	1.00	1.48	1.00	1.84
SEM	0.03	0.05	0.03	0.03	0.15	0.53

	Control	Aβ		Control	Aβ
GDNF	1.08	1.43	***IL-10***	0.70	3.39
	0.63	1.41		1.75	1.33
	0.63	0.73		1.53	1.76
	1.74	1.35		0.56	0.21
	1.12	1.63		0.41	2.10
	0.82	1.18		1.06	0.74
Mean	1.00	1.29	Mean	1.00	1.59
SEM	0.17	0.13	SEM	0.22	0.46
NGFβ	1.44	1.46	***TGFβ1***	0.97	1.26
	0.77	1.28		0.97	1.26
	0.72	0.85		1.08	1.34
	1.36	1.59		1.09	1.21
	0.89	1.66		0.91	1.50
	0.83	1.34		0.98	1.28
Mean	1.00	1.36	Mean	1.00	1.31
SEM	0.13	0.12	SEM	0.03	0.04

Raw PCR data from Section 5.5:

	Control	Thrombin	Aβ
IL-1R1	1.13	0.97	1.05
	0.89	0.96	0.95
	1.18	0.88	1.24
	0.88	0.98	0.89
	1.17	0.92	0.93
	0.76	0.97	1.09

Mean	1.00	0.95	1.03
SEM	0.07	0.02	0.05
IL-1R2	0.75	1.12	0.84
	1.07	1.08	0.77
	1.06	3.07	0.82
	1.13	2.60	0.67
	0.74	2.43	0.73
	1.24	1.50	0.97
Mean	1.00	1.97	0.80
SEM	0.09	0.34	0.04
TNFR1	0.93	0.69	1.09
	0.98	0.65	0.99
	1.13	0.68	1.33
	1.05	0.79	1.00
	1.10	0.65	1.11
	0.80	0.75	1.01
Mean	1.00	0.70	1.09
SEM	0.05	0.02	0.05
TNFR2	0.93	4.00	1.17
	0.91	3.36	1.33
	0.92	4.78	0.99
	0.97	3.92	1.17
	0.97	3.95	0.87
	1.29	3.73	1.08
Mean	1.00	3.96	1.10
SEM	0.06	0.19	0.06
IFNgR1	1.04	1.36	1.06
	0.99	1.41	1.12
	0.91	1.24	1.20
	0.91	1.15	0.96

	1.11	1.11	0.94
	1.03	1.31	1.07
Mean	1.00	1.26	1.06
SEM	0.03	0.05	0.04
IFNgR2	0.87	0.72	0.93
	1.08	0.70	1.18
	0.95	0.57	1.22
	0.90	0.78	1.06
	1.23	0.75	1.14
	0.98	0.66	1.12
Mean	1.00	0.70	1.11
SEM	0.05	0.03	0.04

Raw PCR data from Section 5.8:

	Control	**Thrombin**	**Sal**	**Sal +Throm**	**dbcAMP**	**dbcAMP +Throm**
GDNF	0.85	4.63	15.58	17.44	8.67	14.22
	1.17	5.00	11.62	17.75	9.02	17.17
	0.89	7.27	20.78	24.67	13.28	14.73
	1.22	5.59	19.66	23.67	15.39	20.51
	0.86	5.08	10.01	21.55	10.88	23.41
		6.08	22.42	22.08	15.18	17.31
Mean	1.00	5.61	16.68	21.19	12.07	17.89
SEM	0.08	0.39	2.08	1.23	1.22	1.43
NGFβ	0.92	4.06	2.42	3.74	2.17	5.96
	0.88	3.36	2.53	4.45	2.62	6.94
	0.99	4.51	4.05	6.76	3.73	8.07
	0.88	4.13	3.80	6.16	3.65	8.18
	0.81	4.11	3.06	6.50	3.14	7.35
	1.52	5.13	3.37	5.79	3.46	6.70
Mean	1.00	4.22	3.21	5.57	3.13	7.20
SEM	0.11	0.24	0.27	0.49	0.25	0.35

IL-10	1.30	62.67	0.41	104.57	0.34	137.62
	0.93	72.20	0.68	185.76	0.59	192.44
	1.14	95.39	0.70	335.39	0.77	249.09
	1.23	90.36	0.33	312.80	1.05	234.30
	0.90	55.56	0.52	222.47	0.48	182.32
	0.50	23.47	0.47	170.38	0.54	
Mean	1.00	66.61	0.52	221.90	0.63	199.20
SEM	0.12	10.68	0.06	36.00	0.10	19.80
TGFβ1	0.92	1.01	1.10	1.07	1.38	1.06
	0.81	0.83	1.66	0.88	1.36	1.20
	1.16	0.92	1.65	1.48	1.48	1.46
	1.01	1.16	1.34	1.24	1.43	1.48
	0.97	0.99	1.79	1.59	1.51	1.17
	1.12	0.58	1.49	1.36	1.31	1.35
Mean	1.00	0.92	1.51	1.27	1.41	1.29
SEM	0.05	0.08	0.10	0.11	0.03	0.07

	Control	**Thrombin**	**Salmeterol**	**Salmeterol +Thrombin**
GDNF	0.51	1.55	3.36	2.48
	0.88	1.91	3.48	2.63
	0.98	1.88	3.39	2.56
	0.89	2.25	3.29	2.37
	1.61	1.59	3.85	3.26
	1.12			
Mean	1.00	1.84	3.48	2.66
SEM	0.15	0.13	0.10	0.16
NGFβ	0.50	1.77	1.80	1.97
	1.19	1.91	1.55	2.25
	1.21	1.92	1.83	1.51
	1.08	2.74	1.50	3.00
	1.23	2.68	2.23	2.07

	0.79	2.04	2.20	2.74
Mean	1.00	2.18	1.85	2.26
SEM	0.12	0.17	0.13	0.22
IL-10	1.89	112.65	0.44	125.73
	0.30	120.23	0.71	100.03
	0.12	144.87	0.94	91.92
	2.18	138.61		81.96
	0.68	80.17		145.25
	0.83	83.37		71.63
Mean	1.00	113.30	0.70	102.80
SEM	0.34	11.07	0.14	11.34
TGFβ1	0.91	1.12	0.78	1.07
	1.07	1.22	1.12	1.25
	0.97	1.19	1.40	0.90
	1.08	1.24	1.16	1.26
	0.96	1.46	1.13	1.24
	1.02	1.24	0.92	1.15
Mean	1.00	1.25	1.08	1.15
SEM	0.03	0.05	0.09	0.06

7.3 Abbreviations

6'OHDA	6-hydroxydopamine
AD	Alzheimer's disease
AF-1	accessory factor 1
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APC	antigen presenting cell
ApoE	apolipoprotein E
APP	amyloid precursor protein
AR	adrenergic receptor
AraC	cytosine β-D-arabino-furanoside
ATP	adenosine 5′-triphosphate
Aβ	amyloid-beta
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
Bim	Bcl-2-interacting mediator of cell death
BNZ	N6-Benzoyl-cAMP
BSA	bovine serum albumin
Ca^{2+}	calcium
CaM	calmodulin
CAM	cell adhesion molecule
CamKII	Ca/CaM dependent kinase type II
cAMP	3',5'-cyclic adenosine monophosphate
CD40L	CD40 ligand
cDNA	complementary DNA
CINC	cytokine-induced neutrophil chemoattractant
Cl^-	chlorine
CM	conditioned media
CNS	central nervous system
COMT	catechol-O-methyltransferase
COX	Cycloxygenase
CRE	cAMP-response element
CREB	CRE-binding protein

CSF	cerebrospinal fluid
CT	cycle number
CTF	C-terminal fragment
DA	dopamine
DAG	1,2-diacylglycerol
db-cAMP	dibutyryl-cAMP
DCX	doublecortin
ddH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DOPA	dihydroxyphenylanlanine
DOPAC	dihydroxy-phenyl-acetic acid
DβH	dopamine-β-hydroxylase
EAE	experimental autoimmune encephalomyelitis
EAM	experimental autoimmune myocarditis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial NOS
EPAC	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
EtOH	Ethanol
FBS	foetal bovine serum
FRET	fluorescence resonance energy transfer
GAS	gamma activated sequence
GDNF	glial derived neurotrophic factor
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GFRα	GDNF family receptor α
Gi	inhibitory G-protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gs	stimulatory G-protein
GTP	guanosine triphosphate
H^+	hydrogen
H_2O_2	hydrogen peroxide

H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase-conjugated streptavidin
ICAM	intercellular CAM
ICH	intracerebral haemorrhage
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-10R	IL-10 receptor
IL-1R	IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IL-1RAcP	IL-1R accessory protein
iNOS	inducible NOS
IP-10	IFN-inducible protein-10
IP_3	inositol 1,4,5-triphosphate
IRAK	IL-1 receptor-associated kinase
IRF	IFN regulatory factor
ISG	IFN stimulated genes
JAK	Janus kinase
JNK	Jun N-terminal kinase
K^+	Potassium
KCl	potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
KO	Knockout
LC	locus coeruleus
LDH	lactate dehydrogenase
LOAD	late onset AD
LPS	lipopolysaccharide
LTP	long term potentiation
MAC	membrane attack complexes
MAdCAM	mucosal addressin CAM
MAO	monoamine oxidase

MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant peptide
M-CSF	macrophage-colony stimulating factor
MeOH	methanol
Mg_2SO_4	magnesium sulphate
$MgCl_2$	magnesium chloride
MHC	mitogen histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MND	motor neuron disease
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
MS	multiple sclerosis
NA	noradrenaline
Na^+	sodium
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate monobasic monohydrate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NBM	Neurobasal media
NCAM	neuronal CAM
NET	noradrenergic transporter
NFQ	non-fluorescent quencher
NFT	neurofibrillary tangle
NFκB	nuclear factor of κ binding
NGF	nerve growth factor
NK	natural killer
NMDA	N-methyl-D-aspartate
nNOS	neuronal NOS
NO	nitric oxide

NOS	nitric oxide synthase
NPC	neural precursor cell
NSAID	nonsteroidal anti-inflammatory drug
NT	Neurotrophin
O_2	Oxygen
$p75^{NTR}$	p75 neurotrophin receptor
PAMP	pathogen associated molecular patterns
PAR	protease activated receptor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pCPT	8-pCPT-2'-O-Me-cAMP
PD	Parkinson's disease
PGE2	prostaglandin-E2
PI_3K	phosphatidylinositide-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PNMT	phenylethanolamine N-methyl transferase
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
RQ	relative quantification value
RT-PCR	real time polymerase chain reaction
Sal	Salbutamol
Salm	Salmeterol
SBE	STAT binding element
SEM	standard error of the mean
SNpc	substantia nigra pars compacta
SOCS	suppressor of cytokine signalling
STAT	signal transducers and activators of transcription
TAFI	thrombin activatable fibrinolysis inhibitor
TβR	TGFβ receptor

TGFβ	transforming growth factor-β
Th	helper T cell
TH	tyrosine hydroxylase
TIR	toll/IL-1 receptor
TLR	toll like receptor
TMB	3,3',5,5',-Tetramethyl-benzidine
TNF	tumour necrosis factor
TNFR	TNF receptor
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
Trk	receptor tyrosine kinase
UV	ultra violet
VCAM	vascular CAM
VEGF	vascular endothelial growth factor
VLA	very late antigen
VMAT	vesicular monoamine transporter
Xam	Xamoterol
α-sAPP	α-secretase-cleaved soluble APP
β-sAPP	β-secretase-cleaved soluble APP
ΔCT	CT difference
ΔΔCT	CT difference corrected for β-actin

7.4 List of Abstracts Presented

RAMI (Royal Academy of Medicine in Ireland). Cork, Ireland. June 2007

Thrombin, β-amyloid and IFNγ induce chemokine expression in primary astrocytes and microglia: Implications for neurodegeneration

NEUROTRAIN (Neuroscience Training in Europe - Autumn School). Dubrovnik, Croatia. October 2007

Thrombin, β-amyloid and IFNγ induce chemokine expression in primary astrocytes and microglia: Implications for neurodegeneration

School of Medicine Postgraduate Research Day. Dublin, Ireland. December 2007

Noradrenaline suppresses thrombin-induced chemokine and cell adhesion molecule expression in primary astrocytes and microglia

HRB Research Day (Let's Talk Health Research). Dublin, Ireland. December 2007

Analysing the Brain's Inflammatory Response

International Neuroimmunology Symposium. Dublin, Ireland. March 2008

Noradrenaline suppresses thrombin-induced chemokine and cell adhesion molecule expression in primary astrocytes and microglia

School of Medicine Postgraduate Research Day. Dublin, Ireland. December 2008

The β_2-adrenoceptor agonist salmeterol inhibits thrombin-induced inflammation in primary glial cells: A Role for PKA and EPAC activation by cAMP

BNA (British Neuroscience Association). Liverpool, England. March 2009

The β_2-adrenoceptor agonist salmeterol inhibits thrombin-induced inflammation in primary glial cells: A Role for PKA and EPAC activation by cAMP

PNIRS (Psychoneuroimmunology Research Society). Colorado, USA. June 2009

The β_2-adrenoceptor agonist salmeterol inhibits thrombin-induced inflammation in primary glial cells: A Role for PKA and EPAC activation by cAMP

8. References

Abcouwer, S. F., Shanmugam, S., Gomez, P. F., Shushanov, S., Barber, A. J., Lanoue, K. F., Quinn, P. G., Kester, M. & Gardner, T. W. (2008) Effect of IL-1beta on survival and energy metabolism of R28 and RGC-5 retinal neurons. *Invest Ophthalmol Vis Sci,* 49, 5581-92.

Abdel-Haq, N., Hao, H. N. & Lyman, W. D. (1999) Cytokine regulation of CD40 expression in fetal human astrocyte cultures. *J Neuroimmunol,* 101, 7-14.

Aggarwal, B. B. (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol,* 3, 745-56.

Akama, K. T. & Van Eldik, L. J. (2000) Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. *J Biol Chem,* 275, 7918-24.

Akiyama, H. & McGeer, P. L. (1990) Brain microglia constitutively express beta-2 integrins. *J Neuroimmunol,* 30, 81-93.

Allan, S. M., Tyrrell, P. J. & Rothwell, N. J. (2005) Interleukin-1 and neuronal injury. *Nat Rev Immunol,* 5, 629-40.

Amarenco, P., Bogousslavsky, J., Caplan, L. R., Donnan, G. A. & Hennerici, M. G. (2009) Classification of stroke subtypes. *Cerebrovasc Dis,* 27, 493-501.

Aoki, C. (1992) Beta-adrenergic receptors: astrocytic localization in the adult visual cortex and their relation to catecholamine axon terminals as revealed by electron microscopic immunocytochemistry. *J Neurosci,* 12, 781-92.

Apelt, J. & Schliebs, R. (2001) Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain Res,* 894, 21-30.

Archambault, A. S., Sim, J., McCandless, E. E., Klein, R. S. & Russell, J. H. (2006) Region-specific regulation of inflammation and pathogenesis in experimental autoimmune encephalomyelitis. *J Neuroimmunol,* 181, 122-32.

Aricescu, A. R. & Jones, E. Y. (2007) Immunoglobulin superfamily cell adhesion molecules: zippers and signals. *Curr Opin Cell Biol,* 19, 543-50.

Arien-Zakay, H., Lecht, S., Bercu, M. M., Amariglio, N., Rechavi, G., Galski, H., Lazarovici, P. & Nagler, A. (2009) Interferon-gamma-induced neuronal differentiation of human umbilical cord blood-derived progenitors. *Leukemia,* 23, 1790-800.

Atkins, C. M., Oliva, A. A., Jr., Alonso, O. F., Pearse, D. D., Bramlett, H. M. & Dietrich, W. D. (2007) Modulation of the cAMP signaling pathway after traumatic brain injury. *Exp Neurol,* **208,** 145-58.

Bach, E. A., Aguet, M. & Schreiber, R. D. (1997) The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol,* **15,** 563-91.

Baggiolini, M., Dewald, B. & Moser, B. (1997) Human chemokines: an update. *Annu Rev Immunol,* **15,** 675-705.

Balcaitis, S., Xie, Y., Weinstein, J. R., Andersen, H., Hanisch, U. K., Ransom, B. R. & Moller, T. (2003) Expression of proteinase-activated receptors in mouse microglial cells. *Neuroreport,* **14,** 2373-7.

Ballestas, M. E. & Benveniste, E. N. (1997) Elevation of cyclic AMP levels in astrocytes antagonizes cytokine-induced adhesion molecule expression. *J Neurochem,* **69,** 1438-48.

Balyasnikova, I. V., Pelligrino, D. A., Greenwood, J., Adamson, P., Dragon, S., Raza, H. & Galea, E. (2000) Cyclic adenosine monophosphate regulates the expression of the intercellular adhesion molecule and the inducible nitric oxide synthase in brain endothelial cells. *J Cereb Blood Flow Metab,* **20,** 688-99.

Banati, R. B., Gehrmann, J., Schubert, P. & Kreutzberg, G. W. (1993) Cytotoxicity of microglia. *Glia,* **7,** 111-8.

Banati, R. B. & Graeber, M. B. (1994) Surveillance, intervention and cytotoxicity: is there a protective role of microglia? *Dev Neurosci,* **16,** 114-27.

Banati, R. B. (2003) Neuropathological imaging: in vivo detection of glial activation as a measure of disease and adaptive change in the brain. *Br Med Bull,* **65,** 121-31.

Barry, C. E., Nolan, Y., Clarke, R. M., Lynch, A. & Lynch, M. A. (2005) Activation of c-Jun-N-terminal kinase is critical in mediating lipopolysaccharide-induced changes in the rat hippocampus. *J Neurochem,* **93,** 221-31.

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A. & Schall, T. J. (1997) A new class of membrane-bound chemokine with a CX3C motif. *Nature,* **385,** 640-4.

Becher, B. & Antel, J. P. (1996) Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia. *Glia,* **18,** 1-10.

Benbernou, N., Esnault, S., Shin, H. C., Fekkar, H. & Guenounou, M. (1997) Differential regulation of IFN-gamma, IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway. *Immunology,* **91,** 361-8.

Benzing, W. C. & Mufson, E. J. (1995) Apolipoprotein E immunoreactivity within neurofibrillary tangles: relationship to Tau and PHF in Alzheimer's disease. *Exp Neurol,* 132, 162-71.

Bernardino, L., Xapelli, S., Silva, A. P., Jakobsen, B., Poulsen, F. R., Oliveira, C. R., Vezzani, A., Malva, J. O. & Zimmer, J. (2005) Modulator effects of interleukin-1beta and tumor necrosis factor-alpha on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures. *J Neurosci,* 25, 6734-44.

Bertolino, P., Deckers, M., Lebrin, F. & ten Dijke, P. (2005) Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest,* 128, 585S-590S.

Bette, M., Kaut, O., Schafer, M. K. & Weihe, E. (2003) Constitutive expression of p55TNFR mRNA and mitogen-specific up-regulation of TNF alpha and p75TNFR mRNA in mouse brain. *J Comp Neurol,* 465, 417-30.

Bielarczyk, H., Jankowska-Kulawy, A., Gul, S., Pawelczyk, T. & Szutowicz, A. (2005) Phenotype dependent differential effects of interleukin-1beta and amyloid-beta on viability and cholinergic phenotype of T17 neuroblastoma cells. *Neurochem Int,* 47, 466-73.

Billiau, A. (1996) Interferon-gamma in autoimmunity. *Cytokine Growth Factor Rev,* 7, 25-34.

Binder, G. K. & Griffin, D. E. (2001) Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons. *Science,* 293, 303-6.

Blasko, I., Lederer, W., Oberbauer, H., Walch, T., Kemmler, G., Hinterhuber, H., Marksteiner, J. & Humpel, C. (2006) Measurement of thirteen biological markers in CSF of patients with Alzheimer's disease and other dementias. *Dement Geriatr Cogn Disord,* 21, 9-15.

Blennow, K. (2004) CSF biomarkers for mild cognitive impairment. *J Intern Med,* 256, 224-34.

Block, M. L. & Hong, J. S. (2005) Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol,* 76, 77-98.

Blond, D., Campbell, S. J., Butchart, A. G., Perry, V. H. & Anthony, D. C. (2002) Differential induction of interleukin-1beta and tumour necrosis factor-alpha may account for specific patterns of leukocyte recruitment in the brain. *Brain Res,* 958, 89-99.

Bogatcheva, N. V., Garcia, J. G. & Verin, A. D. (2002) Molecular mechanisms of thrombin-induced endothelial cell permeability. *Biochemistry (Mosc),* 67, 75-84.

Bogdan, C., Paik, J., Vodovotz, Y. & Nathan, C. (1992) Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem,* 267, 23301-8.

Bohatschek, M., Kloss, C. U., Pfeffer, K., Bluethmann, H. & Raivich, G. (2004) B7.2 on activated and phagocytic microglia in the facial axotomy model: regulation by interleukin-1 receptor type 1, tumor necrosis factor receptors 1 and 2 and endotoxin. *J Neuroimmunol,* 156, 132-45.

Bondareff, W., Mountjoy, C. Q., Roth, M., Rossor, M. N., Iversen, L. L., Reynolds, G. P. & Hauser, D. L. (1987) Neuronal degeneration in locus ceruleus and cortical correlates of Alzheimer disease. *Alzheimer Dis Assoc Disord,* 1, 256-62.

Borger, P., Hoekstra, Y., Esselink, M. T., Postma, D. S., Zaagsma, J., Vellenga, E. & Kauffman, H. F. (1998) Beta-adrenoceptor-mediated inhibition of IFN-gamma, IL-3, and GM-CSF mRNA accumulation in activated human T lymphocytes is solely mediated by the beta2-adrenoceptor subtype. *Am J Respir Cell Mol Biol,* 19, 400-7.

Bos, J. L. (2003) Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol,* 4, 733-8.

Bos, J. L. (2006) Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci,* 31, 680-6.

Bouma, B. N. & Mosnier, L. O. (2006) Thrombin activatable fibrinolysis inhibitor (TAFI)--how does thrombin regulate fibrinolysis? *Ann Med,* 38, 378-88.

Braak, H. & Braak, E. (1991) Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. *Brain Pathol,* 1, 213-6.

Briest, W., Homagk, L., Rassler, B., Ziegelhoffer-Mihalovicova, B., Meier, H., Tannapfel, A., Leiblein, S., Saalbach, A., Deten, A. & Zimmer, H. G. (2004) Norepinephrine-induced changes in cardiac transforming growth factor-beta isoform expression pattern of female and male rats. *Hypertension,* 44, 410-8.

Buee, L., Perez-Tur, J., Leveugle, B., Buee-Scherrer, V., Mufson, E. J., Loerzel, A. J., Chartier-Harlin, M. C., Perl, D. P., Delacourte, A. & Hof, P. R. (1996) Apolipoprotein E in Guamanian amyotrophic lateral sclerosis/parkinsonism-dementia complex: genotype analysis and relationships to neuropathological changes. *Acta Neuropathol,* 91, 247-53.

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A. & Hof, P. R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev,* 33, 95-130.

Buntinx, M., Gielen, E., Van Hummelen, P., Raus, J., Ameloot, M., Steels, P. & Stinissen, P. (2004a) Cytokine-induced cell death in human oligodendroglial cell lines. II: Alterations in gene expression induced by interferon-gamma and tumor necrosis factor-alpha. *J Neurosci Res,* 76, 846-61.

Buntinx, M., Moreels, M., Vandenabeele, F., Lambrichts, I., Raus, J., Steels, P., Stinissen, P. & Ameloot, M. (2004b) Cytokine-induced cell death in human oligodendroglial cell lines: I. Synergistic effects of IFN-gamma and TNF-alpha on apoptosis. *J Neurosci Res,* 76, 834-45.

Burns, A., Tomlinson, B. E. & Mann, D. M. (1997) Observations on the brains of demented old people. B.E. Tomlinson, G. Blessed and M. Roth, Journal of the Neurological Sciences (1970) 11, 205-242 and Observations on the brains of non-demented old people. B.E. Tomlinson, G. Blessed and M. Roth, Journal of Neurological Sciences (1968) 7, 331-356. *Int J Geriatr Psychiatry,* 12, 785-90.

Butler, A. R., Flitney, F. W. & Williams, D. L. (1995) NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. *Trends Pharmacol Sci,* 16, 18-22.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., Martino, G. & Schwartz, M. (2006) Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci,* 31, 149-60.

Buznikov, G. A., Nikitina, L. A., Seidler, F. J., Slotkin, T. A., Bezuglov, V. V., Milosevic, I., Lazarevic, L., Rogac, L., Ruzdijic, S. & Rakic, L. M. (2008) Amyloid precursor protein 96-110 and beta-amyloid 1-42 elicit developmental anomalies in sea urchin embryos and larvae that are alleviated by neurotransmitter analogs for acetylcholine, serotonin and cannabinoids. *Neurotoxicol Teratol,* 30, 503-9.

Cacci, E., Ajmone-Cat, M. A., Anelli, T., Biagioni, S. & Minghetti, L. (2008) In vitro neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. *Glia,* 56, 412-25.

Calingasan, N. Y., Erdely, H. A. & Altar, C. A. (2002) Identification of CD40 ligand in Alzheimer's disease and in animal models of Alzheimer's disease and brain injury. *Neurobiol Aging,* 23, 31-9.

Cannon, J. R., Keep, R. F., Hua, Y., Richardson, R. J., Schallert, T. & Xi, G. (2005) Thrombin preconditioning provides protection in a 6-hydroxydopamine Parkinson's disease model. *Neurosci Lett,* 373, 189-94.

Cannon, J. R., Keep, R. F., Schallert, T., Hua, Y., Richardson, R. J. & Xi, G. (2006) Protease-activated receptor-1 mediates protection elicited by thrombin preconditioning in a rat 6-hydroxydopamine model of Parkinson's disease. *Brain Res,* 1116, 177-86.

Cannon, J. R., Hua, Y., Richardson, R. J., Xi, G., Keep, R. F. & Schallert, T. (2007) The effect of thrombin on a 6-hydroxydopamine model of Parkinson's disease depends on timing. *Behav Brain Res,* 183, 161-8.

Cardell, L. O., Uddman, R., Zhang, Y. & Adner, M. (2008) Interleukin-1beta up-regulates tumor necrosis factor receptors in the mouse airways. *Pulm Pharmacol Ther,* 21, 675-81.

Chao, M. V., Rajagopal, R. & Lee, F. S. (2006) Neurotrophin signalling in health and disease. *Clin Sci (Lond),* 110, 167-73.

Charo, I. F. & Ransohoff, R. M. (2006) The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med,* 354, 610-21.

Chen, K., Huang, J., Gong, W., Zhang, L., Yu, P. & Wang, J. M. (2006) CD40/CD40L dyad in the inflammatory and immune responses in the central nervous system. *Cell Mol Immunol,* 3, 163-9.

Chen, Y., Ke, Q., Xiao, Y. F., Wu, G., Kaplan, E., Hampton, T. G., Malek, S., Min, J. Y., Amende, I. & Morgan, J. P. (2005) Cocaine and catecholamines enhance inflammatory cell retention in the coronary circulation of mice by upregulation of adhesion molecules. *Am J Physiol Heart Circ Physiol,* 288, H2323-31.

Cheng, X., Yang, L., He, P., Li, R. & Shen, Y. (2009) Differential Activation of Tumor Necrosis Factor Receptors Distinguishes between Brains from Alzheimer's Disease and Non-Demented Patients. *J Alzheimers Dis.*

Chiarini, A., Dal Pra, I., Whitfield, J. F. & Armato, U. (2006) The killing of neurons by beta-amyloid peptides, prions, and pro-inflammatory cytokines. *Ital J Anat Embryol,* 111, 221-46.

Choi, S. H., Joe, E. H., Kim, S. U. & Jin, B. K. (2003) Thrombin-induced microglial activation produces degeneration of nigral dopaminergic neurons in vivo. *J Neurosci,* 23, 5877-86.

Choi, S. H., Lee, D. Y., Kim, S. U. & Jin, B. K. (2005) Thrombin-induced oxidative stress contributes to the death of hippocampal neurons in vivo: role of microglial NADPH oxidase. *J Neurosci,* 25, 4082-90.

Cinque, P., Bestetti, A., Marenzi, R., Sala, S., Gisslen, M., Hagberg, L. & Price, R. W. (2005) Cerebrospinal fluid interferon-gamma-inducible protein 10 (IP-10, CXCL10) in HIV-1 infection. *J Neuroimmunol,* 168, 154-63.

Clausen, F., Lorant, T., Lewen, A. & Hillered, L. (2007) T lymphocyte trafficking: a novel target for neuroprotection in traumatic brain injury. *J Neurotrauma,* 24, 1295-307.

Colotta, F., Dower, S. K., Sims, J. E. & Mantovani, A. (1994) The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. *Immunol Today,* 15, 562-6.

Combs, C. K., Johnson, D. E., Cannady, S. B., Lehman, T. M. & Landreth, G. E. (1999) Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J Neurosci,* 19, 928-39.

Combs, C. K., Karlo, J. C., Kao, S. C. & Landreth, G. E. (2001) beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J Neurosci,* 21, 1179-88.

Commins, S., Steinke, J. W. & Borish, L. (2008) The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol,* 121, 1108-11.

Cook, A. D., Braine, E. L. & Hamilton, J. A. (2004) Stimulus-dependent requirement for granulocyte-macrophage colony-stimulating factor in inflammation. *J Immunol,* 173, 4643-51.

Corbin, J. G., Kelly, D., Rath, E. M., Baerwald, K. D., Suzuki, K. & Popko, B. (1996) Targeted CNS expression of interferon-gamma in transgenic mice leads to hypomyelination, reactive gliosis, and abnormal cerebellar development. *Mol Cell Neurosci,* 7, 354-70.

Cox, D. J., Racca, C. & LeBeau, F. E. (2008) Beta-adrenergic receptors are differentially expressed in distinct interneuron subtypes in the rat hippocampus. *J Comp Neurol,* 509, 551-65.

Cuello, A. C., Bruno, M. A. & Bell, K. F. (2007) NGF-cholinergic dependency in brain aging, MCI and Alzheimer's disease. *Curr Alzheimer Res,* 4, 351-8.

Davis, C. N., Tabarean, I., Gaidarova, S., Behrens, M. M. & Bartfai, T. (2006) IL-1beta induces a MyD88-dependent and ceramide-mediated activation of Src in anterior hypothalamic neurons. *J Neurochem,* 98, 1379-89.

De Ciuceis, C., Amiri, F., Brassard, P., Endemann, D. H., Touyz, R. M. & Schiffrin, E. L. (2005) Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin II-infused macrophage colony-stimulating factor-deficient mice: evidence for a role in inflammation in angiotensin-induced vascular injury. *Arterioscler Thromb Vasc Biol,* 25, 2106-13.

De Keyser, J., Zeinstra, E., Mostert, J. & Wilczak, N. (2004a) Beta 2-adrenoceptor involvement in inflammatory demyelination and axonal degeneration in multiple sclerosis. *Trends Pharmacol Sci,* 25, 67-71.

De Keyser, J., Zeinstra, E. & Wilczak, N. (2004b) Astrocytic beta2-adrenergic receptors and multiple sclerosis. *Neurobiol Dis,* **15,** 331-9.

de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., Silverman, R. H. & Williams, B. R. (2001) Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol,* **69,** 912-20.

Debeir, T., Gueugnon, J., Vige, X. & Benavides, J. (1996) Transduction mechanisms involved in thrombin receptor-induced nerve growth factor secretion and cell division in primary cultures of astrocytes. *J Neurochem,* **66,** 2320-8.

Dechant, G. & Neumann, H. (2002) Neurotrophins. *Adv Exp Med Biol,* **513,** 303-34.

del Pozo, M. A., Sanchez-Mateos, P., Nieto, M. & Sanchez-Madrid, F. (1995) Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol,* **131,** 495-508.

Dello Russo, C., Boullerne, A. I., Gavrilyuk, V. & Feinstein, D. L. (2004) Inhibition of microglial inflammatory responses by norepinephrine: effects on nitric oxide and interleukin-1beta production. *J Neuroinflammation,* **1,** 9.

Denys, A., Udalova, I. A., Smith, C., Williams, L. M., Ciesielski, C. J., Campbell, J., Andrews, C., Kwaitkowski, D. & Foxwell, B. M. (2002) Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-kappa B in primary human macrophages. *J Immunol,* **168,** 4837-45.

Diem, R., Hobom, M., Grotsch, P., Kramer, B. & Bahr, M. (2003) Interleukin-1 beta protects neurons via the interleukin-1 (IL-1) receptor-mediated Akt pathway and by IL-1 receptor-independent decrease of transmembrane currents in vivo. *Mol Cell Neurosci,* **22,** 487-500.

Dihanich, M., Kaser, M., Reinhard, E., Cunningham, D. & Monard, D. (1991) Prothrombin mRNA is expressed by cells of the nervous system. *Neuron,* **6,** 575-81.

Dinh, C., Hoang, K., Haake, S., Chen, S., Angeli, S., Nong, E., Eshraghi, A. A., Balkany, T. J. & Van De Water, T. R. (2008a) Biopolymer-released dexamethasone prevents tumor necrosis factor alpha-induced loss of auditory hair cells in vitro: implications toward the development of a drug-eluting cochlear implant electrode array. *Otol Neurotol,* **29,** 1012-9.

Dinh, C. T., Haake, S., Chen, S., Hoang, K., Nong, E., Eshraghi, A. A., Balkany, T. J. & Van De Water, T. R. (2008b) Dexamethasone protects organ of corti explants against tumor necrosis factor-alpha-induced loss of auditory hair cells and alters the expression levels of apoptosis-related genes. *Neuroscience,* **157,** 405-13.

Dolga, A. M., Granic, I., Blank, T., Knaus, H. G., Spiess, J., Luiten, P. G., Eisel, U. L. & Nijholt, I. M. (2008a) TNF-alpha-mediates neuroprotection against glutamate-induced excitotoxicity via NF-kappaB-dependent up-regulation of K2.2 channels. *J Neurochem,* 107, 1158-67.

Dolga, A. M., Nijholt, I. M., Ostroveanu, A., Ten Bosch, Q., Luiten, P. G. & Eisel, U. L. (2008b) Lovastatin induces neuroprotection through tumor necrosis factor receptor 2 signaling pathways. *J Alzheimers Dis,* 13, 111-22.

Dong, Y. & Benveniste, E. N. (2001) Immune function of astrocytes. *Glia,* 36, 180-90.

Dorenkamp, M., Riad, A., Stiehl, S., Spillmann, F., Westermann, D., Du, J., Pauschinger, M., Noutsias, M., Adams, V., Schultheiss, H. P. & Tschope, C. (2005) Protection against oxidative stress in diabetic rats: role of angiotensin AT(1) receptor and beta 1-adrenoceptor antagonism. *Eur J Pharmacol,* 520, 179-87.

Downen, M., Amaral, T. D., Hua, L. L., Zhao, M. L. & Lee, S. C. (1999) Neuronal death in cytokine-activated primary human brain cell culture: role of tumor necrosis factor-alpha. *Glia,* 28, 114-27.

Duan, R. S., Yang, X., Chen, Z. G., Lu, M. O., Morris, C., Winblad, B. & Zhu, J. (2008) Decreased fractalkine and increased IP-10 expression in aged brain of APP(swe) transgenic mice. *Neurochem Res,* 33, 1085-9.

Dunn, S. L., Young, E. A., Hall, M. D. & McNulty, S. (2002) Activation of astrocyte intracellular signaling pathways by interleukin-1 in rat primary striatal cultures. *Glia,* 37, 31-42.

Dziewulska, D. & Mossakowski, M. J. (2003) Cellular expression of tumor necrosis factor a and its receptors in human ischemic stroke. *Clin Neuropathol,* 22, 35-40.

Eijkelkamp, N., Cobelens, P. M., Sanders, V. M., Heijnen, C. J. & Kavelaars, A. (2004) Tissue specific effects of the beta 2-adrenergic agonist salbutamol on LPS-induced IFN-gamma, IL-10 and TGF-beta responses in vivo. *J Neuroimmunol,* 150, 3-9.

Eisenhofer, G., Huynh, T. T., Hiroi, M. & Pacak, K. (2001) Understanding catecholamine metabolism as a guide to the biochemical diagnosis of pheochromocytoma. *Rev Endocr Metab Disord,* 2, 297-311.

Eng, L. F., Ghirnikar, R. S. & Lee, Y. L. (2000) Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res,* 25, 1439-51.

Ericsson, A., Liu, C., Hart, R. P. & Sawchenko, P. E. (1995) Type 1 interleukin-1 receptor in the rat brain: distribution, regulation, and relationship to sites of IL-1-induced cellular activation. *J Comp Neurol,* 361, 681-98.

Etminan, M., Gill, S. & Samii, A. (2003) Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. *BMJ,* 327, 128.

Facchinetti, F., Del Giudice, E., Furegato, S., Passarotto, M., Arcidiacono, D. & Leon, A. (2004) Dopamine inhibits responses of astroglia-enriched cultures to lipopolysaccharide via a beta-adrenoreceptor-mediated mechanism. *J Neuroimmunol,* 150, 29-36.

Fan, Y., Zhang, W. & Mulholland, M. (2005) Thrombin and PAR-1-AP increase proinflammatory cytokine expression in C6 cells. *J Surg Res,* 129, 196-201.

Fang, F. C. (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest,* 99, 2818-25.

Farber, J. M. (1997) Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol,* 61, 246-57.

Farina, C., Aloisi, F. & Meinl, E. (2007) Astrocytes are active players in cerebral innate immunity. *Trends Immunol,* 28, 138-45.

Feinstein, D. L. (1998) Suppression of astroglial nitric oxide synthase expression by norepinephrine results from decreased NOS-2 promoter activity. *J Neurochem,* 70, 1484-96.

Feinstein, D. L., Heneka, M. T., Gavrilyuk, V., Dello Russo, C., Weinberg, G. & Galea, E. (2002) Noradrenergic regulation of inflammatory gene expression in brain. *Neurochem Int,* 41, 357-65.

Fenton, J. W., 2nd (1986) Thrombin. *Ann N Y Acad Sci,* 485, 5-15.

Fife, B. T., Kennedy, K. J., Paniagua, M. C., Lukacs, N. W., Kunkel, S. L., Luster, A. D. & Karpus, W. J. (2001) CXCL10 (IFN-gamma-inducible protein-10) control of encephalitogenic CD4+ T cell accumulation in the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol,* 166, 7617-24.

Figiel, I. & Dzwonek, K. (2007) TNFalpha and TNF receptor 1 expression in the mixed neuronal-glial cultures of hippocampal dentate gyrus exposed to glutamate or trimethyltin. *Brain Res,* 1131, 17-28.

Fisher, S. A. & Absher, M. (1995) Norepinephrine and ANG II stimulate secretion of TGF-beta by neonatal rat cardiac fibroblasts in vitro. *Am J Physiol,* 268, C910-7.

Fitzgerald, K. A. & O'Neill, L. A. (2000) The role of the interleukin-1/Toll-like receptor superfamily in inflammation and host defence. *Microbes Infect,* 2, 933-43.

Flavin, M. P., Coughlin, K. & Ho, L. T. (1997a) Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. *Neuroscience,* 80, 437-48.

Flavin, M. P., Ho, L. T. & Coughlin, K. (1997b) Neurotoxicity of soluble macrophage products in vitro--influence of dexamethasone. *Exp Neurol,* 145, 462-70.

Fleetwood, A. J., Lawrence, T., Hamilton, J. A. & Cook, A. D. (2007) Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J Immunol,* 178, 5245-52.

Fontaine, V., Mohand-Said, S., Hanoteau, N., Fuchs, C., Pfizenmaier, K. & Eisel, U. (2002) Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci,* 22, RC216.

Ford, A. L., Goodsall, A. L., Hickey, W. F. & Sedgwick, J. D. (1995) Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J Immunol,* 154, 4309-21.

Forloni, G., Mangiarotti, F., Angeretti, N., Lucca, E. & De Simoni, M. G. (1997) Beta-amyloid fragment potentiates IL-6 and TNF-alpha secretion by LPS in astrocytes but not in microglia. *Cytokine,* 9, 759-62.

Franciosi, S., Choi, H. B., Kim, S. U. & McLarnon, J. G. (2005) IL-8 enhancement of amyloid-beta (Abeta 1-42)-induced expression and production of pro-inflammatory cytokines and COX-2 in cultured human microglia. *J Neuroimmunol,* 159, 66-74.

Friedman, W. J. (2001) Cytokines regulate expression of the type 1 interleukin-1 receptor in rat hippocampal neurons and glia. *Exp Neurol,* 168, 23-31.

Frieri, M. & Capetandes, A. (2008) The effect of enantiomers of beta-agonists on myofibroblast-derived vascular endothelial growth factor and other matrix components in the presence of dust-mite extract. *Allergy Asthma Proc,* 29, 182-8.

Frohman, E. M., Vayuvegula, B., Gupta, S. & van den Noort, S. (1988) Norepinephrine inhibits gamma-interferon-induced major histocompatibility class II (Ia) antigen expression on cultured astrocytes via beta-2-adrenergic signal transduction mechanisms. *Proc Natl Acad Sci U S A,* 85, 1292-6.

Froyland, E., Skjaeret, C., Wright, M. S., Dalen, M. L., Cvancarova, M., Kasi, C. & Rootwelt, T. (2008) Inflammatory receptors and pathways in human NT2-N neurons during hypoxia and reoxygenation. Impact of acidosis. *Brain Res,* 1217, 37-49.

Fu, W., Luo, H., Parthasarathy, S. & Mattson, M. P. (1998) Catecholamines potentiate amyloid beta-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. *Neurobiol Dis,* **5,** 229-43.

Fujimoto, S., Katsuki, H., Ohnishi, M., Takagi, M., Kume, T. & Akaike, A. (2007) Thrombin induces striatal neurotoxicity depending on mitogen-activated protein kinase pathways in vivo. *Neuroscience,* **144,** 694-701.

Furukawa, Y., Tomioka, N., Sato, W., Satoyoshi, E., Hayashi, K. & Furukawa, S. (1989) Catecholamines increase nerve growth factor mRNA content in both mouse astroglial cells and fibroblast cells. *FEBS Lett,* **247,** 463-7.

Galea, E., Heneka, M. T., Dello Russo, C. & Feinstein, D. L. (2003) Intrinsic regulation of brain inflammatory responses. *Cell Mol Neurobiol,* **23,** 625-35.

Galkina, E. & Ley, K. (2007) Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol,* **27,** 2292-301.

Gary, D. S., Bruce-Keller, A. J., Kindy, M. S. & Mattson, M. P. (1998) Ischemic and excitotoxic brain injury is enhanced in mice lacking the p55 tumor necrosis factor receptor. *J Cereb Blood Flow Metab,* **18,** 1283-7.

Gavrilyuk, V., Horvath, P., Weinberg, G. & Feinstein, D. L. (2001) A 27-bp region of the inducible nitric oxide synthase promoter regulates expression in glial cells. *J Neurochem,* **78,** 129-40.

Gavrilyuk, V., Dello Russo, C., Heneka, M. T., Pelligrino, D., Weinberg, G. & Feinstein, D. L. (2002) Norepinephrine increases I kappa B alpha expression in astrocytes. *J Biol Chem,* **277,** 29662-8.

Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M. & Velu, T. (1993) Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J Exp Med,* **177,** 547-50.

German, D. C., Walker, B. S., Manaye, K., Smith, W. K., Woodward, D. J. & North, A. J. (1988) The human locus coeruleus: computer reconstruction of cellular distribution. *J Neurosci,* **8,** 1776-88.

German, D. C., Manaye, K. F., White, C. L., 3rd, Woodward, D. J., McIntire, D. D., Smith, W. K., Kalaria, R. N. & Mann, D. M. (1992) Disease-specific patterns of locus coeruleus cell loss. *Ann Neurol,* **32,** 667-76.

Gibbons, H. M. & Dragunow, M. (2006) Microglia induce neural cell death via a proximity-dependent mechanism involving nitric oxide. *Brain Res,* **1084,** 1-15.

Giulian, D. & Tapscott, M. J. (1988) Immunoregulation of cells within the central nervous system. *Brain Behav Immun,* 2, 352-8.

Giulian, D., Vaca, K. & Corpuz, M. (1993) Brain glia release factors with opposing actions upon neuronal survival. *J Neurosci,* 13, 29-37.

Giulian, D., Li, J., Leara, B. & Keenen, C. (1994) Phagocytic microglia release cytokines and cytotoxins that regulate the survival of astrocytes and neurons in culture. *Neurochem Int,* 25, 227-33.

Giulian, D., Li, J., Bartel, S., Broker, J., Li, X. & Kirkpatrick, J. B. (1995) Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. *J Neurosci,* 15, 7712-26.

Glasgow, E. & Mishra, L. (2008) Transforming growth factor-beta signaling and ubiquitinators in cancer. *Endocr Relat Cancer,* 15, 59-72.

Gomez-Santos, C., Francisco, R., Gimenez-Xavier, P. & Ambrosio, S. (2007) Dopamine induces TNFalpha and TNF-R1 expression in SH-SY5Y human neuroblastoma cells. *Neuroreport,* 18, 1725-8.

Gorbacheva, L. R., Storozhevykh, T. P., Kiseleva, E. V., Pinelis, V. G. & Strukova, S. M. (2005) Proteinase-activated type 1 receptors are involved in the mechanism of protection of rat hippocampal neurons from glutamate toxicity. *Bull Exp Biol Med,* 140, 285-8.

Gorbacheva, L. R., Storozhevykh, T. P., Pinelis, V. G., Ishiwata, S. & Strukova, S. M. (2006) Modulation of hippocampal neuron survival by thrombin and factor Xa. *Biochemistry (Mosc),* 71, 1082-9.

Gough, D. J., Levy, D. E., Johnstone, R. W. & Clarke, C. J. (2008) IFNgamma signaling-does it mean JAK-STAT? *Cytokine Growth Factor Rev,* 19, 383-94.

Grabham, P. & Cunningham, D. D. (1995) Thrombin receptor activation stimulates astrocyte proliferation and reversal of stellation by distinct pathways: involvement of tyrosine phosphorylation. *J Neurochem,* 64, 583-91.

Grainger, D. J. (2007) TGF-beta and atherosclerosis in man. *Cardiovasc Res,* 74, 213-22.

Guan, J. X., Sun, S. G., Cao, X. B., Chen, Z. B. & Tong, E. T. (2004) Effect of thrombin on blood brain barrier permeability and its mechanism. *Chin Med J (Engl),* 117, 1677-81.

Gubba, E. M., Fawcett, J. W. & Herbert, J. (2004) The effects of corticosterone and dehydroepiandrosterone on neurotrophic factor mRNA expression in primary hippocampal and astrocyte cultures. *Brain Res Mol Brain Res,* 127, 48-59.

Guo, G. & Bhat, N. R. (2006) Hypoxia/reoxygenation differentially modulates NF-kappaB activation and iNOS expression in astrocytes and microglia. *Antioxid Redox Signal,* **8,** 911-8.

Halliday, G., Robinson, S. R., Shepherd, C. & Kril, J. (2000) Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms. *Clin Exp Pharmacol Physiol,* 27, 1-8.

Hamill, C. E., Caudle, W. M., Richardson, J. R., Yuan, H., Pennell, K. D., Greene, J. G., Miller, G. W. & Traynelis, S. F. (2007) Exacerbation of dopaminergic terminal damage in a mouse model of Parkinson's disease by the G-protein-coupled receptor protease-activated receptor 1. *Mol Pharmacol,* 72, 653-64.

Hanisch, U. K., van Rossum, D., Xie, Y., Gast, K., Misselwitz, R., Auriola, S., Goldsteins, G., Koistinaho, J., Kettenmann, H. & Moller, T. (2004) The microglia-activating potential of thrombin: the protease is not involved in the induction of proinflammatory cytokines and chemokines. *J Biol Chem,* 279, 51880-7.

Harrington, J. F., Messier, A. A., Levine, A., Szmydynger-Chodobska, J. & Chodobski, A. (2005) Shedding of tumor necrosis factor type 1 receptor after experimental spinal cord injury. *J Neurotrauma,* 22, 919-28.

Hashimoto, M., Ito, T., Fukumitsu, H., Nomoto, H., Furukawa, Y. & Furukawa, S. (2005) Stimulation of production of glial cell line-derived neurotrophic factor and nitric oxide by lipopolysaccharide with different dose-responsiveness in cultured rat macrophages. *Biomed Res,* 26, 223-9.

Hashioka, S., Klegeris, A., Schwab, C. & McGeer, P. L. (2009) Interferon-gamma-dependent cytotoxic activation of human astrocytes and astrocytoma cells. *Neurobiol Aging,* 30, 1924-35.

He, P., Zhong, Z., Lindholm, K., Berning, L., Lee, W., Lemere, C., Staufenbiel, M., Li, R. & Shen, Y. (2007) Deletion of tumor necrosis factor death receptor inhibits amyloid beta generation and prevents learning and memory deficits in Alzheimer's mice. *J Cell Biol,* 178, 829-41.

Heese, K., Hock, C. & Otten, U. (1998) Inflammatory signals induce neurotrophin expression in human microglial cells. *J Neurochem,* 70, 699-707.

Heneka, M. T., Galea, E., Gavriluyk, V., Dumitrescu-Ozimek, L., Daeschner, J., O'Banion, M. K., Weinberg, G., Klockgether, T. & Feinstein, D. L. (2002) Noradrenergic depletion potentiates beta -amyloid-induced cortical inflammation: implications for Alzheimer's disease. *J Neurosci,* 22, 2434-42.

Heneka, M. T., Gavrilyuk, V., Landreth, G. E., O'Banion, M. K., Weinberg, G. & Feinstein, D. L. (2003) Noradrenergic depletion increases inflammatory responses in brain: effects on IkappaB and HSP70 expression. *J Neurochem,* 85, 387-98.

Herrera, A. J., de Pablos, R. M., Carreno-Muller, E., Villaran, R. F., Venero, J. L., Tomas-Camardiel, M., Cano, J. & Machado, A. (2008) The intrastriatal injection of thrombin in rat induced a retrograde apoptotic degeneration of nigral dopaminergic neurons through synaptic elimination. *J Neurochem,* 105, 750-62.

Hua, Y., Keep, R. F., Hoff, J. T. & Xi, G. (2007) Brain injury after intracerebral hemorrhage: the role of thrombin and iron. *Stroke,* 38, 759-62.

Hua, Y., Keep, R. F., Gu, Y. & Xi, G. (2009) Thrombin and brain recovery after intracerebral hemorrhage. *Stroke,* 40, S88-9.

Huang, C., Ma, R., Sun, S., Wei, G., Fang, Y., Liu, R. & Li, G. (2008a) JAK2-STAT3 signaling pathway mediates thrombin-induced proinflammatory actions of microglia in vitro. *J Neuroimmunol,* 204, 118-25.

Huang, C. F., Li, G., Ma, R., Sun, S. G. & Chen, J. G. (2008b) Thrombin-induced microglial activation contributes to the degeneration of nigral dopaminergic neurons in vivo. *Neurosci Bull,* 24, 66-72.

Huang, Y. S. & Yang, Z. C. (2003) [Effects of thrombin peptides and thrombin on the release of TGF-beta and VEGF from human fibroblasts]. *Zhonghua Shao Shang Za Zhi,* 19 Suppl, 5-7.

Igbavboa, U., Johnson-Anuna, L. N., Rossello, X., Butterick, T. A., Sun, G. Y. & Wood, W. G. (2006) Amyloid beta-protein1-42 increases cAMP and apolipoprotein E levels which are inhibited by beta1 and beta2-adrenergic receptor antagonists in mouse primary astrocytes. *Neuroscience,* 142, 655-60.

Inanc, B., Elcin, A. E. & Elcin, Y. M. (2008) Human embryonic stem cell differentiation on tissue engineering scaffolds: effects of NGF and retinoic acid induction. *Tissue Eng Part A,* 14, 955-64.

Inazu, M., Takeda, H. & Matsumiya, T. (2003) Functional expression of the norepinephrine transporter in cultured rat astrocytes. *J Neurochem,* 84, 136-44.

Iosif, R. E., Ekdahl, C. T., Ahlenius, H., Pronk, C. J., Bonde, S., Kokaia, Z., Jacobsen, S. E. & Lindvall, O. (2006) Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. *J Neurosci,* 26, 9703-12.

Ishida, Y., Nagai, A., Kobayashi, S. & Kim, S. U. (2006) Upregulation of protease-activated receptor-1 in astrocytes in Parkinson disease: astrocyte-mediated neuroprotection through increased levels of glutathione peroxidase. *J Neuropathol Exp Neurol,* 65, 66-77.

Istrin, G., Bosis, E. & Solomon, B. (2006) Intravenous immunoglobulin enhances the clearance of fibrillar amyloid-beta peptide. *J Neurosci Res,* **84,** 434-43.

Iwai, T., Ito, S., Tanimitsu, K., Udagawa, S. & Oka, J. (2006) Glucagon-like peptide-1 inhibits LPS-induced IL-1beta production in cultured rat astrocytes. *Neurosci Res,* **55,** 352-60.

Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. (2005) *Immunobiology,* Garland Science Publishing.

Jankovic, J. (2008) Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry,* **79,** 368-76.

Jara, J. H., Singh, B. B., Floden, A. M. & Combs, C. K. (2007) Tumor necrosis factor alpha stimulates NMDA receptor activity in mouse cortical neurons resulting in ERK-dependent death. *J Neurochem,* **100,** 1407-20.

Jessen, K. R. (2004) Glial cells. *Int J Biochem Cell Biol,* **36,** 1861-7.

Johnson, M. (1998) The beta-adrenoceptor. *Am J Respir Crit Care Med,* **158,** S146-53.

Johnson, M. (2001) Beta2-adrenoceptors: mechanisms of action of beta2-agonists. *Paediatr Respir Rev,* **2,** 57-62.

Johnson, M. (2006) Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol,* **117,** 18-24; quiz 25.

Jordan, J., Galindo, M. F., Miller, R. J., Reardon, C. A., Getz, G. S. & LaDu, M. J. (1998) Isoform-specific effect of apolipoprotein E on cell survival and beta-amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. *J Neurosci,* **18,** 195-204.

Justicia, C., Martin, A., Rojas, S., Gironella, M., Cervera, A., Panes, J., Chamorro, A. & Planas, A. M. (2006) Anti-VCAM-1 antibodies did not protect against ischemic damage either in rats or in mice. *J Cereb Blood Flow Metab,* **26,** 421-32.

Kalinin, S., Polak, P. E., Madrigal, J. L., Gavrilyuk, V., Sharp, A., Chauhan, N., Marien, M., Colpaert, F. & Feinstein, D. L. (2006) Beta-amyloid-dependent expression of NOS2 in neurons: prevention by an alpha2-adrenergic antagonist. *Antioxid Redox Signal,* **8,** 873-83.

Kalinin, S., Gavrilyuk, V., Polak, P. E., Vasser, R., Zhao, J., Heneka, M. T. & Feinstein, D. L. (2007) Noradrenaline deficiency in brain increases beta-amyloid plaque burden in an animal model of Alzheimer's disease. *Neurobiol Aging,* **28,** 1206-14.

Kang, J., Park, E. J., Jou, I., Kim, J. H. & Joe, E. H. (2001) Reactive oxygen species mediate A beta(25-35)-induced activation of BV-2 microglia. *Neuroreport,* **12,** 1449-52.

Kaplanski, G., Marin, V., Fabrigoule, M., Boulay, V., Benoliel, A. M., Bongrand, P., Kaplanski, S. & Farnarier, C. (1998) Thrombin-activated human endothelial cells support monocyte adhesion in vitro following expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106). *Blood,* 92, 1259-67.

Kato, H., Kawaguchi, M., Inoue, S., Hirai, K. & Furuya, H. (2009) The effects of beta-adrenoceptor antagonists on proinflammatory cytokine concentrations after subarachnoid hemorrhage in rats. *Anesth Analg,* 108, 288-95.

Katsetos, C. D., Legido, A., Perentes, E. & Mork, S. J. (2003) Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. *J Child Neurol,* 18, 851-66; discussion 867.

Katsuki, H., Okawara, M., Shibata, H., Kume, T. & Akaike, A. (2006) Nitric oxide-producing microglia mediate thrombin-induced degeneration of dopaminergic neurons in rat midbrain slice culture. *J Neurochem,* 97, 1232-42.

Kavelaars, A., van de Pol, M., Zijlstra, J. & Heijnen, C. J. (1997) Beta 2-adrenergic activation enhances interleukin-8 production by human monocytes. *J Neuroimmunol,* 77, 211-6.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K. & Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol,* 167, 5887-94.

Keogh, B., Atkins, G. J., Mills, K. H. & Sheahan, B. J. (2003) Role of interferon-gamma and nitric oxide in the neuropathogenesis of avirulent Semliki Forest virus infection. *Neuropathol Appl Neurobiol,* 29, 553-62.

Khachaturian, A. S., Zandi, P. P., Lyketsos, C. G., Hayden, K. M., Skoog, I., Norton, M. C., Tschanz, J. T., Mayer, L. S., Welsh-Bohmer, K. A. & Breitner, J. C. (2006) Antihypertensive medication use and incident Alzheimer disease: the Cache County Study. *Arch Neurol,* 63, 686-92.

Kielian, T. & Esen, N. (2004) Effects of neuroinflammation on glia-glia gap junctional intercellular communication: a perspective. *Neurochem Int,* 45, 429-36.

Kim, K. Y., Kim, M. Y., Choi, H. S., Jin, B. K., Kim, S. U. & Lee, Y. B. (2002) Thrombin induces IL-10 production in microglia as a negative feedback regulator of TNF-alpha release. *Neuroreport,* 13, 849-52.

Kiseleva, E. V., Storozhevykh, T. P., Pinelis, V. G., Gluza, E. & Strukova, S. M. (2004) Role of thrombin in activation of neurons in rat hippocampus. *Bull Exp Biol Med,* 137, 453-6.

Kogo, J., Takeba, Y., Kumai, T., Kitaoka, Y., Matsumoto, N., Ueno, S. & Kobayashi, S. (2006) Involvement of TNF-alpha in glutamate-induced apoptosis in a differentiated neuronal cell line. *Brain Res,* 1122**,** 201-8.

Kolb, H. & Kolb-Bachofen, V. (1998) Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? *Immunol Today,* 19**,** 556-61.

Koski, C. L., Hila, S. & Hoffman, G. E. (2004) Regulation of cytokine-induced neuron death by ovarian hormones: involvement of antiapoptotic protein expression and c-JUN N-terminal kinase-mediated proapoptotic signaling. *Endocrinology,* 145**,** 95-103.

Krieglstein, K., Strelau, J., Schober, A., Sullivan, A. & Unsicker, K. (2002) TGF-beta and the regulation of neuron survival and death. *J Physiol Paris,* 96**,** 25-30.

Kuno, R., Yoshida, Y., Nitta, A., Nabeshima, T., Wang, J., Sonobe, Y., Kawanokuchi, J., Takeuchi, H., Mizuno, T. & Suzumura, A. (2006) The role of TNF-alpha and its receptors in the production of NGF and GDNF by astrocytes. *Brain Res,* 1116**,** 12-8.

LaDu, M. J., Shah, J. A., Reardon, C. A., Getz, G. S., Bu, G., Hu, J., Guo, L. & van Eldik, L. J. (2000) Apolipoprotein E receptors mediate the effects of beta-amyloid on astrocyte cultures. *J Biol Chem,* 275**,** 33974-80.

LaDu, M. J., Shah, J. A., Reardon, C. A., Getz, G. S., Bu, G., Hu, J., Guo, L. & Van Eldik, L. J. (2001) Apolipoprotein E and apolipoprotein E receptors modulate A beta-induced glial neuroinflammatory responses. *Neurochem Int,* 39**,** 427-34.

LaFerla, F. M., Sugarman, M. C., Lane, T. E. & Leissring, M. A. (2000) Regional hypomyelination and dysplasia in transgenic mice with astrocyte-directed expression of interferon-gamma. *J Mol Neurosci,* 15**,** 45-59.

Laflamme, N., Lacroix, S. & Rivest, S. (1999) An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood-brain barrier in response to a systemic and localized inflammation but not during endotoxemia. *J Neurosci,* 19**,** 10923-30.

Lai, K. B., Sanderson, J. E. & Yu, C. M. (2009) Suppression of collagen production in norepinephrine stimulated cardiac fibroblasts culture: differential effect of alpha and beta-adrenoreceptor antagonism. *Cardiovasc Drugs Ther,* 23**,** 271-80.

Lam, S. Y., Tipoe, G. L., Liong, E. C. & Fung, M. L. (2008) Chronic hypoxia upregulates the expression and function of proinflammatory cytokines in the rat carotid body. *Histochem Cell Biol,* 130**,** 549-59.

Lambertsen, K. L., Clausen, B. H., Babcock, A. A., Gregersen, R., Fenger, C., Nielsen, H. H., Haugaard, L. S., Wirenfeldt, M., Nielsen, M., Dagnaes-Hansen, F., Bluethmann, H., Faergeman, N. J., Meldgaard, M., Deierborg, T. & Finsen, B.

(2009) Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. *J Neurosci,* 29, 1319-30.

Lapchak, P. A. (1996) Therapeutic potentials for glial cell line-derived neurotrophic factor (GDNF) based upon pharmacological activities in the CNS. *Rev Neurosci,* 7, 165-76.

Lara, J., Kusano, K., House, S. & Gainer, H. (2003) Interactions of cyclic adenosine monophosphate, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor treatment on the survival and growth of postnatal mesencephalic dopamine neurons in vitro. *Exp Neurol,* 180, 32-45.

Ledeboer, A., Breve, J. J., Wierinckx, A., van der Jagt, S., Bristow, A. F., Leysen, J. E., Tilders, F. J. & Van Dam, A. M. (2002) Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. *Eur J Neurosci,* 16, 1175-85.

Lee da, Y., Park, K. W. & Jin, B. K. (2006) Thrombin induces neurodegeneration and microglial activation in the cortex in vivo and in vitro: proteolytic and non-proteolytic actions. *Biochem Biophys Res Commun,* 346, 727-38.

Lee, D. Y., Oh, Y. J. & Jin, B. K. (2005) Thrombin-activated microglia contribute to death of dopaminergic neurons in rat mesencephalic cultures: dual roles of mitogen-activated protein kinase signaling pathways. *Glia,* 51, 98-110.

Lee, G. A., Lin, C. H., Jiang, H. H., Chao, H. J., Wu, C. L. & Hsueh, C. M. (2004) Microglia-derived glial cell line-derived neurotrophic factor could protect Sprague-Dawley rat astrocyte from in vitro ischemia-induced damage. *Neurosci Lett,* 356, 111-4.

Lee, J., Kim, S. J., Son, T. G., Chan, S. L. & Mattson, M. P. (2006) Interferon-gamma is up-regulated in the hippocampus in response to intermittent fasting and protects hippocampal neurons against excitotoxicity. *J Neurosci Res,* 83, 1552-7.

Lee, S. J. & Benveniste, E. N. (1999) Adhesion molecule expression and regulation on cells of the central nervous system. *J Neuroimmunol,* 98, 77-88.

Lee, Y. B., Nagai, A. & Kim, S. U. (2002) Cytokines, chemokines, and cytokine receptors in human microglia. *J Neurosci Res,* 69, 94-103.

Leonard, B. E. (1997) *Fundamentals of Psychopharmacology*, John Wiley & Sons.

Li, L., Lu, J., Tay, S. S., Moochhala, S. M. & He, B. P. (2007) The function of microglia, either neuroprotection or neurotoxicity, is determined by the equilibrium among factors released from activated microglia in vitro. *Brain Res,* 1159, 8-17.

Li, Y., Ji, A., Weihe, E. & Schafer, M. K. (2004) Cell-specific expression and lipopolysaccharide-induced regulation of tumor necrosis factor alpha (TNFalpha) and TNF receptors in rat dorsal root ganglion. *J Neurosci,* 24**,** 9623-31.

Liang, C., Xiaonan, L., Xiaojun, C., Changjiang, L., Xinsheng, X., Guihua, J., Xiaobo, H., Yanen, Z., Runyi, S., Huixia, L., Yun, Z. & Mei, Z. (2009) Effect of metoprolol on vulnerable plaque in rabbits by changing shear stress around plaque and reducing inflammation. *Eur J Pharmacol,* 613**,** 79-85.

Liberto, C. M., Albrecht, P. J., Herx, L. M., Yong, V. W. & Levison, S. W. (2004) Pro-regenerative properties of cytokine-activated astrocytes. *J Neurochem,* 89**,** 1092-100.

Liew, F. Y., Xu, D., Brint, E. K. & O'Neill, L. A. (2005) Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol,* 5**,** 446-58.

Lin, C. H., Cheng, F. C., Lu, Y. Z., Chu, L. F., Wang, C. H. & Hsueh, C. M. (2006) Protection of ischemic brain cells is dependent on astrocyte-derived growth factors and their receptors. *Exp Neurol,* 201**,** 225-33.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. & Collins, F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science,* 260**,** 1130-2.

Linda, H., Hammarberg, H., Cullheim, S., Levinovitz, A., Khademi, M. & Olsson, T. (1998) Expression of MHC class I and beta2-microglobulin in rat spinal motoneurons: regulatory influences by IFN-gamma and axotomy. *Exp Neurol,* 150**,** 282-95.

Liu, B. & Hong, J. S. (2003) Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther,* 304**,** 1-7.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, L. & Darnell, J. (2003) *Molecular Cell Biology*, W. H. Freeman and Co.

Loetscher, M., Gerber, B., Loetscher, P., Jones, S. A., Piali, L., Clark-Lewis, I., Baggiolini, M. & Moser, B. (1996) Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med,* 184**,** 963-9.

Lomb, D. J., Desouza, L. A., Franklin, J. L. & Freeman, R. S. (2009) Prolyl hydroxylase inhibitors depend on extracellular glucose and hypoxia-inducible factor (HIF)-2alpha to inhibit cell death caused by nerve growth factor (NGF) deprivation: evidence that HIF-2alpha has a role in NGF-promoted survival of sympathetic neurons. *Mol Pharmacol,* 75**,** 1198-209.

Lu, Y. Z., Lin, C. H., Cheng, F. C. & Hsueh, C. M. (2005) Molecular mechanisms responsible for microglia-derived protection of Sprague-Dawley rat brain cells during in vitro ischemia. *Neurosci Lett,* 373, 159-64.

Lucas, S. M., Rothwell, N. J. & Gibson, R. M. (2006) The role of inflammation in CNS injury and disease. *Br J Pharmacol,* 147 Suppl 1, S232-40.

Lue, L. F. & Walker, D. G. (2002) Modeling Alzheimer's disease immune therapy mechanisms: interactions of human postmortem microglia with antibody-opsonized amyloid beta peptide. *J Neurosci Res,* 70, 599-610.

Luo, W., Wang, Y. & Reiser, G. (2007a) Protease-activated receptors in the brain: receptor expression, activation, and functions in neurodegeneration and neuroprotection. *Brain Res Rev,* 56, 331-45.

Luo, W., Wickramasinghe, S. R., Savitt, J. M., Griffin, J. W., Dawson, T. M. & Ginty, D. D. (2007b) A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. *Neuron,* 54, 739-54.

Luster, A. D. (1998) Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med,* 338, 436-45.

Lynch, J. R., Morgan, D., Mance, J., Matthew, W. D. & Laskowitz, D. T. (2001) Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. *J Neuroimmunol,* 114, 107-13.

Ma, X. C., Gottschall, P. E., Chen, L. T., Wiranowska, M. & Phelps, C. P. (2002) Role and mechanisms of interleukin-1 in the modulation of neurotoxicity. *Neuroimmunomodulation,* 10, 199-207.

Machida, I., Matsuse, H., Kondo, Y., Kawano, T., Saeki, S., Tomari, S., Obase, Y., Fukushima, C. & Kohno, S. (2005) Effects of various anti-asthmatic agents on mite allergen-pulsed murine bone marrow-derived dendritic cells. *Clin Exp Allergy,* 35, 884-8.

Mackay, C. R. (1997) Chemokines: what chemokine is that? *Curr Biol,* 7, R384-6.

Madrigal, J. L., Feinstein, D. L. & Dello Russo, C. (2005) Norepinephrine protects cortical neurons against microglial-induced cell death. *J Neurosci Res,* 81, 390-6.

Madrigal, J. L., Dello Russo, C., Gavrilyuk, V. & Feinstein, D. L. (2006) Effects of noradrenaline on neuronal NOS2 expression and viability. *Antioxid Redox Signal,* 8, 885-92.

Madrigal, J. L., Leza, J. C., Polak, P., Kalinin, S. & Feinstein, D. L. (2009) Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline. *J Neurosci,* 29, 263-7.

Mahul-Mellier, A. L., Strappazzon, F., Petiot, A., Chatellard-Causse, C., Torch, S., Blot, B., Freeman, K., Kuhn, L., Garin, J., Verna, J. M., Fraboulet, S. & Sadoul, R. (2008) Alix and ALG-2 are involved in tumor necrosis factor receptor 1-induced cell death. *J Biol Chem,* 283**,** 34954-65.

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K. & Eisel, U. L. (2004) Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *J Biol Chem,* 279**,** 32869-81.

Marien, M. R., Colpaert, F. C. & Rosenquist, A. C. (2004) Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev,* 45**,** 38-78.

Marin, V., Farnarier, C., Gres, S., Kaplanski, S., Su, M. S., Dinarello, C. A. & Kaplanski, G. (2001) The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. *Blood,* 98**,** 667-73.

Martin, D. S., Lonergan, P. E., Boland, B., Fogarty, M. P., Brady, M., Horrobin, D. F., Campbell, V. A. & Lynch, M. A. (2002) Apoptotic changes in the aged brain are triggered by interleukin-1beta-induced activation of p38 and reversed by treatment with eicosapentaenoic acid. *J Biol Chem,* 277**,** 34239-46.

Matrone, C., Ciotti, M. T., Mercanti, D., Marolda, R. & Calissano, P. (2008) NGF and BDNF signaling control amyloidogenic route and Abeta production in hippocampal neurons. *Proc Natl Acad Sci U S A,* 105**,** 13139-44.

Matyszak, M. K., Denis-Donini, S., Citterio, S., Longhi, R., Granucci, F. & Ricciardi-Castagnoli, P. (1999) Microglia induce myelin basic protein-specific T cell anergy or T cell activation, according to their state of activation. *Eur J Immunol,* 29**,** 3063-76.

McGeer, E. G. & McGeer, P. L. (2003) Inflammatory processes in Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry,* 27**,** 741-9.

McGuire, S. O., Ling, Z. D., Lipton, J. W., Sortwell, C. E., Collier, T. J. & Carvey, P. M. (2001) Tumor necrosis factor alpha is toxic to embryonic mesencephalic dopamine neurons. *Exp Neurol,* 169**,** 219-30.

McNamee, E. N., Ryan, K. M., Kilroy, D. & Connor, T. J. (2010) Noradrenaline induces IL-1ra and IL-1 type II receptor expression in primary glial cells and protects against IL-1beta-induced neurotoxicity. *Eur J Pharmacol,* 626**,** 219-28.

Meda, L., Baron, P., Prat, E., Scarpini, E., Scarlato, G., Cassatella, M. A. & Rossi, F. (1999) Proinflammatory profile of cytokine production by human monocytes and murine microglia stimulated with beta-amyloid[25-35]. *J Neuroimmunol,* 93, 45-52.

Mehler, M. F. & Kessler, J. A. (1997) Hematolymphopoietic and inflammatory cytokines in neural development. *Trends Neurosci,* 20, 357-65.

Mennicken, F., Maki, R., de Souza, E. B. & Quirion, R. (1999) Chemokines and chemokine receptors in the CNS: a possible role in neuroinflammation and patterning. *Trends Pharmacol Sci,* 20, 73-8.

Mills, K. H. & McGuirk, P. (2004) Antigen-specific regulatory T cells--their induction and role in infection. *Semin Immunol,* 16, 107-17.

Min, K. J., Yang, M. S., Jou, I. & Joe, E. H. (2004) Protein kinase A mediates microglial activation induced by plasminogen and gangliosides. *Exp Mol Med,* 36, 461-7.

Miyabayashi, K., Maruyama, M., Yamada, T., Shinoda, C., Hounoki, H., Kanatani, Y., Shinoda, K., Kawagishi, Y., Miwa, T., Suzuki, K., Arai, N., Hayashi, R., Matsui, S., Sugiyama, E. & Kobayashi, M. (2006) Isoproterenol suppresses cytokine-induced RANTES secretion in human lung epithelial cells through the inhibition of c-jun N-terminal kinase pathway. *Biochem Biophys Res Commun,* 350, 753-61.

Mizuta, I., Ohta, M., Ohta, K., Nishimura, M., Mizuta, E. & Kuno, S. (2001) Riluzole stimulates nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor synthesis in cultured mouse astrocytes. *Neurosci Lett,* 310, 117-20.

Mocchetti, I., De Bernardi, M. A., Szekely, A. M., Alho, H., Brooker, G. & Costa, E. (1989) Regulation of nerve growth factor biosynthesis by beta-adrenergic receptor activation in astrocytoma cells: a potential role of c-Fos protein. *Proc Natl Acad Sci U S A,* 86, 3891-5.

Mogi, M., Togari, A., Kondo, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H. & Nagatsu, T. (2000) Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. *J Neural Transm,* 107, 335-41.

Moises, T., Dreier, A., Flohr, S., Esser, M., Brauers, E., Reiss, K., Merken, D., Weis, J. & Kruttgen, A. (2007) Tracking TrkA's trafficking: NGF receptor trafficking controls NGF receptor signaling. *Mol Neurobiol,* 35, 151-9.

Mok, S. A., Lund, K. & Campenot, R. B. (2009) A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. *Cell Res,* 19, 546-60.

Moller, T., Hanisch, U. K. & Ransom, B. R. (2000) Thrombin-induced activation of cultured rodent microglia. *J Neurochem,* 75, 1539-47.

Moncada, S. (1999) Nitric oxide: discovery and impact on clinical medicine. *J R Soc Med,* 92, 164-9.

Montaldo, P. G., Carbone, R., Corrias, M. V., Ferraris, P. C. & Ponzoni, M. (1994) Synergistic differentiation-promoting activity of interferon gamma and tumor necrosis factor-alpha: role of receptor regulation on human neuroblasts. *J Natl Cancer Inst,* 86, 1694-701.

Moore, A. H. & O'Banion, M. K. (2002) Neuroinflammation and anti-inflammatory therapy for Alzheimer's disease. *Adv Drug Deliv Rev,* 54, 1627-56.

Mori, K., Kaneko, Y. S., Nakashima, A., Nagatsu, I., Takahashi, H. & Ota, A. (2005) Peripheral lipopolysaccharide induces apoptosis in the murine olfactory bulb. *Brain Res,* 1039, 116-29.

Morin, D., Sapena, R., Zini, R., Onteniente, B. & Tillement, J. P. (1997) Characterization of beta-adrenergic receptors of freshly isolated astrocytes and neurons from rat brain. *Life Sci,* 60, 315-24.

Morioka, N., Tanabe, H., Inoue, A., Dohi, T. & Nakata, Y. (2009) Noradrenaline reduces the ATP-stimulated phosphorylation of p38 MAP kinase via beta-adrenergic receptors-cAMP-protein kinase A-dependent mechanism in cultured rat spinal microglia. *Neurochem Int,* 55, 226-34.

Mosser, D. M. & Zhang, X. (2008) Interleukin-10: new perspectives on an old cytokine. *Immunol Rev,* 226, 205-18.

Mount, M. P., Lira, A., Grimes, D., Smith, P. D., Faucher, S., Slack, R., Anisman, H., Hayley, S. & Park, D. S. (2007) Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. *J Neurosci,* 27, 3328-37.

Mullen, R. J., Buck, C. R. & Smith, A. M. (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development,* 116, 201-11.

Munch, G., Gasic-Milenkovic, J., Dukic-Stefanovic, S., Kuhla, B., Heinrich, K., Riederer, P., Huttunen, H. J., Founds, H. & Sajithlal, G. (2003) Microglial activation induces cell death, inhibits neurite outgrowth and causes neurite retraction of differentiated neuroblastoma cells. *Exp Brain Res,* 150, 1-8.

Nagahara, A. H., Bernot, T., Moseanko, R., Brignolo, L., Blesch, A., Conner, J. M., Ramirez, A., Gasmi, M. & Tuszynski, M. H. (2009) Long-term reversal of cholinergic neuronal decline in aged non-human primates by lentiviral NGF gene delivery. *Exp Neurol,* 215, 153-9.

Nagai, A., Nakagawa, E., Hatori, K., Choi, H. B., McLarnon, J. G., Lee, M. A. & Kim, S. U. (2001) Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis,* 8, 1057-68.

Nakajima, K., Tohyama, Y., Maeda, S., Kohsaka, S. & Kurihara, T. (2007) Neuronal regulation by which microglia enhance the production of neurotrophic factors for GABAergic, catecholaminergic, and cholinergic neurons. *Neurochem Int,* **50,** 807-20.

Nakamura, A., Johns, E. J., Imaizumi, A., Abe, T. & Kohsaka, T. (1998) Regulation of tumour necrosis factor and interleukin-6 gene transcription by beta2-adrenoceptor in the rat astrocytes. *J Neuroimmunol,* **88,** 144-53.

Nakashima, A., Kumakura, S., Mishima, S., Ishikura, H. & Kobayashi, S. (2005) IFN-alpha enhances TNF-alpha-induced apoptosis through down-regulation of c-Myc protein expression in HL-60 cells. *J Exp Clin Cancer Res,* **24,** 447-56.

Naldini, A., Pucci, A., Carney, D. H., Fanetti, G. & Carraro, F. (2002) Thrombin enhancement of interleukin-1 expression in mononuclear cells: involvement of proteinase-activated receptor-1. *Cytokine,* **20,** 191-9.

Naldini, A., Morena, E., Filippi, I., Pucci, A., Bucci, M., Cirino, G. & Carraro, F. (2006) Thrombin inhibits IFN-gamma production in human peripheral blood mononuclear cells by promoting a Th2 profile. *J Interferon Cytokine Res,* **26,** 793-9.

Nathan, C. (1997) Inducible nitric oxide synthase: what difference does it make? *J Clin Invest,* **100,** 2417-23.

Neumann, H., Schmidt, H., Cavalie, A., Jenne, D. & Wekerle, H. (1997a) Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha. *J Exp Med,* **185,** 305-16.

Neumann, H., Schmidt, H., Wilharm, E., Behrens, L. & Wekerle, H. (1997b) Interferon gamma gene expression in sensory neurons: evidence for autocrine gene regulation. *J Exp Med,* **186,** 2023-31.

Neveu, I., Jehan, F., Jandrot-Perrus, M., Wion, D. & Brachet, P. (1993) Enhancement of the synthesis and secretion of nerve growth factor in primary cultures of glial cells by proteases: a possible involvement of thrombin. *J Neurochem,* **60,** 858-67.

Nguyen, N., Lee, S. B., Lee, Y. S., Lee, K. H. & Ahn, J. Y. (2009) Neuroprotection by NGF and BDNF against neurotoxin-exerted apoptotic death in neural stem cells are mediated through Trk receptors, activating PI3-kinase and MAPK pathways. *Neurochem Res,* **34,** 942-51.

Nguyen, V. T., Walker, W. S. & Benveniste, E. N. (1998) Post-transcriptional inhibition of CD40 gene expression in microglia by transforming growth factor-beta. *Eur J Immunol,* **28,** 2537-48.

Ni, Y., Zhao, X., Bao, G., Zou, L., Teng, L., Wang, Z., Song, M., Xiong, J., Bai, Y. & Pei, G. (2006) Activation of beta2-adrenergic receptor stimulates gamma-secretase activity and accelerates amyloid plaque formation. *Nat Med,* 12**,** 1390-6.

Nicholson, T. E., Dibb, S. & Renton, K. W. (2004) Nitric oxide mediates an LPS-induced depression of cytochrome P450 (CYP1A) activity in astrocytes. *Brain Res,* 1029**,** 148-54.

Nishii, M., Inomata, T., Niwano, H., Takehana, H., Takeuchi, I., Nakano, H., Shinagawa, H., Naruke, T., Koitabashi, T., Nakahata, J. & Izumi, T. (2006) Beta2-Adrenergic agonists suppress rat autoimmune myocarditis: potential role of beta2-adrenergic stimulants as new therapeutic agents for myocarditis. *Circulation,* 114**,** 936-44.

O'Barr, S. & Cooper, N. R. (2000) The C5a complement activation peptide increases IL-1beta and IL-6 release from amyloid-beta primed human monocytes: implications for Alzheimer's disease. *J Neuroimmunol,* 109**,** 87-94.

O'Keefe, G. M., Nguyen, V. T. & Benveniste, E. N. (2002) Regulation and function of class II major histocompatibility complex, CD40, and B7 expression in macrophages and microglia: Implications in neurological diseases. *J Neurovirol,* 8**,** 496-512.

Ohnishi, M., Katsuki, H., Takagi, M., Kume, T. & Akaike, A. (2009) Long-term treatment with nicotine suppresses neurotoxicity of, and microglial activation by, thrombin in cortico-striatal slice cultures. *Eur J Pharmacol,* 602**,** 288-93.

Ohta, K., Ohta, M., Mizuta, I., Fujinami, A., Shimazu, S., Sato, N., Yoneda, F., Hayashi, K. & Kuno, S. (2002) The novel catecholaminergic and serotoninergic activity enhancer R-(-)-1-(benzofuran-2-yl)-2-propylaminopentane up-regulates neurotrophic factor synthesis in mouse astrocytes. *Neurosci Lett,* 328**,** 205-8.

Ohta, K., Kuno, S., Mizuta, I., Fujinami, A., Matsui, H. & Ohta, M. (2003) Effects of dopamine agonists bromocriptine, pergolide, cabergoline, and SKF-38393 on GDNF, NGF, and BDNF synthesis in cultured mouse astrocytes. *Life Sci,* 73**,** 617-26.

Ohtori, S., Takahashi, K., Moriya, H. & Myers, R. R. (2004) TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: studies in murine DRG and spinal cord. *Spine (Phila Pa 1976),* 29**,** 1082-8.

Olanow, C. W. & Tatton, W. G. (1999) Etiology and pathogenesis of Parkinson's disease. *Annu Rev Neurosci,* 22**,** 123-44.

Ott, A., Breteler, M. M., van Harskamp, F., Claus, J. J., van der Cammen, T. J., Grobbee, D. E. & Hofman, A. (1995) Prevalence of Alzheimer's disease and vascular dementia: association with education. The Rotterdam study. *BMJ,* 310, 970-3.

Pannu, R. & Singh, I. (2006) Pharmacological strategies for the regulation of inducible nitric oxide synthase: neurodegenerative versus neuroprotective mechanisms. *Neurochem Int,* 49, 170-82.

Park, K. W. & Jin, B. K. (2008) Thrombin-induced oxidative stress contributes to the death of hippocampal neurons: role of neuronal NADPH oxidase. *J Neurosci Res,* 86, 1053-63.

Parkash, V., Leppanen, V. M., Virtanen, H., Jurvansuu, J. M., Bespalov, M. M., Sidorova, Y. A., Runeberg-Roos, P., Saarma, M. & Goldman, A. (2008) The structure of the glial cell line-derived neurotrophic factor-coreceptor complex: insights into RET signaling and heparin binding. *J Biol Chem,* 283, 35164-72.

Parnavelas, J. G. & Papadopoulos, G. C. (1989) The monoaminergic innervation of the cerebral cortex is not diffuse and nonspecific. *Trends Neurosci,* 12, 315-9.

Paschalis, A., Churchill, L., Marina, N., Kasymov, V., Gourine, A. & Ackland, G. (2009) beta1-Adrenoceptor distribution in the rat brain: an immunohistochemical study. *Neurosci Lett,* 458, 84-8.

Patel, J. R. & Brewer, G. J. (2008) Age-related differences in NFkappaB translocation and Bcl-2/Bax ratio caused by TNFalpha and Abeta42 promote survival in middle-age neurons and death in old neurons. *Exp Neurol,* 213, 93-100.

Pawate, S., Shen, Q., Fan, F. & Bhat, N. R. (2004) Redox regulation of glial inflammatory response to lipopolysaccharide and interferongamma. *J Neurosci Res,* 77, 540-51.

Peek, E. J., Richards, D. F., Faith, A., Lavender, P., Lee, T. H., Corrigan, C. J. & Hawrylowicz, C. M. (2005) Interleukin-10-secreting "regulatory" T cells induced by glucocorticoids and beta2-agonists. *Am J Respir Cell Mol Biol,* 33, 105-11.

Pelidou, S. H., Schultzberg, M. & Iverfeldt, K. (2002) Increased sensitivity to N-methyl-D-aspartate receptor-induced excitotoxicity in cerebellar granule cells from interleukin-1 receptor type I-deficient mice. *J Neuroimmunol,* 133, 108-15.

Peterson, J. W., Bo, L., Mork, S., Chang, A., Ransohoff, R. M. & Trapp, B. D. (2002) VCAM-1-positive microglia target oligodendrocytes at the border of multiple sclerosis lesions. *J Neuropathol Exp Neurol,* 61, 539-46.

Poirier, J., Delisle, M. C., Quirion, R., Aubert, I., Farlow, M., Lahiri, D., Hui, S., Bertrand, P., Nalbantoglu, J., Gilfix, B. M. & Gauthier, S. (1995) Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proc Natl Acad Sci U S A,* 92, 12260-4.

Popko, B. & Baerwald, K. D. (1999) Oligodendroglial response to the immune cytokine interferon gamma. *Neurochem Res,* 24**,** 331-8.

Pradillo, J. M., Romera, C., Hurtado, O., Cardenas, A., Moro, M. A., Leza, J. C., Davalos, A., Castillo, J., Lorenzo, P. & Lizasoain, I. (2005) TNFR1 upregulation mediates tolerance after brain ischemic preconditioning. *J Cereb Blood Flow Metab,* 25**,** 193-203.

Preiss, D. J. & Sattar, N. (2007) Vascular cell adhesion molecule-1: a viable therapeutic target for atherosclerosis? *Int J Clin Pract,* 61**,** 697-701.

Pyo, H., Jou, I., Jung, S. & Joe, E. (1999) cAMP potentiates beta-amyloid-induced nitric oxide release from microglia. *Neuroreport,* 10**,** 37-40.

Qian, L., Hu, X., Zhang, D., Snyder, A., Wu, H. M., Li, Y., Wilson, B., Lu, R. B., Hong, J. S. & Flood, P. M. (2009) beta2 Adrenergic receptor activation induces microglial NADPH oxidase activation and dopaminergic neurotoxicity through an ERK-dependent/protein kinase A-independent pathway. *Glia.*

Quarless, S. A. & Heinrich, G. (1987) Regulation of nerve growth factor gene expression in primary brain monolayer cultures: effects of dibutyryl cyclic AMP and sodium butyrate. *Brain Res,* 388**,** 235-42.

Rahimi, R. A. & Leof, E. B. (2007) TGF-beta signaling: a tale of two responses. *J Cell Biochem,* 102**,** 593-608.

Raivich, G., Liu, Z. Q., Kloss, C. U., Labow, M., Bluethmann, H. & Bohatschek, M. (2002) Cytotoxic potential of proinflammatory cytokines: combined deletion of TNF receptors TNFR1 and TNFR2 prevents motoneuron cell death after facial axotomy in adult mouse. *Exp Neurol,* 178**,** 186-93.

Rami, A., Volkmann, T., Agarwal, R., Schoninger, S., Nurnberger, F., Saido, T. C. & Winckler, J. (2003) beta2-Adrenergic receptor responsiveness of the calpain-calpastatin system and attenuation of neuronal death in rat hippocampus after transient global ischemia. *Neurosci Res,* 47**,** 373-82.

Rang, H. P., Dale, M. M., Ritter, J. M. & Moore, P. (1999) *Pharmacology,* London, Churchill Livingston.

Rao, H. V., Thirumangalakudi, L., Desmond, P. & Grammas, P. (2007) Cyclin D1, cdk4, and Bim are involved in thrombin-induced apoptosis in cultured cortical neurons. *J Neurochem,* 101**,** 498-505.

Robinson, E. & Hudson, A. (2000) Adrenoceptor Pharmacology. *Tocris Reviews,* No.8.

Rogers, J., Strohmeyer, R., Kovelowski, C. J. & Li, R. (2002) Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia,* 40**,** 260-9.

Roitt, I., Brostoff, J. & Male, D. (2001) *Immunology,* London, Mosby.

Rothwell, N. (2003) Interleukin-1 and neuronal injury: mechanisms, modification, and therapeutic potential. *Brain Behav Immun,* 17, 152-7.

Russo-Neustadt, A. & Cotman, C. W. (1997) Adrenergic receptors in Alzheimer's disease brain: selective increases in the cerebella of aggressive patients. *J Neurosci,* 17, 5573-80.

Ryan, K. M. (2009) *Beta2-adrenoceptors as modulators of glial immune function.* Trinity College Dublin

Ryu, J., Pyo, H., Jou, I. & Joe, E. (2000) Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase, and NF-kappa B. *J Biol Chem,* 275, 29955-9.

Saez, E. T., Pehar, M., Vargas, M. R., Barbeito, L. & Maccioni, R. B. (2006) Production of nerve growth factor by beta-amyloid-stimulated astrocytes induces p75NTR-dependent tau hyperphosphorylation in cultured hippocampal neurons. *J Neurosci Res,* 84, 1098-106.

Sanchez, A., Rao, H. V. & Grammas, P. (2009) PACAP38 protects rat cortical neurons against the neurotoxicity evoked by sodium nitroprusside and thrombin. *Regul Pept,* 152, 33-40.

Saperstein, S., Chen, L., Oakes, D., Pryhuber, G. & Finkelstein, J. (2009) IL-1beta augments TNF-alpha-mediated inflammatory responses from lung epithelial cells. *J Interferon Cytokine Res,* 29, 273-84.

Sariola, H. & Saarma, M. (2003) Novel functions and signalling pathways for GDNF. *J Cell Sci,* 116, 3855-62.

Schmidt, M., Evellin, S., Weernink, P. A., von Dorp, F., Rehmann, H., Lomasney, J. W. & Jakobs, K. H. (2001) A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat Cell Biol,* 3, 1020-4.

Schubert, P., Morino, T., Miyazaki, H., Ogata, T., Nakamura, Y., Marchini, C. & Ferroni, S. (2000) Cascading glia reactions: a common pathomechanism and its differentiated control by cyclic nucleotide signaling. *Ann N Y Acad Sci,* 903, 24-33.

Schulte-Herbruggen, O., Hamker, U., Meske, V., Danker-Hopfe, H., Ohm, T. G. & Hellweg, R. (2007) Beta/A4-Amyloid increases nerve growth factor production in rat primary hippocampal astrocyte cultures. *Int J Dev Neurosci,* 25, 387-90.

Schwartz, J. P. & Mishler, K. (1990) Beta-adrenergic receptor regulation, through cyclic AMP, of nerve growth factor expression in rat cortical and cerebellar astrocytes. *Cell Mol Neurobiol,* 10, 447-57.

Schwarz, U. R., Kobsar, A. L., Koksch, M., Walter, U. & Eigenthaler, M. (2000) Inhibition of agonist-induced p42 and p38 mitogen-activated protein kinase phosphorylation and CD40 ligand/P-selectin expression by cyclic nucleotide-regulated pathways in human platelets. *Biochem Pharmacol,* 60, 1399-407.

Sebastiani, G., Morissette, C., Lagace, C., Boule, M., Ouellette, M. J., McLaughlin, R. W., Lacombe, D., Gervais, F. & Tremblay, P. (2006) The cAMP-specific phosphodiesterase 4B mediates Abeta-induced microglial activation. *Neurobiol Aging,* 27, 691-701.

Semkova, I. & Krieglstein, J. (1999) Neuroprotection mediated via neurotrophic factors and induction of neurotrophic factors. *Brain Res Brain Res Rev,* 30, 176-88.

Shen, Q., Zhang, R. & Bhat, N. R. (2006) MAP kinase regulation of IP10/CXCL10 chemokine gene expression in microglial cells. *Brain Res,* 1086, 9-16.

Shen, Y., Li, R. & Shiosaki, K. (1997) Inhibition of p75 tumor necrosis factor receptor by antisense oligonucleotides increases hypoxic injury and beta-amyloid toxicity in human neuronal cell line. *J Biol Chem,* 272, 3550-3.

Sheng, W. S., Hu, S., Kravitz, F. H., Peterson, P. K. & Chao, C. C. (1995) Tumor necrosis factor alpha upregulates human microglial cell production of interleukin-10 in vitro. *Clin Diagn Lab Immunol,* 2, 604-8.

Sheng, W. S., Hu, S., Ni, H. T., Rowen, T. N., Lokensgard, J. R. & Peterson, P. K. (2005) TNF-alpha-induced chemokine production and apoptosis in human neural precursor cells. *J Leukoc Biol,* 78, 1233-41.

Sherer, T. B., Spitsbergen, J. M., Steers, W. D. & Tuttle, J. B. (1997) Thrombin regulates nerve growth factor secretion from vascular, but not bladder smooth muscle cells. *Cell Tissue Res,* 289, 155-61.

Sherer, T. B., Clemow, D. B. & Tuttle, J. B. (2000) Calcium homeostasis and nerve growth factor secretion from vascular and bladder smooth muscle cells. *Cell Tissue Res,* 299, 201-11.

Shin, R. W., Ogino, K., Kondo, A., Saido, T. C., Trojanowski, J. Q., Kitamoto, T. & Tateishi, J. (1997) Amyloid beta-protein (Abeta) 1-40 but not Abeta1-42 contributes to the experimental formation of Alzheimer disease amyloid fibrils in rat brain. *J Neurosci,* 17, 8187-93.

Siegmund, B., Eigler, A., Hartmann, G., Hacker, U. & Endres, S. (1998) Adrenaline enhances LPS-induced IL-10 synthesis: evidence for protein kinase A-mediated pathway. *Int J Immunopharmacol,* 20, 57-69.

Sokolova, E. & Reiser, G. (2008) Prothrombin/thrombin and the thrombin receptors PAR-1 and PAR-4 in the brain: localization, expression and participation in neurodegenerative diseases. *Thromb Haemost,* 100, 576-81.

Souza, D. G., Guabiraba, R., Pinho, V., Bristow, A., Poole, S. & Teixeira, M. M. (2003) IL-1-driven endogenous IL-10 production protects against the systemic and local acute inflammatory response following intestinal reperfusion injury. *J Immunol,* **170,** 4759-66.

Stevens, B. (2003) Glia: much more than the neuron's side-kick. *Curr Biol,* **13,** R469-72.

Stewart, A. G., Tomlinson, P. R. & Wilson, J. W. (1997) Beta 2-adrenoceptor agonist-mediated inhibition of human airway smooth muscle cell proliferation: importance of the duration of beta 2-adrenoceptor stimulation. *Br J Pharmacol,* **121,** 361-8.

Stoll, G., Jander, S. & Schroeter, M. (2000) Cytokines in CNS disorders: neurotoxicity versus neuroprotection. *J Neural Transm Suppl,* **59,** 81-9.

Streit, W. J., Graeber, M. B. & Kreutzberg, G. W. (1988) Functional plasticity of microglia: a review. *Glia,* **1,** 301-7.

Streit, W. J., Mrak, R. E. & Griffin, W. S. (2004) Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation,* **1,** 14.

Stuchbury, G. & Munch, G. (2005) Alzheimer's associated inflammation, potential drug targets and future therapies. *J Neural Transm,* **112,** 429-53.

Stuehr, D. J. (1999) Mammalian nitric oxide synthases. *Biochim Biophys Acta,* **1411,** 217-30.

Suidan, H. S., Niclou, S. P. & Monard, D. (1996) The thrombin receptor in the nervous system. *Semin Thromb Hemost,* **22,** 125-33.

Suo, Z., Tan, J., Placzek, A., Crawford, F., Fang, C. & Mullan, M. (1998) Alzheimer's beta-amyloid peptides induce inflammatory cascade in human vascular cells: the roles of cytokines and CD40. *Brain Res,* **807,** 110-7.

Szaba, F. M. & Smiley, S. T. (2002) Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion in vivo. *Blood,* **99,** 1053-9.

Szczepanik, A. M., Funes, S., Petko, W. & Ringheim, G. E. (2001) IL-4, IL-10 and IL-13 modulate A beta(1--42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. *J Neuroimmunol,* **113,** 49-62.

Takahashi, H. K., Iwagaki, H., Mori, S., Yoshino, T., Tanaka, N. & Nishibori, M. (2004) Beta 2-adrenergic receptor agonist induces IL-18 production without IL-12 production. *J Neuroimmunol,* **151,** 137-47.

Takei, Y. & Laskey, R. (2008) Tumor necrosis factor alpha regulates responses to nerve growth factor, promoting neural cell survival but suppressing differentiation of neuroblastoma cells. *Mol Biol Cell,* 19**,** 855-64.

Tanaka, K. F., Kashima, H., Suzuki, H., Ono, K. & Sawada, M. (2002) Existence of functional beta1- and beta2-adrenergic receptors on microglia. *J Neurosci Res,* 70**,** 232-7.

Tansey, M. G., McCoy, M. K. & Frank-Cannon, T. C. (2007) Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp Neurol,* 208**,** 1-25.

Taylor, D. L., Jones, F., Kubota, E. S. & Pocock, J. M. (2005) Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microglial-derived Fas ligand. *J Neurosci,* 25**,** 2952-64.

Thirumangalakudi, L., Rao, H. V. & Grammas, P. (2009) Involvement of PGE2 and PGDH but not COX-2 in thrombin-induced cortical neuron apoptosis. *Neurosci Lett,* 452**,** 172-5.

Titheradge, M. A. (1999) Nitric oxide in septic shock. *Biochim Biophys Acta,* 1411**,** 437-55.

Tokay, T., Hachem, R., Masmoudi-Kouki, O., Gandolfo, P., Desrues, L., Leprince, J., Castel, H., Diallo, M., Amri, M., Vaudry, H. & Tonon, M. C. (2008) Beta-amyloid peptide stimulates endozepine release in cultured rat astrocytes through activation of N-formyl peptide receptors. *Glia,* 56**,** 1380-9.

Tokumine, J., Kakinohana, O., Cizkova, D., Smith, D. W. & Marsala, M. (2003) Changes in spinal GDNF, BDNF, and NT-3 expression after transient spinal cord ischemia in the rat. *J Neurosci Res,* 74**,** 552-61.

Tomlinson, P. R., Wilson, J. W. & Stewart, A. G. (1994) Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br J Pharmacol,* 111**,** 641-7.

Tomlinson, P. R., Wilson, J. W. & Stewart, A. G. (1995) Salbutamol inhibits the proliferation of human airway smooth muscle cells grown in culture: relationship to elevated cAMP levels. *Biochem Pharmacol,* 49**,** 1809-19.

Tosaki, T., Kamiya, H., Yasuda, Y., Naruse, K., Kato, K., Kozakae, M., Nakamura, N., Shibata, T., Hamada, Y., Nakashima, E., Oiso, Y. & Nakamura, J. (2008) Reduced NGF secretion by Schwann cells under the high glucose condition decreases neurite outgrowth of DRG neurons. *Exp Neurol,* 213**,** 381-7.

Town, T., Tan, J. & Mullan, M. (2001) CD40 signaling and Alzheimer's disease pathogenesis. *Neurochem Int,* 39**,** 371-80.

Traver, S., Salthun-Lassalle, B., Marien, M., Hirsch, E. C., Colpaert, F. & Michel, P. P. (2005) The neurotransmitter noradrenaline rescues septal cholinergic neurons in culture from degeneration caused by low-level oxidative stress. *Mol Pharmacol,* **67,** 1882-91.

Troadec, J. D., Marien, M., Darios, F., Hartmann, A., Ruberg, M., Colpaert, F. & Michel, P. P. (2001) Noradrenaline provides long-term protection to dopaminergic neurons by reducing oxidative stress. *J Neurochem,* 79, 200-10.

Tsai, S. Y., Schluns, K. S., Le, P. T. & McNulty, J. A. (2001) TGF-beta1 and IL-6 expression in rat pineal gland is regulated by norepinephrine and interleukin-1beta. *Histol Histopathol,* **16,** 1135-41.

Tsakiri, N., Kimber, I., Rothwell, N. J. & Pinteaux, E. (2008a) Differential effects of interleukin-1 alpha and beta on interleukin-6 and chemokine synthesis in neurones. *Mol Cell Neurosci,* **38,** 259-65.

Tsakiri, N., Kimber, I., Rothwell, N. J. & Pinteaux, E. (2008b) Interleukin-1-induced interleukin-6 synthesis is mediated by the neutral sphingomyelinase/Src kinase pathway in neurones. *Br J Pharmacol,* **153,** 775-83.

Tsuda, M., Masuda, T., Kitano, J., Shimoyama, H., Tozaki-Saitoh, H. & Inoue, K. (2009) IFN-gamma receptor signaling mediates spinal microglia activation driving neuropathic pain. *Proc Natl Acad Sci U S A,* **106,** 8032-7.

Turgeon, V. L. & Houenou, L. J. (1999) Prevention of thrombin-induced motoneuron degeneration with different neurotrophic factors in highly enriched cultures. *J Neurobiol,* **38,** 571-80.

Twig, G., Graf, S. A., Messerli, M. A., Smith, P. J., Yoo, S. H. & Shirihai, O. S. (2005) Synergistic amplification of beta-amyloid- and interferon-gamma-induced microglial neurotoxic response by the senile plaque component chromogranin A. *Am J Physiol Cell Physiol,* **288,** C169-75.

Ubogu, E. E., Cossoy, M. B. & Ransohoff, R. M. (2006) The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol Sci,* **27,** 48-55.

van Buul, J. D. & Hordijk, P. L. (2008) Endothelial signalling by Ig-like cell adhesion molecules. *Transfus Clin Biol,* **15,** 3-6.

van Kooten, C. & Banchereau, J. (1997) Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol,* **9,** 330-7.

van Marle, G., Henry, S., Todoruk, T., Sullivan, A., Silva, C., Rourke, S. B., Holden, J., McArthur, J. C., Gill, M. J. & Power, C. (2004) Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology,* 329, 302-18.

Van Oosterhout, A. J., Stam, W. B. & Nijkamp, F. P. (1990) Effect of lymphokines on beta-adrenoceptor stimulation of human PBMC. *Agents Actions Suppl,* 31, 163-7.

Van Oosterhout, A. J., Stam, W. B., Vanderschueren, R. G. & Nijkamp, F. P. (1992) Effects of cytokines on beta-adrenoceptor function of human peripheral blood mononuclear cells and guinea pig trachea. *J Allergy Clin Immunol,* 90, 340-8.

Verhoeckx, K. C., Doornbos, R. P., van der Greef, J., Witkamp, R. F. & Rodenburg, R. J. (2005a) Inhibitory effects of the beta-adrenergic receptor agonist zilpaterol on the LPS-induced production of TNF-alpha in vitro and in vivo. *J Vet Pharmacol Ther,* 28, 531-7.

Verhoeckx, K. C., Gaspari, M., Bijlsma, S., van der Greef, J., Witkamp, R. F., Doornbos, R. P. & Rodenburg, R. J. (2005b) In search of secreted protein biomarkers for the anti-inflammatory effect of beta2-adrenergic receptor agonists: application of DIGE technology in combination with multivariate and univariate data analysis tools. *J Proteome Res,* 4, 2015-23.

Verity, A. N., Wyatt, T. L., Lee, W., Hajos, B., Baecker, P. A., Eglen, R. M. & Johnson, R. M. (1999) Differential regulation of glial cell line-derived neurotrophic factor (GDNF) expression in human neuroblastoma and glioblastoma cell lines. *J Neurosci Res,* 55, 187-97.

Vikman, K., Robertson, B., Grant, G., Liljeborg, A. & Kristensson, K. (1998) Interferon-gamma receptors are expressed at synapses in the rat superficial dorsal horn and lateral spinal nucleus. *J Neurocytol,* 27, 749-59.

Vincent, V. A., Tilders, F. J. & Van Dam, A. M. (1997) Inhibition of endotoxin-induced nitric oxide synthase production in microglial cells by the presence of astroglial cells: a role for transforming growth factor beta. *Glia,* 19, 190-8.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C. L. & Marinovich, M. (2003) Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J Neurosci,* 23, 8692-700.

von Bernhardi, R. & Eugenin, J. (2004) Microglial reactivity to beta-amyloid is modulated by astrocytes and proinflammatory factors. *Brain Res,* 1025, 186-93.

Waeber, C., Rigo, M., Chinaglia, G., Probst, A. & Palacios, J. M. (1991) Beta-adrenergic receptor subtypes in the basal ganglia of patients with Huntington's chorea and Parkinson's disease. *Synapse,* 8, 270-80.

Wagner, R., Myers, R. R. & O'Brien, J. S. (1998) Prosaptide prevents hyperalgesia and reduces peripheral TNFR1 expression following TNF-alpha nerve injection. *Neuroreport,* 9, 2827-31.

Wallukat, G. (2002) The beta-adrenergic receptors. *Herz,* 27, 683-90.

Walsh, D. T., Montero, R. M., Bresciani, L. G., Jen, A. Y., Leclercq, P. D., Saunders, D., AN, E. L.-A., Gbadamoshi, L., Gentleman, S. M. & Jen, L. S. (2002) Amyloid-beta peptide is toxic to neurons in vivo via indirect mechanisms. *Neurobiol Dis,* 10, 20-7.

Wang, H., Zhou, M., Brand, J. & Huang, L. (2007a) Inflammation activates the interferon signaling pathways in taste bud cells. *J Neurosci,* 27, 10703-13.

Wang, Q. W., Rowan, M. J. & Anwyl, R. (2009) Inhibition of LTP by beta-amyloid is prevented by activation of beta2 adrenoceptors and stimulation of the cAMP/PKA signalling pathway. *Neurobiol Aging,* 30, 1608-13.

Wang, X., Chen, S., Ma, G., Ye, M. & Lu, G. (2005) Involvement of proinflammatory factors, apoptosis, caspase-3 activation and Ca2+ disturbance in microglia activation-mediated dopaminergic cell degeneration. *Mech Ageing Dev,* 126, 1241-54.

Wang, X., Fu, S., Wang, Y., Yu, P., Hu, J., Gu, W., Xu, X. M. & Lu, P. (2007b) Interleukin-1beta mediates proliferation and differentiation of multipotent neural precursor cells through the activation of SAPK/JNK pathway. *Mol Cell Neurosci,* 36, 343-54.

Wang, X. F., Huang, L. D., Yu, P. P., Hu, J. G., Yin, L., Wang, L., Xu, X. M. & Lu, P. H. (2006a) Upregulation of type I interleukin-1 receptor after traumatic spinal cord injury in adult rats. *Acta Neuropathol,* 111, 220-8.

Wang, X. F., Yin, L., Hu, J. G., Huang, L. D., Yu, P. P., Jiang, X. Y., Xu, X. M. & Lu, P. H. (2006b) Expression and localization of p80 interleukin-1 receptor protein in the rat spinal cord. *J Mol Neurosci,* 29, 45-53.

Wang, Y., Luo, W. & Reiser, G. (2007c) Activation of protease-activated receptors in astrocytes evokes a novel neuroprotective pathway through release of chemokines of the growth-regulated oncogene/cytokine-induced neutrophil chemoattractant family. *Eur J Neurosci,* 26, 3159-68.

Waterhouse, B. D., Moises, H. C. & Woodward, D. J. (1998) Phasic activation of the locus coeruleus enhances responses of primary sensory cortical neurons to peripheral receptive field stimulation. *Brain Res,* 790, 33-44.

Waterhouse, B. D., Mouradian, R., Sessler, F. M. & Lin, R. C. (2000) Differential modulatory effects of norepinephrine on synaptically driven responses of layer V barrel field cortical neurons. *Brain Res,* 868, 39-47.

Wei, F., Guo, W., Zou, S., Ren, K. & Dubner, R. (2008) Supraspinal glial-neuronal interactions contribute to descending pain facilitation. *J Neurosci,* 28, 10482-95.

Wei, R. & Jonakait, G. M. (1999) Neurotrophins and the anti-inflammatory agents interleukin-4 (IL-4), IL-10, IL-11 and transforming growth factor-beta1 (TGF-beta1) down-regulate T cell costimulatory molecules B7 and CD40 on cultured rat microglia. *J Neuroimmunol,* 95, 8-18.

Weigert, A. & Brune, B. (2008) Nitric oxide, apoptosis and macrophage polarization during tumor progression. *Nitric Oxide,* 19, 95-102.

Weinstein, J. R., Hong, S., Kulman, J. D., Bishop, C., Kuniyoshi, J., Andersen, H., Ransom, B. R., Hanisch, U. K. & Moller, T. (2005) Unraveling thrombin's true microglia-activating potential: markedly disparate profiles of pharmaceutical-grade and commercial-grade thrombin preparations. *J Neurochem,* 95, 1177-87.

Wen, W., Sanelli, T., Ge, W., Strong, W. & Strong, M. J. (2006) Activated microglial supernatant induced motor neuron cytotoxicity is associated with upregulation of the TNFR1 receptor. *Neurosci Res,* 55, 87-95.

Wickramasinghe, S. R., Alvania, R. S., Ramanan, N., Wood, J. N., Mandai, K. & Ginty, D. D. (2008) Serum response factor mediates NGF-dependent target innervation by embryonic DRG sensory neurons. *Neuron,* 58, 532-45.

Williams, M. A., Turchan, J., Lu, Y., Nath, A. & Drachman, D. B. (2005) Protection of human cerebral neurons from neurodegenerative insults by gene delivery of soluble tumor necrosis factor p75 receptor. *Exp Brain Res,* 165, 383-91.

Wirjatijasa, F., Dehghani, F., Blaheta, R. A., Korf, H. W. & Hailer, N. P. (2002) Interleukin-4, interleukin-10, and interleukin-1-receptor antagonist but not transforming growth factor-beta induce ramification and reduce adhesion molecule expression of rat microglial cells. *J Neurosci Res,* 68, 579-87.

Wolf, S. C., Sauter, G., Preyer, M., Poerner, T., Kempf, V. A., Risler, T. & Brehm, B. R. (2007) Influence of nebivolol and metoprolol on inflammatory mediators in human coronary endothelial or smooth muscle cells. Effects on neointima formation after balloon denudation in carotid arteries of rats treated with nebivolol. *Cell Physiol Biochem,* 19, 129-36.

Wu, D. T., Woodman, S. E., Weiss, J. M., McManus, C. M., D'Aversa, T. G., Hesselgesser, J., Major, E. O., Nath, A. & Berman, J. W. (2000) Mechanisms of leukocyte trafficking into the CNS. *J Neurovirol,* 6 Suppl 1, S82-5.

Wu, T. C. (2007) The role of vascular cell adhesion molecule-1 in tumor immune evasion. *Cancer Res,* 67, 6003-6.

Xi, G., Keep, R. F. & Hoff, J. T. (2006) Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol,* 5, 53-63.

Xia, M. Q., Bacskai, B. J., Knowles, R. B., Qin, S. X. & Hyman, B. T. (2000) Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. *J Neuroimmunol,* 108, 227-35.

Xue, M., Fan, Y., Liu, S., Zygun, D. A., Demchuk, A. & Yong, V. W. (2009) Contributions of multiple proteases to neurotoxicity in a mouse model of intracerebral haemorrhage. *Brain,* 132, 26-36.

Yamaguchi, H. (2005) Illustration of dynamic changes in Alzheimer pathology: from mild cognitive impairment to terminal stage. *Neuropathology,* 25, 285-7.

Yamamoto, M., Kiyota, T., Horiba, M., Buescher, J. L., Walsh, S. M., Gendelman, H. E. & Ikezu, T. (2007) Interferon-gamma and tumor necrosis factor-alpha regulate amyloid-beta plaque deposition and beta-secretase expression in Swedish mutant APP transgenic mice. *Am J Pathol,* 170, 680-92.

Yan, P., Liu, N., Kim, G. M., Xu, J., Li, Q., Hsu, C. Y. & Xu, X. M. (2003) Expression of the type 1 and type 2 receptors for tumor necrosis factor after traumatic spinal cord injury in adult rats. *Exp Neurol,* 183, 286-97.

Yang, L., Lindholm, K., Konishi, Y., Li, R. & Shen, Y. (2002) Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. *J Neurosci,* 22, 3025-32.

Yang, S., Song, S., Hua, Y., Nakamura, T., Keep, R. F. & Xi, G. (2008) Effects of thrombin on neurogenesis after intracerebral hemorrhage. *Stroke,* 39, 2079-84.

Yew, K. S. & Cheng, E. (2009) Acute stroke diagnosis. *Am Fam Physician,* 80, 33-40.

Yin, L., Ohtaki, H., Nakamachi, T., Kudo, Y., Makino, R. & Shioda, S. (2004) Delayed expressed TNFR1 co-localize with ICAM-1 in astrocyte in mice brain after transient focal ischemia. *Neurosci Lett,* 370, 30-5.

Yoo, Y. M., Kim, Y. J., Lee, U., Paik, D. J., Yoo, H. T., Park, C. W., Kim, Y. B., Lee, S. G., Kim, W. K. & Yoo, C. J. (2003) Neurotrophic factor in the treatment of Parkinson disease. *Neurosurg Focus,* 15, ECP1.

Yoshida, A., Elner, S. G., Bian, Z. M., Kunkel, S. L., Lukacs, N. W. & Elner, V. M. (2001) Thrombin regulates chemokine induction during human retinal pigment epithelial cell/monocyte interaction. *Am J Pathol,* 159, 1171-80.

Yoshimura, A., Mori, H., Ohishi, M., Aki, D. & Hanada, T. (2003) Negative regulation of cytokine signaling influences inflammation. *Curr Opin Immunol,* 15, 704-8.

Yu, J. T., Tan, L., Ou, J. R., Zhu, J. X., Liu, K., Song, J. H. & Sun, Y. P. (2008) Polymorphisms at the beta2-adrenergic receptor gene influence Alzheimer's disease susceptibility. *Brain Res,* 1210, 216-22.

Zafra, F., Lindholm, D., Castren, E., Hartikka, J. & Thoenen, H. (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci,* 12, 4793-9.

Zetterstrom, M., Sundgren-Andersson, A. K., Ostlund, P. & Bartfai, T. (1998) Delineation of the proinflammatory cytokine cascade in fever induction. *Ann N Y Acad Sci,* **856,** 48-52.

Zhang, X. J., Wang, X., Xiong, L. Z., Fan, J., Duan, X. L. & Wang, B. R. (2007) Up-regulation of IL-1 receptor type I and tyrosine hydroxylase in the rat carotid body following intraperitoneal injection of IL-1beta. *Histochem Cell Biol,* **128,** 533-40.

Zhou, Z., Peng, X., Insolera, R., Fink, D. J. & Mata, M. (2009) Interleukin-10 provides direct trophic support to neurons. *J Neurochem,* **110,** 1617-27.

Zhu, Y., Culmsee, C., Roth-Eichhorn, S. & Krieglstein, J. (2001) Beta(2)-adrenoceptor stimulation enhances latent transforming growth factor-beta-binding protein-1 and transforming growth factor-beta1 expression in rat hippocampus after transient forebrain ischemia. *Neuroscience,* **107,** 593-602.

Zietlow, R., Dunnett, S. B. & Fawcett, J. W. (1999) The effect of microglia on embryonic dopaminergic neuronal survival in vitro: diffusible signals from neurons and glia change microglia from neurotoxic to neuroprotective. *Eur J Neurosci,* **11,** 1657-67.