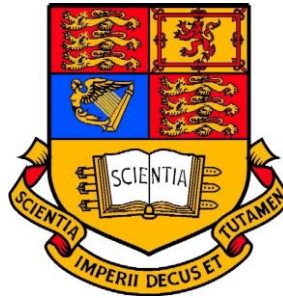


Imperial College London

Faculty of Medicine

Division of Brain Sciences



**Elucidating the role of microglial group III metabotropic
glutamate receptors in microglial inflammation: focus on
Parkinson's disease and neuroprotection**

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**A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in
the Faculty of Medicine, Imperial College London**

Declaration of originality

I hereby declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Abstract

Microglia, the resident immune cells in the central nervous system, are implicated in the progressive neuronal death seen in Parkinson's disease. Microglia are responsible for host defence by mounting an inflammatory response against pathogens and injury. However, if the inflammatory response is not controlled, collateral neuronal damage may occur, leading to neuronal degeneration.

Microglia express metabotropic glutamate receptors (mGluR), and their activation has important effects on microglial inflammation. mGluR are a class of G-protein coupled receptors responsible for modulating glutamate transmission. They are divided into three main groups, according to their signalling mechanisms and sequence homology. However, activation of individual receptors within a single group can have varying consequences on microglial biology and microglia-driven neurotoxicity. Therefore, it is important to characterize the effects of each receptor on microglial inflammation if they are to be developed as a therapeutic target against neurodegenerative diseases.

This thesis focuses on the effects of individual microglial group III mGluR on microglial inflammation *in vitro*. We have used an immortalized mouse microglial cell line whose inflammatory response to bacterial endotoxin and mGluR expression were validated by comparison with primary rat microglia. We have found that activating individual receptors within the group III mGluR has differing effects on microglial inflammation, either reducing or increasing various inflammation parameters. Importantly, these effects translated into either a neuroprotective or neurotoxic microglial phenotype against cultured dopaminergic neurons. Furthermore, the signalling mechanisms engaged by microglia group III mGluR were shown to differ from the canonical mGluR signalling mechanism most often found in neurons.

This work therefore adds to the knowledge of group III metabotropic glutamate receptor biology and its potential as a therapeutic strategy against neurodegenerative diseases, highlighting the importance of differences between receptors putatively sharing signalling mechanisms.

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Abbreviations

5-HT	5-hydroxytyramine
6-OHDA	6-hydroxydopamine
AC	adenylyl cyclase
AIS	adaptive immune system
AMN082	N,N'-Bis(diphenylmethyl)-1,2-ethanediamine dihydrochloride
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPA_r	AMPA receptor
AMP	adenosine monophosphate
AP-1	activator protein-1
APDC	ammonium pyrrolidinedithiocarbamate (NFκB inhibitor)
ATP	adenosine triphosphate
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BG	basal ganglia
c/w	cells per well
cAMP	cyclic adenosine monophosphate
CRD	cysteine rich domain
CREB	cyclic AMP response element binding protein
CX3CL1	cx3c chemokine ligand 1
DA	dopamine
DAG	diacyl glycerol
DAT	dopamine transporters
DCGIV	(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine (mGlu2/3 agonist)
DHPG	(S)-3,5-Dihydroxyphenylglycine (mGlu5 agonist)

D-MEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
EPSC	excitatory post-synaptic current
ERK	extracellular
ETC	electron transport chain
GABA	gamma aminobutyric acid
GDNF	glial derived neurotrophic factor
GEF	guanine exchange factor
GPe	external globus pallidus
GPI	internal globus pallidus
HRP	horse radish peroxidase
iGluR	ionotropic glutamate receptor
ICC	immunocytochemistry
ICV	intracerebroventricular
IHC	immunohistochemistry
IIS	innate immune system
IL	interleukin
iNOS	inducible nitric oxide synthase
IFN	interferon
IKK	inhibitor of kappa kinase
IP3	inositol trisphosphate
IRAK	IL-1R associated kinase
IRF-3	interferon regulatory factor-3
JAK	janus kinase

L-AP4	L-(+)-2-Amino-4-phosphonobutyric acid
LB	Lewy bodies
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
L-SOP	L-Serine- O -phosphate
LTP	long term potentiation
LY367385	(S)-(+)- α -Amino-4-carboxy-2-methylbenzeneacetic acid
MAC	membrane attack complex
MAO-B	monoamine oxidase B
MAPK	mitogen activated protein kinase
MCM	microglial conditioned medium
mGluR	metabotropic glutamate receptor
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPEP	2-methyl-6-(phenylethynyl) pyridine hydrochloride
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSOP	(RS)- α -methylserine- O -phosphate
MyD-88	myeloid differentiation primary-response protein 88
NADH	nicotinamide adenine dinucleotide
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NK cells	natural killer cells
NMDA	N-methyl-o-aspartate
NMDAR	NMDA receptor
NO	nitric oxide

NSAID	non-steroidal anti-inflammatory drug
PPAR	peroxisome proliferator activated receptor
PD	Parkinson's disease
PFAS	proto-fibrillary alpha-synuclein
PHCCC	N-Phenyl-7-(hydroxyimino) cyclopropa [b] chromen-1a-carboxamide
PINK-1	PTEN-induced putative kinase 1
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidylserine
Rb	Rabbit
Rt	Rat
ROS	reactive oxygen species
SEM	standard error mean
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
STN	subthalamic nucleus
TH	tyrosine hydroxylase
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumour necrosis factor alpha
TRAF-6	tumor-necrosis factor receptor-associated factor-6
VFD	Venus flytrap domain
VTA	ventral tegmental area
VU0155041	cis-2-[[[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid

Chapter I

Introduction

1.1 Parkinson's disease overview

Parkinson's disease (PD) is the second most common neurodegenerative disease behind Alzheimer's disease. In the western world, PD affects ~1% of people over 60 years old (Nussbaum and Ellis, 2003), and ~3 % of people over 80 years old (de Lau and Breteler, 2006). PD primarily leads to deterioration of motor skills, causing, among other symptoms, a characteristic tremor of the extremities. At late disease stages, the majority of PD patients lose their ability to exist independently and become completely dependent on carers. Although a number of risk factors have been linked to development of PD, the pathogenic mechanisms still remain uncertain.

All current PD therapies aim to reduce symptoms, with no therapy able to halt disease progression. Given that the population age in the western world is predicted to increase dramatically in the next few decades, the number of people affected by PD will reach unmanageable levels, placing a great burden on both national and personal finances. Therefore, there is a great moral and economical pressure to find efficient therapies that halt disease progression and reverse the neurological damage.

1.2 History of PD

The symptoms of PD have been recognized since ancient times, with the millennium old Indian traditional medicine *Ayurveda* recognizing it as an independent condition known as *Kampavata*. However, it was not until 1817, when the English physician James Parkinson wrote '*An essay on the Shaking Palsy*', that a comprehensive medical characterization was first published. Parkinson described the shaking palsy as '*involuntary tremulous motion, with lessened muscular power, in parts not in action...; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured*'. It was rightly hypothesized that the origins of the disease laid in a nervous disorder. However, it was wrongly believed that the location of this disorder rested in the spinal cord and the medulla oblongata. In addition, due to the lack of

observable disruption to the senses or intellect, it was believed the brain itself was spared injury. In 1861, the French physician Jean-Martin Charcot further characterized PD symptoms, clearly identifying rigidity, slowness of movement and postural instability, currently thought of as the cardinal motor-symptoms in PD along with tremor at rest.

Greater insight of PD allowed physicians to efficiently distinguish PD from other neurological disorders. However, it was not until the 20th century that anatomical and biochemical features were able to be related to PD. Early in the 20th century, autopsies revealed neurodegeneration of the pigmented neurons of the midbrain, allowing for an anatomical target to be established (Tetriakoff, 1919). In 1960, Ehringer and Hornykiewicz noted reduced levels of the neurotransmitter dopamine (DA) in the striatum of PD patients (Hornykiewicz, 2002). This discovery finally provided scientists with a molecular deficiency that could be targeted to treat the disease.

1.3 Pathology of PD

1.3.1 Anatomical changes

Anatomically, PD is defined by:

- i) chronic degeneration of dopamine-producing cells in the substantia nigra pars compacta (SNc) nucleus of the basal ganglia
- ii) appearance of intracellular proteinaceous aggregates termed Lewy bodies (LB)

1.3.1.1 Dopaminergic cell death

A clear depigmentation can be seen in the midbrain of PD patients as the neuromelanin-containing dopaminergic cells of the SNc die off, leading to depletion of dopamine in the striatum and emergence of motor symptoms (see section 1.4). Although the SNc is not the only dopaminergic nucleus in the midbrain, other neighbouring dopaminergic areas, such as the ventral tegmental area (VTA) and the retrorubral area, are largely spared from degeneration. Furthermore,

degeneration of the SNc follows a particular caudo-rostral and ventro-dorsal pattern (Damier *et al.*, 1999), pointing to different susceptibility to degeneration even within the SNc. Exactly why the SNc is particularly vulnerable to degeneration in PD and why certain SNc neurons have a higher propensity to die off is not currently known. However, several differences between neurons have been found which may account for this susceptibility. It has been noted that SNc cell loss correlates well with the level of expression of the DA transporter (DAT). Studies in healthy patients have shown that the neurons of the SNc express higher levels of DAT mRNA than neurons in the VTA. In addition, surviving neurons in the SNc of PD patients show less DAT mRNA expression compared to SNc neurons in healthy patients (Uhl *et al.*, 1994), either suggesting decreased neuronal function or increased vulnerability to degeneration in neurons expressing high DAT. Therefore, high levels of DAT may cause cells to have increased DA metabolism, which could lead to increased levels of oxidative stress and impair mitochondrial dysfunction (Obeso *et al.*, 2010). Other factors put forward to explain the variable neuronal vulnerability include differences in calcium ion flux control (Esteves *et al.* 2009) and reliance on L-type calcium channels for pace making activity (Chan *et al.*, 2007), as well as the different transcription factor profile of neurons in the SNc (Alavian *et al.*, 2008).

1.3.1.2. Non-dopaminergic cell death

PD patients may also exhibit non-motor symptoms, including autonomic dysfunction, sleep disorders, behavioural changes and sensory abnormalities (Jankovic, 2008; Fenelon *et al.*, 2000). Unlike motor symptoms, it is thought non-motor symptoms are caused by degeneration of other neuronal regions apart from the SNc, such as norepinephrine neurones in the locus coeruleus, cholinergic neurones of the nucleus basalis of Meynert and serotonergic neurones of the dorsal raphe nucleus (Jenner, 2006) (see section 1.3.2).

1.3.1.3 *Lewy bodies*

LB, the second defining criteria in PD, were first recognized by the German scientist Frederic Lewy and consist of eosinophilic intracellular aggregates of various proteins, including alpha-synuclein, ubiquitin and chaperone proteins. LB may be found throughout the brain, but their appearance has been suggested to follow a particular pattern in PD (see section 1.3.3). Although their presence is considered a hallmark to define PD, their involvement in pathogenesis and disease progression remains uncertain (see section 1.3.3 and 1.5.3).

1.3.2 *Biochemical changes*

Interestingly, PD motor symptoms do not manifest until 60-80% of striatal DA has been lost (Hornykiewicz, 1998), indicating the involvement of compensatory mechanisms for neurotransmission during nigral cell loss. Biochemical changes in the remaining dopaminergic nigro-striatal synapses include an increase in dopamine metabolism to synthesise and release higher levels of DA into the striatum (evidenced by an increase in the ratio of homovanillic acid, a DA metabolite, to DA in the striatum of PD patients), as well as an increase in expression of D2 dopamine receptors in the post-synaptic membrane to maximise the effects of DA. Loss of dopaminergic innervation to the striatum also causes imbalances in other neurotransmitter systems. For instance, gamma-aminobutyric acid (GABA), a neurotransmitter essential for striatal neuron communication, is elevated in the brains of PD patients (Kish *et al.*, 1986), and correlates with DA loss (*e.g.* the putamen striatal region had both the highest levels of GABA and the lowest levels of DA). Furthermore, extra-striatal regions spared DA loss, have normal levels of GABA. Increase of striatal GABA is thought to occur due to loss of dopamine inhibition through the actions of the inhibitory D2 receptor.

In addition to an overall increase in striatal GABA, loss of DA may lead to dysregulation of firing pattern of striatal neurons. For instance, Dehorter *et al.* (2009) have shown that in *ex vivo*

striatal tissue slices deprived of DA, GABAergic striatal neurons shift their firing pattern from a tonic, low amplitude, low frequency pattern to an oscillatory, high amplitude, high frequency pattern. This shift in activity would render the striatum less efficient to regulate incoming excitatory cortical inputs, thereby affecting motor control.

Although DA undeniably plays a central role in PD, the involvement of other neurotransmitters in symptom onset has been established in recent years, as well as recognition that these non-dopaminergic systems may be affected years before dopaminergic nuclei degenerate. In particular, it has been shown that olfaction, REM sleep and constipation are affected early in PD. Hyposmia (deficient sense of smell) occurring in PD has been well documented over the past two decades (reviewed by Hawkes *et al.*, 2007). Evidence points to the lack of involvement of DA in hyposmia emergence. Firstly, PD-related hyposmia is unresponsive to DA replacement therapy (Roth *et al.*, 1998), namely apomorphine administration. Secondly, parkinsonism caused by the DA-specific toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is not accompanied by hyposmia, as compared to control subjects and patients with idiopathic PD (Doty *et al.*, 1992). Finally, though there is neuronal degeneration in the olfactory bulb during PD, dopaminergic neurons are spared. In fact, dopaminergic neuron number may increase in the olfactory bulb during PD compared to control subjects (Huisman *et al.*, 2004). Therefore, a deficiency in non-dopaminergic neurotransmitter systems seems to be responsible for hyposmia in PD.

Similarly, REM sleep disorder in PD seems to be attributed mainly to non-dopaminergic systems, in particular serotonergic systems (Suzuki *et al.*, 2011).

1.3.3 Disease progression: Braak staging

The usual age of onset of PD is ~60 years old, though a small subset of cases develop PD earlier in life (~40 year old). A reliable disease progression scheme is hard to establish, since PD progression varies greatly from patient to patient, disease diagnosis usually does not occur until motor symptoms begin to manifest (at which point the disease is at a relatively advanced stage), and comprehensive anatomical and pathological examination can only be carried out after the patient's death. Despite these constraints, Braak *et al.* (2003) have suggested a six-stage model for PD progression based on immunostaining of aggregated alpha synuclein, representing LB, in post-mortem tissue of PD and control cases. The main predictions of this model are a pathological initiation at the medulla oblongata of the brain stem and the olfactory bulb, spreading to the caudal raphe nucleus and the melanin-containing cells of the midbrain. At latter stages, pathology spreads to the basal forebrain and macroscopically detectable loss of substantia nigra pigmentation begins to appear. During the final stages, LB pathology spreads to cortical areas, and the substantia nigra becomes virtually bare of melanin containing neurons, and therefore the characteristic pigmentation becomes absent. There is also a marked loss of extranigral melanin containing neurons.

A salient point of this model is the fact that pathology begins in non-dopaminergic areas, thereby excluding dopamine and dopamine oxidation as a primary pathogenic factor in PD. In addition, Braak's study confirms that pathology begins years before onset of motor symptoms, though non-motor symptoms may accompany brain stem LB pathology.

The Braak staging system is supported by other studies examining different LB-associated pathologies (*e.g.* Dickson *et al.*, 2010; Parkinnen *et al.*, 2003). However, several short-comings and contradictions of the staging scheme have been flagged up by various researchers (Zimprich *et al.*, 2004; Burke *et al.*, 2008). Therefore, the observed LB emergence pattern in the brain may reflect the specific brain regions' propensity to develop LB (*e.g.* due to levels of alpha-synuclein

expression) following an underlying pathogenic event in the brain, and may not represent either the main pathogenic factor or correlate with the severity of underlying pathology.

1.4 The basal ganglia

The basal ganglia (BG) are a set of subcortical nuclei which are primarily involved in modulating motor control and which are particularly affected in PD (reviewed by Redgrave *et al.*, 2010).

1.4.1 Organization of the basal ganglia

The basal ganglia can be divided into four different nuclei: the striatum, which itself is divided into the caudate, putamen and nucleus accumbens, separated by the internal capsule; the subthalamic nucleus (STN); the globus pallidus, divided into the internal (GPi) and external (GPe) segments; and substantia nigra, divided into the pars compacta region (SNc) and pars reticulata (SNr).

The main input centre in the BG is the striatum, which receives primarily glutamatergic excitatory cortical efferents transmitting motor information. The main output centres are the GPi and the SNr, which send inhibitory projections to the thalamus and brain stem (motor associated areas) (Bezard *et al.*, 2001). Therefore, BG output ultimately leads to reduction of neuronal activity in its target motor areas.

1.4.2 Dopaminergic control of the basal ganglia

In addition to cortical control, the striatum is also under the direct control of the SNc, which sends dopaminergic efferents to the striatum. Dopamine signals through two main types of receptor, namely the excitatory D1 receptors and the inhibitory D2 receptors. Striatal neurons primarily express one type of receptor over the other (Gerfen *et al.*, 1990). Hence, nigral dopamine will either excite or inhibit a striatal neuron. D1 expressing neurones project towards the GPi and to the SNr

(termed the direct pathway), while D2 expressing neurons project towards the GPe (termed the indirect pathway). Therefore, dopamine released into the striatum will activate the direct pathway and inhibit the indirect pathway, thereby reducing the activity of the BG output centre (**fig. 1.1**). Thus, upon depletion of nigral-derived striatal dopamine, as is the case in PD, there is an overactivation of the BG output centres, leading to excessive inhibition of thalamocortical pathways and motor dysregulation. This concept has been demonstrated to be true in a rodent model of PD by Mallet *et al.* (2006) who showed electrophysiologically that striatonigral neurons (*i.e.* the direct pathway) from hemiparkinsonian rats had a decreased activity, while striatopallidal neurons (*i.e.* indirect pathway) increased their activity upon dopamine depletion. In addition, electrophysiological recordings in humans have shown that the activity of the STN is enhanced during PD (Steigerwald *et al.*, 2008). Furthermore, this model of BG organization and function has been further validated by a recent study utilizing optogenetics (Kravitz *et al.*, 2010) which clearly demonstrated the opposing roles of the direct and indirect pathway in motor function, for the first time empirically demonstrating activation of the indirect pathway leads to motor disruption in free moving mice, while activation of the direct pathway completely alleviates motor symptoms in parkinsonian mice.

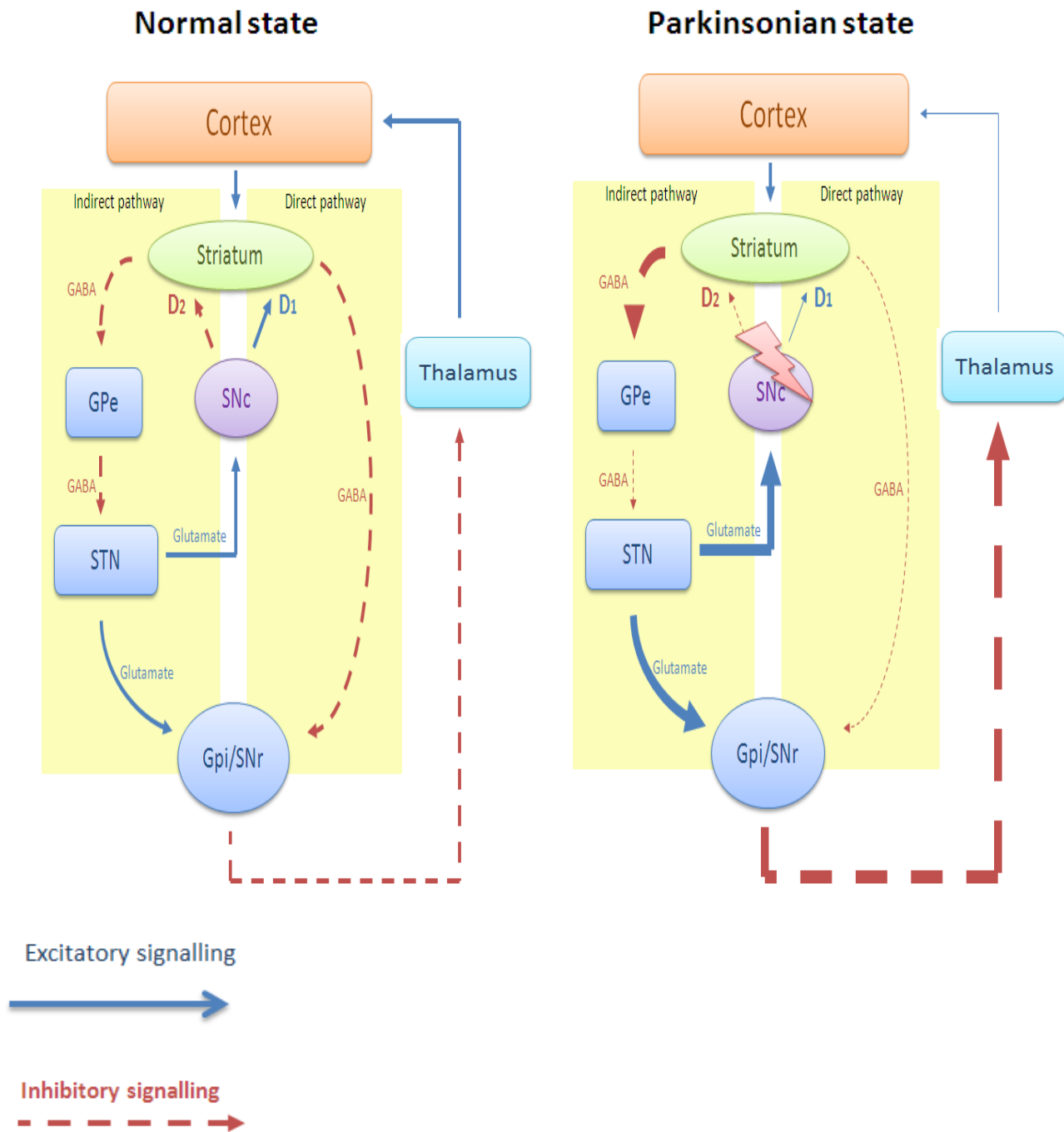


Figure 1.1 Basal ganglia organization in the normal and Parkinsonian state. The basal ganglia's main input centre is the striatum, which receives motor control signals primarily from the cortex. The main output centre is comprised by the globus pallidus internus (GPI) and the substantia nigra pars reticulata (SNr) and works to inhibit thalamocortical signalling. The substantia nigra pars compacta (SNc) aides to control basal ganglia activity by modulation of the output centre through the direct and indirect pathways which inhibit or enhance the output centre's signalling, respectively. In Parkinson's disease, loss of the substantia nigra leads to decrease of the direct pathway and increase of the indirect pathway, resulting in overactivation of the output centre and emergence of motor symptoms. In addition, increased subthalamic nucleus (STN) glutamatergic signalling to the SNc increases nigral cell death through excitotoxic mechanisms.

1.4.3 Short-comings of basal ganglia structure and function models

Although the overall direct/indirect pathway organization of the BG has been established using various techniques, there are several observations that do not fit this model. Firstly, rodent and primate studies have shown that a subset of striatal neurons (5-10%) express mRNA for both D1 and D2 receptors (Le Moine and Bloch, 1995; Aubert *et al.*, 2000). Secondly, anatomical studies in rodents and primates have shown that single striatal projections may arborize to innervate both sections of the globus pallidus, hence transmitting through both the direct and indirect pathways (Barroso-Chinea *et. al*, 2010 and references therein). Thirdly, anatomical data shows that the cortex can directly innervate the STN, thereby circumventing striatal control of the indirect pathway. Therefore, undoubtedly the basal ganglia circuitry will prove to be more complex than the current models suggest.

1.5 Aetiology of PD

Although the causes of PD symptoms have been well characterized, the triggers which initiate cell death and the mechanisms by which progressive cell death is maintained have not been clearly identified. Among the most prominent suspects of cell toxicity are oxidative stress, mitochondrial dysfunction, alpha-synuclein, excitotoxicity and inflammation. Evidence of their involvement comes both from environmental factors as well as genetic susceptibilities, and each will be discussed in turn in the following sections.

1.5.1 Oxidative stress

Cell death through oxidative stress results from free radicals (*i.e.* molecules with an unpaired electron in their outer orbital) interacting with and damaging lipids, proteins and DNA within cells. Free radicals can be formed from a wide range of sources, including metabolic reactions, cellular processes such as the electron transport chain, and redox enzymatic activity. Particularly important

for PD is the fact that oxidation of DA is known to be able to induce the production of free radicals (Graham, 1978) through the formation of DA-quinones capable of interacting with cysteine amino acids, thereby disrupting proper protein function. In fact, Miyazaki *et al.* (2007) have shown that *in vitro* DA administration to neurons induces the production of quinones, as well as lowering cell viability. Furthermore, it has been found that there are elevated levels of oxidative damage to lipids and proteins in brains of PD patients, as evidenced by the increase in lipid hydroperoxides and reactive carbonyls, respectively (reviewed by Jenner, 2003).

Intracellular redox reactions can also induce the production of free radicals. For instance, the reaction between iron and hydrogen peroxide (known as the Fenton reaction) can yield highly reactive hydroxyl radicals ($\text{OH}\cdot$). This reaction may be particularly important in PD, since metabolism of DA by the enzyme monoamine oxidase (MAO) yields hydrogen peroxide (Hrometz *et al.*, 2004). In addition, elevated iron levels have been shown to exist specifically in the substantia nigra of PD patients (Graham *et al.*, 2000). Lipid damage by hydroxyl radicals causes cell membranes to become less stable, rendering the cells more prone to rupturing. In addition, protein damage by hydroxyl radicals can lead to structure destabilization, rendering them more susceptible to aggregation and degradation. These observations provide mechanisms to explain the high susceptibility of SNc dopaminergic neurones to cell death.

An indirect method to assess oxidative stress *in vivo* is the measurement of glutathione, a small peptide containing a thiol group. The majority of glutathione exists in a reduced state (G-SH), with the thiol group containing a hydrogen atom. Glutathione helps protect against oxidative stress by becoming oxidized (G-SS-G) by free radicals, thereby donating an electron to the free radicals and neutralizing them. Therefore, a decreased level of reduced glutathione suggests increased oxidative stress. Post-mortem studies have revealed that indeed there is a decreased level of reduced glutathione in the brains of PD patients compared to control subjects (Pearce *et al.*, 1997). Furthermore, this reduction was specific to the substantia nigra, with no alterations seen in other

brain areas (Sian *et al.*, 1994). In addition, decreased levels of reduced glutathione can also be found in the substantia nigra of patients with incidental Lewy body disease (Owen *et al.*, 1996), a pathologic state considered as pre-symptomatic PD, indicating oxidative stress is an early pathologic event.

The role of oxidative damage in PD is further highlighted by the fact that mutations in the peptidase protein DJ-1 have been linked to cases of familial PD (Bonifati *et al.*, 2003). Although the function of DJ-1 has not been fully elucidated, *in vitro* studies have shown DJ-1 provides protection against oxidative stress, both directly and indirectly. For instance, Canet-Aviles *et al.* (2004) have shown that DJ-1 serves as a free radical scavenger *in vitro*, thereby providing direct protection against oxidative stress. An indirect protective mechanism against oxidative stress has been demonstrated by Zhou and Freed (2005). This study showed that DJ-1 is able to induce an increase in expression of glutathione in dopaminergic cells, with concomitant reduction in reactive oxygen species. Furthermore, overexpressing DJ-1 protected dopaminergic cells against hydrogen peroxide and 6-OHDA-induced toxicity.

Therefore, deficits in DJ-1 function would decrease the cell's ability to scavenge free radicals, thereby rendering the cell more prone to oxidative stress.

Finally, organochlorinated pesticides, which are thought as a risk factor for PD due to their presence in the substantia nigra of PD patients (Mao *et al.*, 2008), are known to induce production of reactive oxygen species in microglia (Mao *et al.*, 2008), thereby further supporting the role of oxidative stress in PD pathology.

1.5.2 Mitochondrial dysfunction

A second mechanism put forward as responsible for degeneration of dopaminergic is mitochondrial dysfunction. The role of mitochondrial dysfunction in the pathogenesis of PD was first suggested after the discovery that the activity of mitochondrial complex-I protein is reduced in

the substantia nigra of PD patients (Schapira *et al.*, 1989a, b), although there may be complex-I deficiencies in other brain areas, such as the frontal cortex (Parker *et al.*, 2008). The mitochondrial deficiency theory is backed by the fact that several environmental risk factors for PD, including the pesticide rotenone (Hancock *et al.*, 2008; Dhillon *et al.*, 2008) and the synthetic molecule MPTP (Schapira, 2006), are known to interact with the Complex-I protein of mitochondria and disrupt the electron transport chain (ETC) responsible for ATP production. Both MPTP and rotenone have been crucial in the development of animal models of PD. Animals treated with these compounds develop SNc-specific neuronal degeneration, mimicking PD. Furthermore, at least in the case of MPTP, intoxicated animals respond similarly to dopaminergic therapies, both regarding the beneficial short term actions as well as the negative long term consequences (*i.e.* dystonia) (reviewed by Winklhofer and Haass, 2010 and references therein), lending credibility to the comparability between the actions of these toxins and idiopathic PD.

Electron transport chain disruption through mitochondrial deficiencies may lead to cell death through various mechanisms (reviewed by Sherer *et al.*, 2002), including leakage of free radicals from the mitochondrial membranes and energy (*i.e.* ATP) depletion. Energy depletion affects important cellular processes required for cell survival, without which cell death occurs through the activation of apoptotic pathways. In addition, loss of ATP can lead to the cell becoming more vulnerable to excitotoxicity (see section 1.5.4).

The role of mitochondria in the pathogenesis of PD is further supported by genetic mutations in mitochondrial proteins which cause familial PD. One such mutation occurs in the protein PTEN-induced putative kinase 1 (PINK1), a ubiquitously expressed serine/threonine kinase which is translocated to the mitochondria upon expression. Mutations in the gene encoding PINK1 have been shown to lead to early onset PD (Valente *et al.*, 2004). *In vitro* studies have shown that PINK1 mutations do not alter either protein expression/translocation or mitochondrial viability under normal conditions. However, upon induction of cellular stress, PINK1 mutated cells are less able to

maintain membrane potential compared to controls (Valente *et al.*, 2004) and produce higher levels of reactive oxygen species (Wang *et al.*, 2011a). Therefore, PINK1 mutations alter the cells' capacity to cope with elevated stress levels. Hence, in individuals with PINK1 mutations, neurons will be more prone to cell death in regions with elevated oxidative stress levels, as might be the case in the SNc of early stages PD.

1.5.3 Alpha-synuclein

A third pathogenic factor suggested for PD is the protein alpha synuclein. Alpha-synuclein is a 140 amino acid protein abundantly expressed in the central nervous system. Although its functions remain unclear, a role in synaptic dynamics has been suggested due to its enrichment in presynaptic terminals and vesicles (Tofaris and Spillantini, 2007). Alpha-synuclein may play a role in SNc cell degeneration through accumulation and aggregation of damaged/ irregular proteins in the cytosol (*i.e.* LB), leading to cellular stress and toxicity. The presence of LB, whose principal component is alpha-synuclein, in PD brains was thought to link directly to cell death, further backing alpha-synuclein inclusions as a causative factor for PD. Indeed, deficient alpha-synuclein function and degradation seems to play an important role in PD, since inhibitors of the proteasome (*e.g.* lactacystin) are able to produce an animal phenotype which closely resembles PD pathology, including motor symptoms, neuronal degeneration and alpha-synuclein accumulation (McNaughton *et al.*, 2002). Furthermore, the most frequent autosomal recessive mutation causing familial PD affects parkin (Kiedel *et al.*, 1998), a protein implicated in the ubiquitin-proteasome system through ubiquitin ligation of target proteins, one of which has been identified as alpha-synuclein (Shimura *et al.*, 2001). Similarly, mutations in the gene encoding alpha-synuclein have been linked to familial PD (Polymeropoulos *et al.*, 1997).

However, it has been proposed that LB formation is actually a neuroprotective measure against toxicity due to proto-fibrillary alpha-synuclein (pfA-synuclein). Indeed, pfA-synuclein has been

shown to be able to form pores on cell membranes and induced DA leakage from vesicles (Volles *et al.*, 2001; Ding *et al.*, 2002), which has the potential to result in increased oxidative stress from dopamine metabolism (Xu *et al.*, 2002). Although oxidative stress due to DA metabolism has increasingly lost favour as a contributor to PD pathology (reviewed by Ahlskog, 2007), pfA-synuclein may still be toxic by leakage of essential cellular components. Interestingly, molecular studies have shown DA to be able to associate with alpha-synuclein and prevent fibril protein formation, instead promoting the accumulation of the proto-fibrillar protein conformation (Conway *et al.*, 2001), suggesting a reason for dopaminergic cells' increased susceptibility to degeneration in PD.

Therefore, alpha-synuclein mutants prone to form protofibrils or aberrant clearance of alpha-synuclein by the ubiquitin-proteasome system, either due to pharmacological inhibition or inherent defects in the system, may lead to PD-like neurodegeneration, either by direct mechanisms (*e.g.* membrane porosity or sequestration of anti-apoptotic proteins (Xu *et al.*, 2002)) or indirectly through increased cell stress.

1.5.4 Excitotoxicity

Previous studies have established the ability of glutamate, the brain's principal excitatory neurotransmitter, to induce cell death both *in vitro* and *in vivo* (Schubert *et al.*, 2001; Olney, 1969), through a mechanism termed excitotoxicity. Excitotoxicity results from excessive glutamatergic signalling, leading to massive sodium and calcium influx mediated, primarily, by the ionotropic glutamate receptor NMDA receptor (see section 1.8.1). Excessive sodium entry causes chloride ions to passively enter the cell to maintain ionic equilibrium, in turn shifting the osmotic gradient and causing entry of water. High levels of intracellular water causes osmotic swelling and cell rupture. Although sodium entry contributes to early cell toxicity, it does not seem to be the main causative factor for cell death, as removal of sodium and chloride ions in *in vitro* studies inhibits cell swelling

but not cell death (Choi, 1987). The main cause of excitotoxicity therefore appears to be calcium entry. Excessive intracellular calcium concentration induces cell death through overactivation of calcium-dependent proteins such as calpain and PK-C, which may damage proteins, DNA and lipids (reviewed by Doble, 1999), and activate apoptotic pathways. Furthermore, elevated intracellular calcium may lead to mitochondrial dysfunction due to excessive calcium sequestration, resulting in ATP deficiency and increased oxidative stress (see section 1.5.1). In addition, mitochondrial dysfunction causes the Na/K ATPase pump to be unable to maintain resting cell membrane potential, thereby releasing NMDAR from their voltage-induced magnesium block (Henneberry *et al.*, 1989) and allowing lower (even physiologic) concentrations of glutamate to activate the NMDAR. Therefore, mitochondrial damage results in a feedforward loop to exacerbate excitotoxicity.

The involvement of excitotoxicity in PD comes from observations of increased concentration of glutamate in the SNc of PD animal models (Meredith *et al.*, 2009). Furthermore, PD leads to overactivity of the STN (Bevan, 2002; Steigerwald *et al.*, 2008), which sends glutamatergic projections to the SNc. In accordance with this, deep brain stimulation (*i.e.* inhibition) of the STN in PD animal models as well as PD patients results in reversal of parkinsonian symptoms (Limousin *et al.*, 1995; Benazzouz *et al.*, 2003; Volkman, 2007). It has been noted too, that surgical ablation of the STN protects neurons of the SNc against various toxic insults, including MPTP in monkeys (Wallace *et al.*, 2007) and 6-OHDA in rats (Carvalho *et al.*, 2001). Overactivity of the STN therefore provides a mechanism by which an initial SNc cell death may be perpetuated through a feedforward loop of excitotoxicity.

1.5.5 Inflammation

Experimental as well as post-mortem data point to a clear involvement of inflammation in the pathology of PD as well as other neurodegenerative diseases. Because inflammation and its

modulation will be a central theme of this thesis, a separate section (section 1.6) will be dedicated to discussing its physiological role and its involvement in PD pathogenesis.

1.6 Inflammation

1.6.1 Overview

Inflammation is the body's immune response against injury and infection to promote pathogen removal and healing. It is composed of two main systems, namely adaptive immunity and innate immunity.

1.6.2 Adaptive immunity

The adaptive immune system (AIS) is a complex defence mechanisms found only on jawed vertebrates. Its main role is to mount a tailored defensive response against specific pathogens and to build a 'memory' of pathogen recognition to aid in more efficient and robust future immune responses. The AIS is able to recognize particular pathogens through specific immune proteins, termed antibodies, capable of recognizing unique 'labels', termed epitopes, on the pathogens' surface. During normal periods, the AIS generates a wide range of lymphocytes (immune cells), each expressing a different epitope receptor. Therefore, the AIS is ready to recognize virtually any pathogen infiltrating the host. Once the AIS has detected a specific pathogen, clonal replication of lymphocytes expressing the appropriate antibody leads to a robust proliferation of cells capable of generating antibodies able to identify the invading organism. The antibodies then attach to the pathogen and neutralize it or mark it for destruction. The main cellular components of the AIS are T-lymphocytes, responsible for destroying infected cells, and B-lymphocytes, responsible for producing antibodies. Both cells are generated in the bone marrow, though T-lymphocytes travel to the thymus to undergo maturation.

1.6.3 Innate immunity

In addition to the AIS, there exists a more 'primitive' immune system, termed the innate immune system (IIS). The IIS is considered more primitive than the AIS since it is found in all multicellular organisms (Hoffmann *et al.*, 1999), including insects and plants. In addition, the IIS does not have a pathogen identification memory, therefore each immune response will be of equal magnitude even if the pathogen has been encountered before. The IIS can be considered the first line of defence against infection. However, the AIS may enhance the actions of the IIS (*e.g.* T-lymphocytes may release cytokines to increase the phagocytic activity of macrophages, while B-lymphocytes may produce antibodies which instruct macrophages to phagocytose tagged microbes); therefore both systems can be thought of as acting cooperatively.

1.6.3.1 Cellular components of IIS

The main cellular components of the IIS are natural killer (NK) cells, neutrophils and macrophages, along with the epithelial cells which form a protective physical barrier. NK cells are a subset of bone marrow derived lymphocytes whose main role is killing infected cells through induction of apoptosis. In addition, they are able to secrete the cytokine interferon gamma ($\text{IFN}\gamma$) to enhance phagocytic activity of macrophages. Neutrophils form the most abundant population of immune cells. Unlike NK cells, they do not kill infected cells. However, they can become phagocytic and remove circulating pathogens. Neutrophils are differentiated from macrophages in that their nucleus is segmented into several connected lobules, giving rise to the term polymorphonuclear leukocytes, as well as having a much shorter life-span (~6 hrs). In contrast, macrophages, the professional phagocytic cells, are mononuclear. Peripheral macrophages are generated in the bone marrow as monocytic precursors. After leaving the bone marrow, they migrate to their target tissues and develop into mature resident macrophages. Like neutrophils, macrophages are able to phagocytose pathogens as well as cellular debris. However, macrophages

are also able to act as antigen presenting cells, thereby acting as a bridge between the AIS and the ISS through interaction with T-lymphocytes. In addition, macrophages may secrete cytokines to recruit and activate further immune cells to a site of infection.

1.6.3.2 Pathogen recognition by macrophages

Identification of pathogens by the ISS is not as specific as in the AIS (*i.e.* macrophages and NK cells are not able to recognize specific epitopes expressed in individual pathogen species). Therefore, the defensive actions of the IIS are not tailored to each pathogen species. However, the ISS is able to differentiate between different kinds of pathogens (*e.g.* bacteria vs. virus). This section will deal only with pathogen recognition by macrophages, as these will be the focus of this thesis.

1.6.3.2.1 Membrane receptors

Macrophages rely on membrane receptors to detect non-self organisms, including mannose and opsonin receptors, which are crucial for pathogen phagocytosis. However, the most important macrophage receptors in terms of inducing microbicidal activity are a group of membrane bound proteins termed Toll-like receptors (TLR). TLR are homologous of the *Drosophila* protein Toll (Medzhitov *et al.*, 1997), which is involved in fruitfly morphogenesis and immunity (Takeda *et al.*, 2003). TLR exploit the fundamental cellular structural differences between microbial and multicellular organisms to recognize pathogens. Hence, they are termed pattern recognition receptors. TLR are characterized by the presence of an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor (TIR) domain crucial for signal transduction. To date, 18 different forms of TLR have been identified in vertebrates, though their expression differs between species (Roach *et al.*, 2005). TLR can be roughly divided into seven subgroups according to protein sequence homology: TLR1 subgroup consists of TLR1, 2, 6, 10 and 14; TLR7 subgroup is composed of TLR 7, 8, and 9; TLR11 consists of TLR11, 12, 13, 23, 22, 23; the TLR3, TLR4, TLR5

and TLR15 receptors are sufficiently dissimilar to all other TLR to be categorized each in its own subgroup.

Each receptor is able to recognize different patterns of pathogen families. For instance, TLR2 recognizes peptidoglycans found in Gram-positive bacteria (Yoshimura *et al.*, 1999), while TLR4 is able to recognize lipopolysaccharide (LPS), a compound found in the cell wall of Gram-negative bacteria (Hoshino *et al.*, 1999). TLR are also able to recognize intracellular pathogen patterns. For instance, TLR3 is able to recognize double stranded RNA (Alexopoulou *et al.*, 2001) found only in viruses during their replicative cycle. Similarly, TLR9 is able to recognize unmethylated cytosine-guanine DNA patterns found in bacteria (Hemmi *et al.* 2000). Cytosine-guanine DNA sequences are severely reduced in vertebrates, and when they do occur they are methylated in the cytosine residue.

The signal transduction mechanisms of all TLR are highly similar, since they all share the intracellular TIR domain necessary for signal transduction. The main signalling pathway for TLR-4 involves interaction of TLR with the adaptor protein myeloid differentiation primary-response protein 88 (MyD-88) upon pathogen recognition (**fig 1.2**) (Akira *et al.*, 2004; Takeda *et al.* 2005). MyD-88 has a C-terminal TIR domain responsible for interacting with TLR and an N-terminal death domain responsible for recruitment of the serine/threonine kinase IL-1R-associated kinase-4 (IRAK-4) for signal transduction. IRAK-4 subsequently phosphorylates and activates IRAK-1, which in turn associates with tumour-necrosis factor receptor-associated factor-6 (TRAF-6), a ubiquitin ligase (Sun *et al.*, 2004). Activation of TRAF-6 leads to two distinct gene expression pathways. TRAF-6 may activate MAP kinases leading to activation of the transcription factor activator protein-1 (AP-1) (Funakoshi-Tago *et al.*, 2003). Alternatively, TRAF-6 may lead to activation of the inhibitor of kappa kinase (IKK) through formation of polyubiquitin chains, without the requirement of the protease system (Deng *et al.*, 2000). IKK ultimately leads to activation of the transcription factor nuclear factor kappa B (NFκB) through phosphorylation and promotion of degradation of the protein inhibitor of kappa B (IκB).

In addition, a MyD-88-independent signalling pathway also exists for TLR. This has been demonstrated in MyD-88 deficient mice where early after TLR activation there is a lack of cytokine production. However, after a delay there is activation of NF κ B and c-Jun N-terminal kinase (JNK) (Kawai *et al.*, 1999). The activation of MyD-88-independent pathway leads to activation of the transcription factor interferon-regulatory factor-3 (IRF-3) and late-phase NF κ B activity, both of which lead to expression of interferon- β (IFN- β) and associated genes (Takeda and Akira, 2004).

1.6.3.2.2 Complement system

In addition to membrane receptors, the IIS may utilize a set of soluble and cell-associated proteins, termed the complement system, to detect pathogen infiltration (reviewed by Veerhuis *et al.*, 2011). The complement system is comprised of inactive precursor proteins which, upon pathogen recognition, are activated through protease cleavage cascades. The complement system can be activated through three different pathways: the alternative, the lectin and the classical pathway. Activation through the alternative pathway relies on spontaneous activation of the complement protein C3. If a pathogen surface is not in the vicinity of C3, it reverts back to its stable and inactive structure. However, if a pathogen is in the vicinity of C3, the active C3 fragment will bind to its surface and initiate the complement cascade. The lectin pathway on the other hand requires direct pathogen recognition. The lectin protein recognizes and binds terminal mannose residues on microbial glycoproteins and glycolipids, thereby initiating the complement cascade. In a similar manner, the classical pathway requires a complement protein termed C1 to detect and bind antigen-antibody complexes formed on the pathogen's surface to initiate the complement cascade. Therefore, the classical pathway acts as a link between the AIS and the complement system.

Activation of the complement system ultimately leads to generation of proteins responsible for opsonising pathogens to facilitate their phagocytosis, chemotactic recruitment of immune cells, as well as assembling the 'membrane attack complex' (MAC) which forms transmembrane pores on pathogens and induces their lysis.

The complement system is self-amplifying, hence an initial trigger results in an enhanced response by the system. However, if this activation is not properly controlled, the complement system may form MAC on host cells, resulting in lysis of healthy cells.

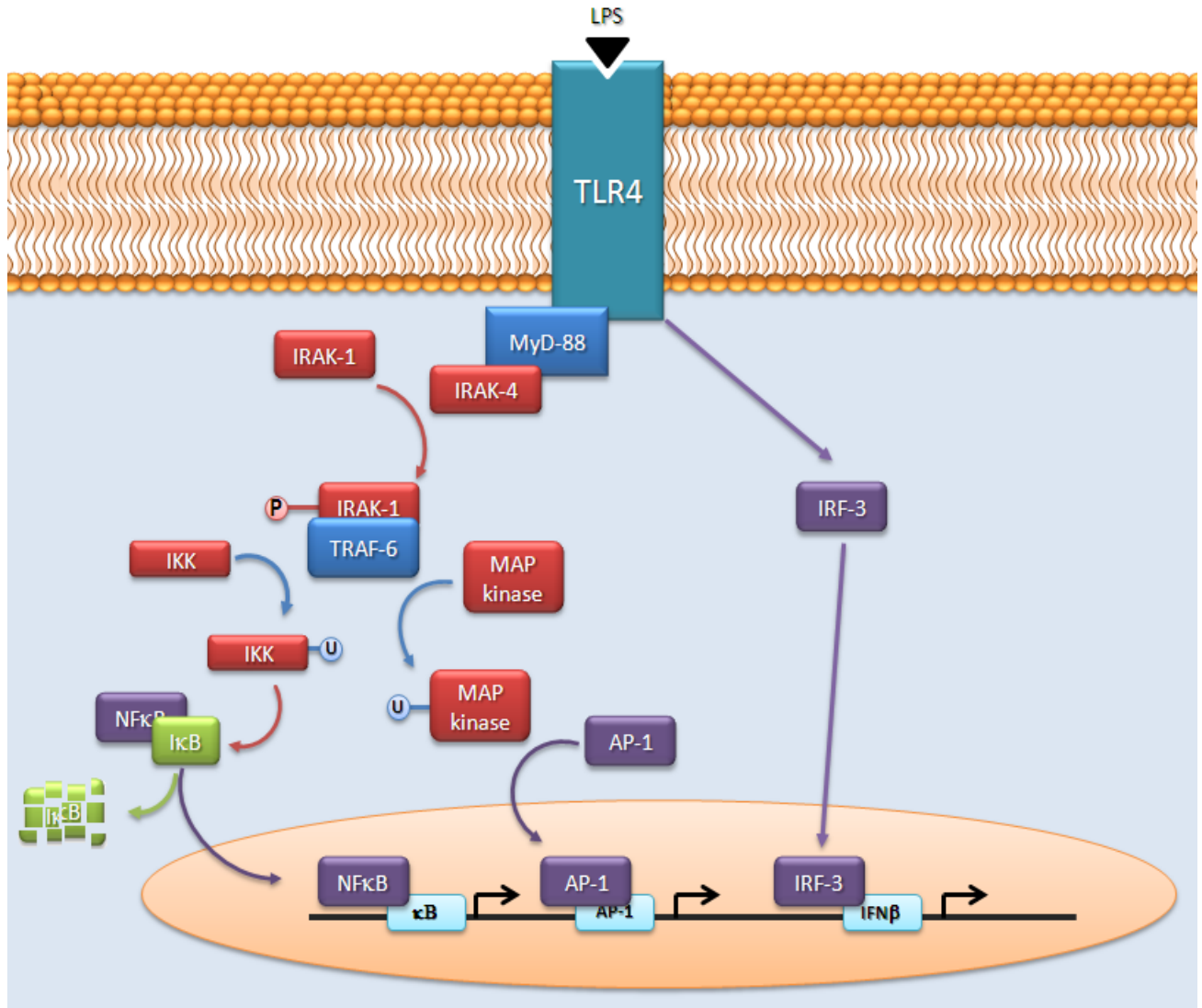


Figure 1.2 LPS signalling through the TLR-4 receptor. Activation of TLR-4 by LPS induces two main signalling pathways, namely a MyD-88-dependent and a MyD-88-independent pathway. MyD-88 dependent pathway leads to activation of the transcription factor NFκB through degradation of the inhibitory protein IκB. NFκB activation leads to expression of genes such as iNOS, TNFα, IL-6/1β. In addition, MyD-88-dependent pathway activation may lead to activation of the transcription factor AP-1 through activation of MAP kinases. AP-1 activation leads to expression of alternative genes necessary for inflammation. Alternatively, activation of the MyD-88-independent pathway leads to activation of the transcription factor IRF-3 and expression of IFNβ, which in turn induces expression of IFNβ-associated genes.

1.6.3.2.3 Class II major histocompatibility complex

The class II major histocompatibility complex (MHC-II) proteins work as a cross-talk point between the AIS and the IIS. MHC-II proteins are found in professional antigen presenting cells, including macrophages and dendritic cells. Upon phagocytosis of a pathogen, macrophages will process its protein load and create peptide fragments which serve as antigens. Antigens are bound by intracellular MHC-II proteins and transported to the cell's surface. Presentation of the antigen on the macrophage's surface allows T-lymphocytes to recognize the antigen. Antigen recognition activated T-lymphocytes, which in turn promote an upregulation of the IIS response as well as mounting an AIS response. Therefore, the presence of MHC-II proteins on a macrophage's surface may be considered a marker for pathogen presence and macrophage reactivity.

1.6.4 Microglia

Microglia were first identified as a cellular component of the central nervous system (CNS) by the Spanish neurologist Pio del Rio-Hortega early in the 20th century through a modified silver carbonate staining. Among the properties which he attributed to these newly identified cells were their amoeboid morphology during migration, transformation into a ramified morphology in the mature brain, and an ability to revert to an amoeboid morphology following a pathological event (Del Rio-Hortega, 1932). Today, microglia are known as the resident macrophage in the CNS and known to perform a wide range of functions, from brain development and neuronal synapse maintenance to tissue homeostasis and antimicrobial protection.

1.6.4.1 Origins of microglia

The debate of where microglial cells originate from has been an intense one. Early studies suggested an ectodermal origin with a common progenitor for microglia and astrocytes (*e.g.* Fedoroff *et al.*, 1997). The current consensus, however, is that the main bulk of microglia arise from

embryonic and early post-natal migration of myeloid precursor cells into the developing CNS (Kettenmann *et al.*, 2011; Ginhoux *et al.*, 2010; Beers *et al.*, 2006). A number of studies reported a significant migration of macrophages from the periphery to the CNS during adulthood (*e.g.* Hickey, 1991). However, more detailed studies established this migration to require disruption of the blood-brain barrier, and demonstrated that in the normal, uninjured brain there is negligible migration from the periphery (Ajami *et al.*, 2007; Mildner *et al.*, 2007). Therefore, it seems that microglia colonize the CNS early in development and the population is maintained during adulthood without significant contributions from circulating peripheral macrophages.

1.6.4.2 Microglial physiology

Microglia constitute ~10% of the brain's cells and are responsible for innate immunity and cellular maintenance through removal of cell debris, as well as partially regulating brain development by aiding in the programmed elimination of neural cells (Marin-Teva *et al.*, 2004). During their migrating stage, microglia adopt an amoeboid morphology, using vessels and white matter tracts as guiding structures. During this stage they colonize the entire CNS, with each cell occupying a defined territory with little overlap between cells. Once they reach their 'assigned' space, they transform into a ramified phenotype (also known, somewhat misleadingly, as a 'resting' phenotype) (reviewed by Kettenmann *et al.*, 2011).

In the adult brain, microglia exist in an inactivated and ramified state in which their cell bodies remain static with very little migration, while their branches are highly motile, extending and retracting processes in a time-scale of minutes, thereby allowing them to constantly monitor their surroundings (Nimmerjahn *et al.*, 2005). The physiological role of resting microglia is hard to establish clearly, since these cells will react to even subtle effects of experimental condition. However, under physiological conditions, microglia are thought to act as CNS 'electricians' to monitor and modify neuronal synapses. Indeed, Wake *et al.* (2009) have shown that resting

microglia specifically extend their ramifications towards and make direct contact with neuronal synapses *in vivo*. Furthermore, they showed that the time-lapse of microglial association with synapses was markedly increased during neuronal ischaemia (from 5 minutes in control conditions to 1 hour during ischemia), suggesting a prolonged window of action for microglia to modify neuronal synapses (either to try to restore synaptic function or remove faulty synapses). In support of this, previous studies have shown that microglia do indeed have the capacity to remove neuronal synapses by preferentially removing pre-synaptic boutons in the cortex (Trapp *et al.*, 2007). The Wake *et al.* study, by inducing an ischaemic environment, probably overestimates the physiologic role of microglia (since ischaemia will also affect microglia directly). However, it provides a mechanism by which microglia may detect faulty synapses and repair or remove them (aiding in synaptic plasticity and transmission efficiency). A further interesting finding of the Wake *et al.* study is the fact that microglia seem to sample neuronal synapses at a rate which is proportional to the neurons' activity. Therefore, microglia may be able to determine neuronal firing rate, perhaps through detection of release of neurotransmitters such as glutamate.

Microglia are also responsible for aiding brain development through phagocytosis of neurons undergoing programmed cell death (apoptosis), as well as directing neuronal migration and differentiation. Marin-Teva *et al.* (2004) have shown that Purkinje neurons undergoing caspase-3 mediated apoptosis in the mouse cerebellum are in close contact with microglia. In addition, the authors showed that microglia are able to phagocytose and destroy apoptotic cells through the production of superoxide ions. Microglia have also been shown to be capable of aiding neuronal migration and differentiation in the rodent brain (Aarum *et al.*, 2003).

Despite uncertainty about their exact physiological role, it is well established that microglia can respond strongly to a pathologic CNS environment (see also section 1.6.4.5). Upon detection of altered brain homeostasis (*e.g.* injury, foreign body infiltration or ischemia), microglia become activated and undergo a series of morphological and functional changes, including retraction and

thickening of their processes and cell body enlargement, ultimately adopting an amoeboid morphology (Haynes *et al.*, 2006). In addition, their protein expression profile changes dramatically. For instance, upon activation, microglia begin to express the surface molecules CD14 and MHC-II (Block *et al.*, 2007) for efficient antigen processing, as well as pro-inflammatory proteins such as TNF α and iNOS for efficient microbicidal function. Furthermore, altered brain homeostasis can lead to increased microglial numbers through increased cellular migration following a chemotactic gradient towards the site of insult (Haynes *et al.*, 2006) or increased local cellular proliferation (Vela *et al.*, 2002).

1.6.4.3 Neuronal control of microglial phenotype

Although the consequences of microglial activation have been extensively studied in the past decade, knowledge of how microglia are kept in a resting state has lagged behind. However, accumulating evidence points to a central role for neuron-glia communication in modulating microglial phenotype.

1.6.4.3.1 'Off' signals

It has been recently established that the type-1 membrane glycoprotein CD200, which belongs to the immunoglobulin superfamily and is expressed by neurons (Lyons *et al.*, 2007; Webb and Barclay, 1984), plays a key role in modulating inflammation. CD200 signals through interaction with a structurally similar membrane protein, termed CD200 receptor (CD200R). CD200R is expressed in microglia but not neurons (Chitnis *et al.*, 2007; Lyons *et al.*, 2007) and induces intracellular signalling through kinase cascades. Genetic studies have shown that mice with a CD200 knock-out mutation have an enhanced inflammatory response to neuronal injury and are more susceptible to developing autoimmune diseases (Hoek *et al.*, 2000). Conversely, overexpression of CD200 in mice confers resistance to autoimmune disease onset, which is

abrogated by administration of an anti-CD200 antibody (Chitnis *et al.*, 2007). *In vitro* studies indicate neuroprotection afforded by CD200 is due to decreased detrimental microglial activation, since *in vitro* activation of CD200R leads to decreased macrophage/microglial expression of the pro-inflammatory cytokines TNF α and IL-6 (Jenmalm *et al.*, 2006) and decreased MHC-II expression (Lyons *et al.*, 2007). However, CD200R may not regulate all possible activation pathways in macrophages, since its activation is able to reduce pro-inflammatory activation due to IFN- γ and amyloid- β , but not LPS (Jenmalm *et al.*, 2006; Lyons *et al.*, 2007).

A second neuronal factor capable of controlling microglial phenotype is the chemokine CX3C chemokine ligand 1 (CX3CL1, also known as fractalkine). Fractalkine and its G-protein coupled receptor CX3CR1 are preferentially expressed in neurons and microglia, respectively, in both humans and rodents (Hatori *et al.*, 2002; Biber *et al.*, 2007; Harrison *et al.*, 1998; Cardona *et al.*, 2006; Hulshof *et al.*, 2003). However, expression of both proteins can be induced in each cell type under specific conditions (*e.g.* inflammation) (Mizuno *et al.*, 2003; Hatori *et al.*, 2002 and references therein). A central role for fractalkine in preventing microglial activation has been shown both in *in vitro* and *in vivo* studies. Hence, *in vitro* treatment of primary rodent microglia with fractalkine reduces LPS-induced expression of the cytokines TNF α and IL-6, as well as a reduction in NO production (as assessed by decreased nitrite concentration) and expression of its synthesising enzyme iNOS. In addition, this reduction in expression of inflammatory factors translated into neuroprotection in neuron-microglia transwell co-cultures (Mizuno *et al.*, 2003). Conversely, *in vitro* treatment of primary rat microglia with a fractalkine-neutralizing antibody enhances LPS-stimulated release of TNF α into the cell culture medium, which translates into increased neuronal survival in primary neuron/microglia co-cultures (Zujovic *et al.*, 2000). The anti-inflammatory and neuroprotective effects of CX3CL1/CX3CR1 interaction have also been corroborated in *in vivo* studies. Using an MPTP mouse PD model, as well as an intraperitoneal LPS injection, Cardona *et al.* (2006) have shown that genetic ablation of the fractalkine receptor CX3CR1 specifically in

microglia induced a greater morphological change towards an activated state in SNc microglia, which was accompanied by enhanced neuronal damage/loss. Furthermore, ablation of the fractalkine receptor was shown to lead to sustained microglial reactivity and neurotoxicity following systemic LPS treatment, as transfer of LPS-activated microglia from CX3CR1^{-/-} rats into naïve rat brains led to a robust increase in apoptotic neurons in the host animals. Similarly, dysregulation of fractalkine/CX3CR1 signalling in the rodent brain through neutralization of fractalkine activity by fractalkine-neutralizing antibodies increases expression of TNF α and oxidative stress following an intracranial LPS challenge (Zujovic *et al.*, 2001). Interestingly, dysregulation of fractalkine/CX3CR1 signalling does not appear to affect microglial activation following facial motor nerve transection in mice (Jung *et al.*, 2000), while microglial activity is enhanced by CD200-CD200R signalling dysregulation in a similar nerve transection model (Hoek *et al.*, 2000), again indicating neuronal control of microglial activation is a complex phenomenon employing a wide range of mechanisms.

A further piece of evidence pointing to neuronal control of microglial activity comes from observations that peripheral neuronal damage can lead to activation of microglia in the CNS (Banati *et al.*, 2001). Human patients with amputated limbs had increased microglial activation in the thalamus region innervated by the amputated nerves. This periphery-to-centre neuronal control of microglia activity could aid in brain plasticity through stripping of unused thalamic synapses or complete removal of obsolete neurons.

1.6.4.3.2 'On' signals

In addition to promoting microglial quiescence, neurons have been shown to be able to modulate microglia in the opposite direction, *i.e.* to induce microglial reactivity. A well characterized family of molecules capable of activating microglia is the matrix metalloproteinase (MMP) family. MMP are proteolytic enzymes capable of degrading extracellular macromolecules for various physiological

activities, including tissue remodelling and wound healing (reviewed by Rosenberg, 2002 and 2009). MMP are expressed in the CNS predominantly by neurons (Kim *et al.*, 2005; Vecil *et al.*, 2000), and their expression can be enhanced by apoptotic signalling (Kim *et al.*, 2010). The most widely studied MMP with regard to microglial activation has been MMP-3. *In vitro* administration of recombinant MMP-3 to the microglial cell line BV2 results in induction of TNF α protein expression, which is reversed by co-administration of an MMP-3 inhibitor. This increase in cytokine expression by MMP-3 is mimicked by administration of medium derived from serum deprived PC12 neurons (*i.e.* apoptotic neurons). Crucially, the increase in cytokine expression by the neuronal medium is abrogated if the microglia are pre-treated with an MMP-3 inhibitor (Kim *et al.*, 2005), showing that MMP-3 secreted by apoptotic neurons plays a crucial role in microglial activation. Similarly, it has been shown *in vitro* that degenerating primary rodent dopaminergic neurons release active MMP-3, and treatment of primary rodent microglia with recombinant MMP-3 results in microglial activation as determined by increased levels of TNF α and superoxide production (Kim *et al.*, 2005 and 2007). The MMP-3/microglia interaction seems to play a crucial role in neurodegeneration since incubation of PC12 neurons with medium derived from MMP-3 treated microglia results in enhanced cell death (Kim *et al.*, 2005). Similarly, MPTP administration results in significantly lower dopaminergic cell death in MMP-3 null mice compared to wild type mice, accompanied by decreased production of superoxide and decreased expression of the microglial activation marker CD11b (Kim *et al.*, 2007). The same authors also showed that induction of microglial cytokine expression by MMP-3 is dependent on activation of mitogen activated protein (MAP) kinases and subsequent activation of NF κ B.

In addition to enzymes, microglial activity can also be modulated by engagement of their neurotransmitter receptors. For instance, it is known that activation of the purine receptor P2Y₆ can induce a phagocytic profile on primary rodent microglia *in vitro* (Koizumi *et al.*, 2007). In addition, purines can enhance release of cytokines (Shigemoto-Mogami *et al.*, 2001), though this effect seems

to be reversed if the microglia are activated with LPS (Ogata *et al.*, 2003). Similarly, the excitatory amino acid glutamate has also been shown to modulate microglial reactivity. However, its effects are complex, since the effects on microglial reactivity depend on which glutamate receptor is engaged (see section 1.8.2.3).

1.6.4.4 Microglia and Parkinson's disease

Over the past decade, *in vitro* and *in vivo* experiments, as well as human post-mortem studies have shown that microglial activation is associated with PD. Firstly, though found throughout the brain, microglial density is heterogeneously distributed. In the adult rodent, microglial density is higher in the substantia nigra compared to other brain areas (Lawson *et al.*, 1990; Block *et al.*, 2007; Kim *et al.*, 2000). Furthermore, the SNc shows higher microglial density compared to the neighbouring dopaminergic ventral tegmental area (Qin *et al.*, 2007), an area that is relatively spared from degeneration in PD and animal PD models. However, the comparison between these findings and microglial distribution in human brains has been called into question by human post-mortem studies which demonstrate microglial density in the normal adult human substantia nigra to be comparable to other areas relatively spared in PD (*e.g.* hippocampus and thalamus) (Mittelbronn *et al.*, 2001; Graeber *et al.*, 2010). Notwithstanding this, studies in neuron-glia co-cultures taken from different rodent brain regions have shown that neurons derived from the substantia nigra are more susceptible to LPS toxicity *in vitro* (Kim *et al.*, 2000), pointing to a more marked inflammatory response in the substantia nigra either through more reactive microglia or a higher microglial density.

The second piece of evidence linking microglia to PD is microgliosis seen in PD patients. Immunohistochemical studies have shown an increase in microgliosis (assessed by level of MHC-II immunopositive cells) found in the SNc of PD patients compared to age matched controls (McGeer *et al.*, 1988), even at early stages of the disease (Ouchi *et al.*, 2005). Furthermore, reactive microglia

can be found in the substantia nigra years after an initial MPTP insult both in animal models and human patients (McGeer *et al.*, 2003; Vingerhoets *et al.*, 1994; Langston *et al.*, 1999).

Thirdly, epidemiological studies have demonstrated a link between use of the anti-inflammatory non-steroidal drug Ibuprofen and decreased risk of developing PD (Gao *et al.*, 2011).

However, the actual role of microglia remains unclear, with arguments having been put forward for a neuroprotective as well as a neurotoxic role (Graeber and Streit, 2010).

1.6.4.5 Microglia: friend and foe

1.6.4.5.1 Neuroprotective effects

Although the evidence for microglia involvement in PD is strong, the exact role microglia play in PD pathogenesis and progression is hard to establish due to the various neuroprotective and neurotoxic actions which microglia can elicit. For instance, microglia are able to express trophic factors such as glial-derived neurotrophic factor (GDNF) (Suzuki *et al.*, 2001; Lee *et al.*, 2004). The physiological role of endogenous GDNF has been hard to establish, since knock out of the GDNF gene leads to early post-natal death due to lack of kidney function (Sanchez *et al.*, 1996). However, using a conditional knock out technique, Pascual *et al.* have shown that GDNF is essential for the survival of adult dopaminergic neurons, since its ablation in adult mice leads to progressive loss of dopaminergic neurons, accompanied by a worsening hypokinetic phenotype (Pascual *et al.*, 2008), two aspects shared with PD. The role of GDNF in neuronal survival is also highlighted by studies with neurodegenerative models. For instance, GDNF deficiency specifically renders nigral neurons susceptible to degeneration after an inflammatory insult in mice (Granholt *et al.*, 2011). In addition, administration of exogenous GDNF is able to prevent nigral degeneration following an MPTP insult, with accompanying improvements in motor function (Kordower *et al.*, 2000). Therefore, GDNF may function to inhibit apoptotic signalling in dopaminergic neurons and its production might be enhanced in microglia upon detection of neuronal injury to increase neuronal

survival. In support of this, *in vitro* studies have shown that microglia are able to up-regulate GDNF in response to increased extracellular glutamate (Liang *et al.*, 2010), a sign of neuronal injury, or under ischaemic conditions (Lee *et al.*, 2004). Expression of GDNF protein and mRNA is also enhanced in microglia after *in vitro* treatment with the activating factors IFN γ (Suzuki *et al.*, 2001) and LPS (Tanaka *et al.*, 2008), respectively. However, the increase in GDNF mRNA by LPS treatment may not be functionally protective, since GDNF protein secretion from neonatal rat primary microglia was shown to actually be reduced by LPS treatment (Matsushita *et al.*, 2008).

In addition, microglia are capable of producing anti-inflammatory cytokines such as IL-10 (Sheng *et al.*, 1995), thereby down-regulating inflammation and its detrimental side-effects. The production of anti-inflammatory cytokines is often accompanied by synthesis of tissue remodelling enzymes, therefore it appears microglia are able to adopt an alternative microglial activation state which would help resolve inflammation and aid in tissue repair and wound healing (reviewed by Loane and Byrnes, 2010; Colton, 2009).

A microglial neuroprotective mechanism more relevant to PD involves reduction of excitotoxicity. *In vitro* studies have shown that microglia are able to uptake glutamate, thereby reducing the extracellular glutamate concentration (Nakajima *et al.*, 2001) and preventing excitotoxic damage (see section 1.5.4). In addition, microglia are able to detect excitotoxic damage to neurons and increase phagocytosis of damaged cell debris, as well as increasing the production of anti-oxidant enzymes without an increase in production of cytotoxic factors (Noda *et al.*, 2011).

Finally, the potential for activated microglia to act as neuroprotective agents is supported by studies which show that LPS-activated microglia promote dopaminergic cell survival in neo-natal mice challenged with MPTP, though this activation proved toxic in aged MPTP-treated mice (Sawada *et al.*, 2007) and adult (10 week old) naïve mice (Arai *et al.*, 2004).

1.6.4.5.2 Neurotoxic effects

Despite the beneficial effects of activated microglia, microglia are also able to cause cellular damage through release of cytotoxic factors such as reactive oxygen species (ROS) capable of inducing oxidative stress (see section 1.5.1), pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α and IL-6 capable of activating apoptotic pathways, and the synthesis of nitric oxide (NO), which can react with hydrogen peroxide to produce the highly reactive molecule peroxynitrite (Taylor *et al.*, 2005; Nakamura *et al.*, 1999; Kreutzberg, 1996; Boje *et al.*, 1992). In addition, NO production is able to inhibit the electron transport chain proteins NADH:ubiquinone reductase (Complex I) (Riobo *et al.*, 2001) and Cytochrome c Oxidase (Complex IV) (Cleeter *et al.*, 1994; Sarti *et al.*, 2003), leading to cell death through energy deficiency and free radical formation (see section 1.5.2 and 1.5.1, respectively). A link between nitric oxide, microglia and Parkinson's disease has been suggested by histochemical post-mortem studies of PD patients. Hunot *et al.* (1996) showed that there is higher expression of the enzyme inducible nitric oxide (iNOS, responsible for NO synthesis from L-arginine) in the mesencephalon of PD patients compared to control subjects. Furthermore, iNOS expression seemed to be restricted to glial cell with an activated macrophage morphology. However, the number of patients examined in this study was relatively low (n = 4 PD).

Several studies have shown that medium from microglia activated with the bacterial component LPS causes cell death in cultured neurons with up-regulation of pro-inflammatory cytokines and ROS (Taylor *et al.*, 2003; Farso *et al.*, 2009). This toxicity can also be elicited by disease-related compounds such as amyloid- β and chromogranin-A (Taylor *et al.*, 2002 and 2003). Furthermore, a recent study has established a mechanism of microglial activation in PD. Zhang *et al.* (2011) have shown that neuromelanin, which is present in dopaminergic cells and therefore is released extracellularly as the cells degenerate, is phagocytosed by microglia and is able to directly activate microglia *in vitro*, leading to the production of nitric oxide, superoxide and hydrogen peroxide,

which results in the demise of cultured nigral neurons. Additional dopaminergic neuron-related compounds have been shown to activate microglia *in vitro*. For instance, alpha-synuclein has been shown to induce production of superoxide and intracellular ROS in cultured primary microglia in a dose dependent manner (Zhang *et al.*, 2005). The actions of alpha-synuclein were shown to require microglial phagocytic activity, since cytochalasin-D, an inhibitor of phagocytosis, decreased ROS production. Interestingly, alpha-synuclein was not able to induce production of nitric oxide or pro-inflammatory cytokines, as has been shown by other inflammogens, demonstrating microglial reactivity is not a black or white process, but rather a complex spectrum of phenotypes differentially elicited depending on inflammogen identity or concentration.

The toxic hypothesis of microglia is further supported by studies which show that intra-nigral infusion of LPS *in vivo* leads to microglial reactivity and toxicity of dopaminergic neurones (Liu *et al.*, 2000). Furthermore, systemic administration of LPS in mice can lead to activation of microglia accompanied by a delayed (7 month) and progressive degeneration of dopaminergic neurons in the substantia nigra (Qin *et al.*, 2007). Degeneration in the neighbouring dopaminergic VTA was not noticed, demonstrating the SNc dopaminergic neurons are particularly vulnerable to inflammatory actions. Another study of systemic LPS administration has shown that there is an increase in pro-inflammatory cytokine production in the brain of rats as early as 8 hours post-injection (Jeong *et al.*, 2010). However, this study showed there is no obvious degeneration of dopaminergic cells at 8 days post-injection. The delay in degeneration in the study by Qin *et al.* (2007) suggests a toxicity amplification cycle, where a primary inflammatory process leads to neuronal damage and hence further inflammation, must occur before neuronal degeneration is seen.

In addition, animal studies have shown that inhibition of microglial activation is able to attenuate cell death. For instance, it has been shown that cell death caused by administration of the dopaminergic-specific toxin 6-OHDA into the striatum is reduced by prior treatment with minocycline (He *et al.*, 2001), an anti-microbial compound with independent anti-inflammatory

actions. Minocycline was able to reduce the number of microglia with retracted processes and reduced the density of CD11b staining in the SNc, indicating reduced microglial activation. In addition, minocycline induced an increase in surviving TH-positive SNc neurons. Similarly, minocycline is able to protect neurons against an MPTP insult (Wu *et al.*, 2002). Mice treated with minocycline soon after MPTP administration (18 mg/kg) have decreased SNc degeneration as determined by TH-positive cell numbers. In addition, minocycline administration soon after a less severe MPTP dose (16 mg/kg) is able to completely prevent SNc cell death. Furthermore, at this lower MPTP dose, minocycline was able to increase the density of TH-positive fibers in the striatum, indicating actual preservation of the nigrostriatal pathway. Neuroprotection by minocycline was accompanied by reduced levels of nitrotyrosine formation (indicating reduced levels of NO-related damage), IL-1 β expression, and activity of iNOS and NADPH-oxidase (enzyme responsible for reactive oxygen species formation), all of which are molecules associated with microglial activation. Microglia's central role in the minocycline-induced neuroprotection was further established by the fact that minocycline did not affect either MPTP's metabolism or astrocytic activity. Neuroprotection by minocycline is replicated in animal studies employing peroxisome proliferator activated receptor (PPAR)- γ agonists (Sadeghian, 2008). PPAR- γ is a nuclear hormone receptor capable of inhibiting activity of a wide range of pro-inflammatory transcription factors such as NF κ B and AP-1 (reviewed by Kielian and Drew, 2003), as well as reducing microglial production of nitrite, TNF α and IL-1 and 6 (Storer *et al.*, 2005). PPAR- γ activation in mice after a 6-OHDA lesion is able to completely prevent reduction in striatal DA concentration and nigral TH-positive cell number, with parallel complete prevention of microglial reactivity (as assessed by lack of MHC-II positive and phagocytic microglia).

Direct evidence of increased inflammation in PD comes from post-mortem studies showing higher levels of the pro-inflammatory cytokines IL-1 β and IL-6 (Blum-Degen *et al.*, 1995), and TNF α (Boka *et al.*, 1994), in the cerebrospinal fluid and substantia nigra of PD patients.

The involvement of inflammation in PD pathogenesis is further supported by epidemiological studies showing a reduced risk (~25%) of developing PD with regular use of non-aspirin non-steroidal anti-inflammatory drugs (NSAID), specially ibuprofen (Gao *et al.*, 2011; Chen *et al.*, 2005). The reason for lack of protection using aspirin is unclear. *In vitro*, it is known that ibuprofen protects neurons against excitotoxicity to a greater extent than aspirin (Casper *et al.*, 2000), though it acts as a weaker free radical scavenger than aspirin (Asanuma *et al.*, 2001). Therefore, the efficacy of each NSAID in protecting against PD could be down to how well it penetrates the blood-brain barrier (BBB). It is known that NSAIDs in principle are able to cross the BBB. However, if attached to plasma proteins such as albumin, their crossing potential is reduced (Parepally *et al.*, 2006). Therefore, the efficacy of each NSAID might rest on how prone it is to binding plasma proteins and therefore how well is able to cross the BBB. Crucially however, Ibuprofen has been shown to be able to directly decrease microglial reactivity (Jin *et al.*, 2008).

Therefore, upon excessive and chronic microglial activation, the neuroprotective actions of microglia may be eclipsed by the increased production of cytotoxic factors, thereby turning microglia into deleterious agents. Hence, modulating microglial reactivity may be an attractive therapeutic target to prevent and possibly reverse neurodegeneration in PD.

1.7 Treatments

As of today there is no cure for PD. Several therapeutic strategies exist to alleviate motor symptoms, mostly based on dopamine replacement and modulation of basal ganglia activity. Of these, the most widely employed is L-DOPA, an exogenous dopamine precursor converted to dopamine by the action of dopamine decarboxylase in presynaptic neuronal terminals. However, though effective during the first 5-10 years of treatment, L-DOPA therapy becomes increasingly inadequate with disease progression and leads to the emergence of motor fluctuations and dyskinesias, including uncontrolled and debilitating erratic movements and abnormal muscle

contractions (Bezard *et al.*, 2001) in up to 50% of patients (Rascol *et al.*, 2000; Ahlskog and Muenter, 2001) due to dysregulated dopamine signalling.

However, dopamine receptor agonism may be employed as a front line therapy to delay the use of L-DOPA. It has been shown that activation of these receptors with dopamine agonists is able to induce antiparkinsonian effects similar to L-DOPA. In addition, certain dopamine agonists have been shown to produce less dyskinesias in both PD patients and animal models in the first years of therapy compared to L-DOPA (Jenner, 2001; Schapira, 2006).

Dopamine signalling may also be modulated with the use of monoamine oxidase-B (MAO-B) inhibitors, such as Selegiline. MAO is a ubiquitous mitochondrial membrane enzyme responsible for metabolising amines and hence terminating dopamine transmission. In addition, MAO-B inhibitors may directly inhibit dopamine re-uptake and promote its synthesis by blocking presynaptic autoreceptors (Elmert and Bertoni, 2008 and references within). However, as total levels of dopamine drop following nigral degeneration, MAO-B inhibitors lose their efficacy, requiring additional therapies to alleviate symptoms.

A potential non-dopaminergic therapy against PD which has shown promise is administration of the neurotrophic factor GDNF, which is a crucial factor for dopaminergic cell survival (see section 1.6.4.5.1). In addition, post-mortem studies have detected reduced levels of GDNF protein in melanin-containing neurons of PD patients compared to age-matched controls (Chaudhan *et al.*, 2000). Furthermore, microglial activation has been shown to decrease GDNF protein secretion (Tanaka *et al.*, 2008; Matsuichita *et al.*, 2008).

Early studies into the administration of GDNF as a therapeutic strategy failed to show any beneficial clinical effects, as well as showing no anatomical amelioration during post-mortem analysis (Kordower *et al.*, 1999; Nutt *et al.*, 2003). The short-comings of these trials were probably due to the method of administration, resulting in lack of sufficient GDNF concentration in the

appropriate areas (*i.e.* in the striatum and the substantia nigra), as well as wide dispersal of the administered GDNF into non-target areas containing receptors for the neurotrophic factor.

More recently, a clinical trial employing a different GDNF delivery method demonstrated greater clinical beneficial effects (Gill *et al.*, 2003), including near complete lack of akinesia and improved PD symptom rating with no overt unwanted side effects, and signs of neuroprotection. However, other studies have put the results of this trial into question (Lang *et al.*, 2006).

1.8 Glutamatergic signalling and neuroprotection

With the exception of exogenous GDNF, all currently available therapies against PD are palliative and hence focus on treating the symptoms. However, as the disease progresses, their efficacy is lost. In addition, prolonged use of current therapies (*e.g.* L-DOPA) results in severe detrimental side-effects (see *e.g.* section 1.7.1). Therefore, there is an urgent need to develop therapies which are capable of not only providing symptomatic relief, but in addition have the ability to halt disease progression and ideally reverse cell death.

A promising target for the development of neuroprotective therapies is glutamatergic signalling. Glutamate is closely associated with PD, since it plays a central role in the functioning of basal ganglia. The subthalamic nucleus of the BG, which is overactive in PD (Bevan *et al.*, 2002; Steigerwald, 2008), sends glutamatergic projections to the main output nucleus and hence contributes to the emergence of motor symptoms. In addition, glutamatergic STN axons also project to the substantia nigra (Smith *et al.*, 1996). It is hypothesised that, due to STN overactivity, these axons release excessive levels of glutamate into the SNc, which might induce cell death through excitotoxic mechanisms (see section 1.5.4). Therefore, reducing glutamatergic STN signalling would provide symptomatic relief (Aziz *et al.*, 1991; Brochie *et al.*, 1991) as well as reducing cell death, thereby slowing disease progression. Indeed, animal studies have shown that disrupting STN

activity can induce neuroprotection in the substantia nigra against the dopaminergic specific toxin 6-OHDA, alongside beneficial behavioural effects (Piallat *et al.*, 1996 and 1999).

1.8.1 Ionotropic glutamate receptors

1.8.1.1 Structure and function

Glutamatergic signalling is primarily mediated through glutamate binding to ionotropic glutamate receptors (iGluR). Ionotropic glutamate receptors are multimeric ligand-gated ion channels whose general structure consists of a large N-terminal extracellular domain, four hydrophobic membrane segments, from which a large extracellular segment extends between membrane domain 3 and 4, and an intracellular C-terminal. Agonist binding occurs through interaction with the N-terminal domain as well as the extracellular segment extending from the membrane domains, while the second membrane domain forms the ion pore (reviewed by Ozawa *et al.*, 1998 and Kew and Kemp, 2005). iGluR can be divided into three main groups according to their pharmacological profile: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (AMPA), 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors and N-methyl-D-aspartate (NMDA) receptors (NMDAR). iGluR have great molecular diversity due to varying receptor subunit composition, as well as the many subunit variants resulting from alternative splicing and RNA editing. To date, four AMPAR subunits (GluR1-4), five kainate receptor subunits (GluR5-7, KA1-2) and seven NMDAR subunits (NR1, NR2A-D, NR3A-B) have been cloned (Kew and Kemp, 2005). The specific subunit composition of each receptor confers a different mechanism of action for each receptor (*e.g.* Chatterton *et al.*, 2002). Therefore, while AMPAR are mainly permeable to sodium and potassium ions (with a small subset being weakly permeable to calcium, depending on their subunit make up), NMDAR are permeable both to sodium and potassium, as well as being highly permeable to calcium ions (reviewed by Ozawa *et al.*, 1998). Therefore, NMDAR are thought of as the main excitotoxic mediators.

NMDAR are also unique in that they require two co-agonists, namely glycine and glutamate, to induce channel opening (Clements *et al.*, 1991). NMDAR activation is responsible for fast excitatory neurotransmission in the CNS, though its activation and deactivation kinetics are much slower than for AMPAR (Ozawa *et al.*, 1998). Under resting potentials, the receptor's pore is blocked in a voltage-dependent manner by a magnesium ion (Mg^{2+}), therefore transmission is impaired until the arrival of a depolarizing potential. Upon membrane depolarization and agonist binding, the NMDAR channel opens and high amounts of calcium ions enter the cell. One of the main roles of NMDAR-induced calcium influx is regulating proper synaptic plasticity. Indeed, it is known that NMDAR are crucial for the long term potentiation (LTP) component of memory (Lamsa *et al.*, 2007). The role for NMDAR in normal memory and cognition function is highlighted by the fact that NMDAR subunits and associated intracellular molecules are affected in individuals with schizophrenia (Clinton and Meador-Woodruff, 2004), a disease characterized by severe cognitive and memory dysfunction.

1.8.1.2 Ionotropic glutamate receptors as therapeutic targets

Glutamatergic modulation through antagonism of NMDA receptors has been attempted as a therapeutic strategy against PD with moderate success in preclinical studies. NMDAR antagonism with the polyamine antagonist ifenprodil has been shown to reduce parkinsonian symptoms in the reserpine-treated rat PD model (Nash *et al.*, 1999). The effects were attributed to a decrease in the activity of the indirect pathway of the basal ganglia, which is overactive in PD patients. Similarly, the NMDAR antagonist MK-801 and memantine have been shown to have antiparkinsonian effects (as measured by increased locomotion and rearing) in mice rendered parkinsonian by MPTP administration (Fredriksson *et al.*, 2001). Furthermore, NMDAR antagonism has been shown to be able to protect dopaminergic neurons in various animal models of PD. For instance, systemic administration of MK-801 is able to reduce neuronal loss in the SNc following administration of

MPP⁺ (the active metabolite of MPTP) (Turski *et al.*, 1991), an effect attributed to reduced excitotoxicity. Decreased neuronal loss may be accompanied by decreased striatal dopamine loss following MK-801 and MPTP administration (Tabatabaei *et al.*, 1992), suggesting true protection of the nigrostriatal dopaminergic pathway.

However, NMDA antagonism in clinical settings has proven highly inadequate due to the emergence of intolerable side effects such as psychosis (Kornhuber and Weller, 1997), memory loss (Rockstroh *et al.*, 1996) and hallucinations (Muir and Lees, 1995). These effects are attributed to two main characteristics of NMDA receptors: firstly, their ubiquitous distribution (Monaghan *et al.*, 1989; Daggett *et al.*, 1998; Meoni *et al.*, 1998) causes NMDAR antagonists to affect the nervous system in general without specific and local actions, thereby producing unpredictable side effects. Secondly, NMDA receptors are intimately associated with long-term potentiation (LTP), which is crucial for plasticity and learning (Pare, 2004).

Hence, for anti-glutamatergic neuroprotective strategies to be clinically successful, they need to target specific areas of the brain, as well as not completely abolish glutamatergic signalling in order to maintain the basal cognitive function of glutamate. A promising therapeutic target fitting these characteristics are the metabotropic glutamate receptors (mGluRs) due to their more select distribution and their modulatory function in glutamatergic signalling.

1.8.2 Metabotropic glutamate receptors

1.8.2.1 Structure and function

The second major class of glutamate receptors, metabotropic glutamate receptors (mGluR), have become a promising target for PD therapy due to their more selective and less widespread actions compared to iGluR distribution (Messenger *et al.*, 2002). mGluR comprise of a varied family of G-protein coupled receptors (GPCR) divided into three main groups according to their signal transduction mechanisms and genetic coding (reviewed by Conn and Pin, 1997). All three groups

share a general structure comprising a large N-terminal extracellular domain and a C-terminal intracellular domain linked by a seven transmembrane domain (7-TMD). The ligand binding domain in mGluR is a bilobed structure resting on the extremity of the N-terminal chain, termed the Venus Flytrap Domain (VFD). A cleft formed between the two lobes of the VFD is responsible for binding glutamate and confers agonist specificity to each receptor. In support of this, chimeric receptor studies have shown that exchange of the VFD of receptor 'A' for the VFD of receptor 'B' allowed receptor B-specific agonists to activate receptor A (Tones *et al.*, 1995; Takahashi *et al.*, 1993). Once an agonist is bound to the VFD, the two lobes adopt a closed conformation, causing a structural change able to transduce the ligand's signal. It has been shown that for the VFD to be able to relay signals, a further region of the N-terminal domain is required, namely the cysteine-rich domain (CRD). The CRD is located between the VFD and the 7-TMD, and encompasses an 80 amino acid sequence rich in cysteine residues (Pin *et al.*, 2003). The CRD is required to relay ligand signals, but it is not needed to bind the ligands themselves. Instead, it seems the CRD undergoes a further structural change upon ligand binding, namely a cysteine-cysteine interaction between residues in the CRD and VFD, which results in the signal being transmitted to the intracellular region. Accordingly, mutagenic studies have shown that mutation of a key cysteine residue on the VFT resulted in a receptor which was correctly transported to the cell membrane and was able to bind agonists, but lacked the capacity to induce intracellular signalling (Rondard *et al.*, 2006). Relaying of signals from the VFT domain to the intracellular domains is thought to be due to stabilization of an active conformation of the 7-TMD. The 7-TMD is responsible for binding G proteins through interactions between the G protein and an intracellular loop of the 7-TMD. Therefore, upon ligand binding and stabilization of an active conformation of the 7-TMD, the G protein machinery would be activated to initiate an intracellular signalling cascade. As discussed below, each group of mGluR is associated with a particular G protein depending on the composition of the receptor's 7-TMD intracellular loops, therefore receptor activation leads to different

intracellular signalling cascades for each group of receptors. However, the mechanism of signal transduction by mGluR is complicated by the fact that these receptors exist as dimers (*e.g.* Romano *et al.*, 1996). Studies have shown that an mGluR monomer is able to induce signal transduction, but that its efficacy is increased if paired with another mGluR monomer (reviewed by Brock *et al.*, 2007). Therefore, dimers may work synergistically by each monomer stabilizing the active conformation of the other. Furthermore, there is evidence that mGluR may form heterodimers (*e.g.* mGlu2/mGlu4 dimers) (Doumazane *et al.*, 2010). This opens the possibility that an agonist traditionally viewed as specific for one type of receptor may induce signalling typical of a different receptor.

1.8.2.1.1 Group I mGluR

The first group of mGluR, group I, comprises the receptors mGlu 1 and 5. Group I receptors are coupled to a $G_{q/s}$ protein to promote the production of diacyl glycerol (DAG) and inositol trisphosphate (IP3) and activation of protein kinase C (PKC) upon glutamate binding. Post-synaptically they increase cellular excitability and augment NMDAR-mediated currents (Pisani *et al.*, 2001) (reviewed by Conn *et al.*, 2005). Pre-synaptically they facilitate glutamate release from glutamatergic terminals. Therefore, overall, group I mGluR activation results in increased synaptic excitability. However, activation of mGluR1 has also been reported to inhibit neuronal firing when the receptor is transiently activated (Fiorillo *et al.*, 1998). Neuronal excitation inhibition was attributed to calcium-dependent potassium currents hyperpolarizing dopaminergic neurons, further complicating mGluR signalling. Because of their link with NMDA currents, group I receptors are thought to play a modulating role in LTP. Indeed, it has been noted that blockade of group I activity decreases LTP in the striatum (Gubellini *et al.*, 2004). Group I receptors are linked to NMDA receptors through a protein scaffolding localizing both receptors at the synapse's post-synaptic

density. In *in vivo* studies, it has been shown that mGluR-1 deficient mice have impaired learning ability coupled with inefficient LTP (Aiba *et al.*, 1994).

Table 1.1 Classification and effects of metabotropic glutamate receptors

Receptor Class	Receptor type	G-protein coupling	Activation effect
I	mGluR 1 mGluR 5	G _{q/s}	Activate phospholipase-C, increase intracellular Ca ²⁺ , inhibit K ⁺ outflow, induce neuronal firing
II	mGluR 2 mGluR 3	G _i	Inhibit adenylate cyclase activity, decrease intracellular cAMP, increase hyperpolarizing potassium currents, prevent neuronal firing
III	mGluR 4 mGluR 6 mGluR 7 mGluR 8	G _i	Inhibit adenylate cyclase activity, decrease intracellular cAMP, prevent neuronal firing

Immunohistochemical studies on rat tissue (Testa *et al.*, 1998; Tallaksen-Greene *et al.*, 1998) have shown mGlu1 protein expression in the neostriatum, which appeared to localize to post-synaptic membranes. In the globus pallidus and the SNr, expression was shown to be clustered along dendrites, while staining in the subthalamic nucleus appeared in coarse fibres. In the SNc, mGluR 1 expression has been detected in presynaptic membranes in close proximity to corticostriatal projections (Smith *et al.*, 2000). Similarly, mRNA expression studies (Testa *et al.*, 1995) and immunohistochemical studies in the rat (Tallaksen-Greene *et al.*, 1998) have shown selective mGlu 5 mRNA expression in the striatum, depending on the identity of striatal cells.

Table 1.2 Metabotropic glutamate receptor mRNA expression in the rodent brain.

	mGlu 1	mGlu 5	mGlu 2	mGlu 3	mGlu4	mGlu 7	mGlu 8
Globus Pallidus	+	+	-	++	+	+	+
Nucleus Accumbens	+	+++++	-	+++	++++	+++++	+++
SNc	+++++	++	-	++	+	+	++
SNr	+	+	+	+	+	+	+
Striatum	++	++++	-	+++	+++	++++	++
STN	++	+++	+++	++	++	++	++
Thalamus	+++	+	-	+	+++++	+++	+

Adapted from Messenger *et al.*, 2002

1.8.2.1.2 Group II mGluR

The second group of mGluR, group II, comprises the receptors mGlu 2 and 3. Group II receptors are linked to a G_i protein, therefore inhibit adenylyl cyclase activity and drive down cAMP production upon glutamate binding (Conn and Pin, 1997). In addition, group II receptors are able to inhibit the firing of voltage gated calcium channels and stimulate hyperpolarising potassium conductance, therefore prevent calcium and membrane depolarization-dependent transmitter release (Conn *et al.*, 2005). Hence, group II receptors act post- and pre-synaptically to inhibit neuronal excitation and further release of transmitter, respectively, thereby preventing excessive and potentially harmful neurotransmission. This role is backed by evidence showing group II receptor activation causes inhibition of cortical signalling to the striatum and reduces STN signalling to the SNr and SNc (Gubellini *et al.*, 2004; Murray *et al.*, 2002; Wigmore *et al.*, 1998). Genetic knock-out studies point to the involvement of group II receptors in LTD. Yokoi *et al.* (1996) reported an impairment of hippocampal LTD in mice lacking mGluR2. The group II receptors are further differentiated from the other groups in that they have the highest sensitivity to glutamate, being activated by concentrations 10-100 times lower than for the other two groups (Cartmell and Schoepp, 2000).

Immunohistochemical studies in rats (Testa *et al.*, 1998) have shown group II receptors are expressed widely in the basal ganglia, with a predominant pre-synaptic localisation on corticostriatal and subthalamonigral neurons.

Table 1.3 Metabotropic glutamate receptor protein expression in brain regions across species

(R, rat; Mk, monkey; H, human; Ms, mouse).

	mGlu 1	mGlu 5	mGlu 2/3	mGlu 2	mGlu4	mGlu 6	mGlu 7	mGlu 8
Gl. Pallidus	R Mk	Mk	R	H	Ms	---	R	R
SNC	R Mk	R Mk	R	---	R	---	---	---
SNr	R Mk	R Mk	R	H	R	---	R	---
Striatum	R Mk	R Mk	R	H	R Ms	---	R	R
STN	R	R	R	H	---	---	---	---
Retina	---	---	---	---	---	H R	---	R

Unquantified positive expression
Strong positive expression
Moderate positive expression
Low positive expression
 --- Negative/undetermined expression

Zhanq *et al.*, 2009; Awad *et al.*, 2002; Smith *et al.*, 2000; Hubert *et al.*, 2001; Testa *et al.*, 1998; Phillips *et al.*, 2000; Makoff *et al.*, 1996; Kinoshita *et al.*, 1996; Corti *et al.*, 2002; Bradley *et al.*, 1999.

1.8.2.1.3 Group III mGluR

The third group of mGluR, group III, comprise the receptors mGlu 4, 6, 7 and 8. Like group II receptors, group III receptor activation induces inhibition of adenylyl cyclase through G_i signalling and prevents cAMP formation, thereby inhibiting neurotransmitter release and post-synaptic membrane depolarization. In accordance with this, studies have shown that activation of group III receptors in striatopallidal and subthalamonigral brain slice preparations inhibits striatal signalling to the globus pallidus and subthalamic signalling to the SNC, respectively, through a pre-synaptic

mechanism (Valenti *et al.*, 2003 and 2005). In addition, *in vivo* studies have shown that activation of group III receptors elicits long term depression in the hippocampus of rats (Naie *et al.*, 2006).

The expression of group III mRNA and protein has been less extensively studied than for the other two groups. However, studies have established pre-synaptic expression of mGluR 7 on striatopallidal, subthalamonigral and striatonigral projections in the rat brain (Kosinski *et al.*, 1999). Furthermore, mGluR7 expression has been localized to a specific synaptic region termed the active zone (Kinoshita *et al.*, 1998). Since mGluR7 has the lowest sensitivity for glutamate of all receptors, being activated only by glutamate concentrations nearly 1000x higher (Cartmell and Schoepp, 2000), it may function as a break to prevent glutamatergic overstimulation. In line with this hypothesis, studies in mice lacking mGluR 7 receptors have shown that these animals are specially sensitive to epileptic seizures (Sansig *et al.*, 2001).

The expression of mGluR4 has also been shown to be primarily presynaptic. mGluR 4 is strongly expressed in the cerebellum, with expression also evident in the hippocampus, olfactory bulb and basal ganglia. Knock-out studies have shown that mice lacking the mGluR4 receptor have impaired cerebellar synaptic plasticity and spatial memory performance (reviewed by Niswender *et al.*, 2010).

Although the expression of mGlu8 mRNA has a wide distribution in the CNS of both humans and rodents (Kinoshita *et al.*, 1996; Robbins *et al.*, 2007) as well as in the periphery, it is thought to be lower than that for mGlu4 and 7. Like mGluR4 and 7, its location is primarily pre-synaptic, though expression has been detected on post-synaptic membranes as well (reviewed by Niswender *et al.*, 2010). Activation of mGluR8 with synthetic ligands can induce anti-convulsant properties in mice (Moldrich *et al.*, 2001), though its main role seems to be regulation of anxiety. Accordingly, mice lacking mGluR8 show increased anxiety levels with no differences in sensorimotor functions (Robbins *et al.*, 2007).

Of all the mGluR, mGlu 6 shows the most restricted expression. So far, its expression has only been shown in the retina, where it is expressed in ON bipolar retinal cells (Niswender *et al.*, 2010). Therefore, impairment in mGlu 6 function results in visual deficits.

Further studies need to be carried out to comprehensively map expression of all three mGluR groups, as well as assess changes in mGluR expression (of all three groups) during pathological conditions.

Studies have shown that modulation of all three groups of mGluR is able to induce symptomatic relief in animal models. It has been noted that antagonists or agonists of group I or group II/III receptors, respectively, reverse motor symptoms in animal models of PD. For instance, MacInnes *et al.* (2004) have shown the general group III agonist L-SOP is able to reverse akinesia in DA depleted rats through specific modulation of group III receptors, since the group III antagonist M-SOP is able to prevent this effect. Although the mechanisms of L-SOP's effects were not established, subsequent studies by the same authors (MacInnes *et al.*, 2008) showed L-SOP administration reduced GABA release into the GPe in an M-SOP dependent manner, thereby restoring the tonic pallidal inhibition of STN activity and normalizing BG activity.

1.8.2.2 Neuroprotection through mGluR modulation

In addition to ameliorating motor symptoms, mGluR ligands have been shown to protect nigral dopaminergic neurones from degeneration in certain animal models. Studies within our research group have shown that group I receptor blockade by the mGlu1 and 5 antagonists LY367385 and MPEP, respectively, was able to reduce dopaminergic SNc cell death by *in vivo* administration of 6-OHDA (Vernon *et al.*, 2007a). Neuroprotection was concentration dependent, suggesting a receptor-mediated mechanism, and, importantly for therapeutic prospects, MPEP was still neuroprotective even when administered 7 days post-lesion. In a similar manner Vernon *et al.* (2007b) have shown neuroprotection of SNc dopaminergic neurons upon activation of group III mGluR. In a 6-OHDA

animal model of PD, loss of TH-positive neurones in the SNc was attenuated by administration of the group III general agonist L-AP4. Importantly, SNc neuronal survival was accompanied by an increase in striatal dopamine, indicating the surviving SNc neurons still had functioning nigrostriatal projections. Despite the efficacy of L-AP4, it was noted that the neuroprotective effects had a bell-shaped concentration dependence. This could either imply activation of non-group III receptors by L-AP4 at high doses, or desensitization and internalization of group III receptors. This dosage limitation is important, since even the most efficacious L-AP4 dose did not induce full neuroprotection. Similarly, this research group has shown *in vivo* neuroprotection by mGlu8 and mGlu4 activation with the compounds (S)-DCPG and PHCCC, respectively (unpublished data). Importantly, neuroprotection was seen even with systemic administration of compounds, as well as in various animal models (*i.e.* 6-OHDA and lactacystin-induced neurodegeneration).

The neuroprotective effect of the various mGluR ligands is thought to occur by preventing excitotoxicity through downregulation of glutamatergic subthalamo-nigral signalling. In support of this, various *ex vivo* studies utilizing brain slice preparations have established the ability of group II and III agonists to reduce glutamatergic signalling from the STN onto SNc dopaminergic neurones (Katayama *et al.*, 2003; Wigmore *et al.*, 1998, Valenti *et al.*, 2005). The exact role of each type of mGluR were not elucidated, but the specific involvement of mGlu4 and mGlu8 was established through sub-type selective agonism. However, to the best of our knowledge, no *in vivo* studies have unequivocally established modulation of subthalamo-nigral modulation by mGluR ligands.

1.8.2.3 Non-neuronal mGluR: localization and effects

The above studies provide evidence that *in vivo* neuroprotection by mGluR ligands occurs through decreased subthalamo-nigral signalling. However, the fact that mGluR receptors exist in glial cells opens up the possibility of alternative neuroprotective mechanisms which might synergize with the reduced excitotoxicity mechanism proposed for mGluR ligand neuroprotection. Taylor *et*

al. (2003, 2002) have shown both mRNA and protein expression (*in vitro*) of mGlu 2, 3, 4 and 8 in primary microglia from neonatal rats. Interestingly, they were also able to detect mRNA and protein for mGlu6 (thought to occur only in the retina), while not being able to detect mGlu7. Similarly, Biber *et al.*, (1999) have shown astrocytic expression of mGlu5 and 3 mRNA in cultures from neonatal rats. Human tissue studies have revealed astrocytic and microglial expression of mGlu1, mGlu2/3, mGlu4, mGlu5 and mGlu8 (Geurts *et al.*, 2003, 2005). Importantly, these studies showed an up regulation of glial group III mGluR during brain lesions, suggesting a role for these receptors in regulation of the inflammatory process.

Glial modulation by mGluR ligands may involve a decrease in the production of cytotoxic agents such as nitric oxide and pro-inflammatory cytokines, an increase in trophic support through production of trophic factors, and/or decreased excitotoxicity through enhanced glial glutamate uptake.

Indeed, several *in vitro* studies have shown that mGluR activity can modulate the activation profile of primary microglia. Farso *et al.* (2009) have shown that the group I agonist (S)-3,5-Dihydroxyphenylglycine (DHPG) is able to reduce microglial activation, measured as a reduction in production of NO, pro-inflammatory cytokine TNF α , and glutamate release. In a similar manner, Byrnes *et al.* (2009) have shown that activation of microglial mGlu5 decreases the production of NO, TNF α and, importantly, reactive oxygen species in a concentration and receptor-dependent manner. Although the reduction in neurotoxin production was modest, it was sufficient to confer neuroprotection to neurons co-cultured with activated microglia. In addition, *in vivo* activation of mGlu5 has been shown to induce brain derived neurotrophic factor (BDNF) expression (Matarredone *et al.*, 2001). Although group I agonists show the potential to inhibit microglial activation, they might not be desirable therapeutic candidates since 1) their modulation of microglial reactivity was modest and 2) would cause an increase in neuronal excitability, thereby exacerbating excitotoxicity and motor symptoms (Aguirre *et al.*, 2001).

The effect of group II mGluR activation on microglial activation has also been studied. Taylor *et al.* (2002, 2005) have shown that group II activation with DCGIV increases microglial reactivity and renders microglia neurotoxic. Neurotoxicity was attributed to enhanced TNF α production, which activates the pro-apoptotic caspase-3 pathway in neurons. Furthermore, group II activation induced apoptosis of the microglial cells. The effect of DCGIV was specifically mediated by group II receptors, since antagonism at these receptors prevented neurotoxicity. Furthermore, Pinteaux-Jones *et al.* (2008) have shown that whilst activation of microglial mGlu2 augments the neurotoxic microglial phenotype induced by myelin stimulation, activation of microglial mGlu3 actually reduced neurotoxicity.

Studies with group III agonists have been more promising than for group II agonists. It has been shown that activation of microglial group III mGluR prevents microglia from adopting a neurotoxic profile (Taylor *et al.*, 2005). The protective effect of group III activation was specifically microglial, since direct addition of the agonist to neurons treated with medium from activated microglia did not increase survival. Importantly, group III activation also prevented microglial apoptosis upon activation. The exact mechanism by which activation of group III receptors induces a non-toxic profile in microglia is not known. Although a reduction in neurotoxic factors such as NO or pro-inflammatory cytokines seems likely, it is also possible that increased production of neurotrophic factors (*e.g.* GDNF) supports neuronal viability upon microglial insult. An increase in glutamate uptake might be considered *in vivo* (Nakajima *et al.*, 2001), but seems an unlikely mechanism for the above studies. Indeed, microglia are known to produce GDNF (Lee *et al.*, 2004; Suzuki *et al.*, 2001). Administration of GDNF can protect neurones against a wide range of insults. Boscia *et al.* (2009) have shown neuroprotection of hippocampal neurons against *in vitro* NDMA excitotoxicity. Furthermore, GDNF has also been shown to be protective towards nigral neurons *in vivo* against 6-OHDA toxicity in rats (Kearns *et al.*, 1995), proteasomal dysfunction in mice (Du *et al.*, 2008) and MPTP toxicity in mice (Schober *et al.*, 2007) and monkeys (Kordower *et al.*, 2000). Interestingly,

the study by Kordower not only established neuroprotection, but in addition functional recovery was seen, suggesting a neuroregenerative role for lenti-virally delivered GDNF.

The hypothesis that microglial mGluR activation induces GDNF expression is backed by studies showing neuronal mGluR activation leads to GDNF mRNA and protein production in neurons (Battaglia *et al.*, 2009). Although signalling mechanisms between neurons and glia might lead to different protein expression, *in vitro* experiments have shown that activation of astroglial group II mGluR also induces expression of neurotrophic factors (Bruno *et al.*, 1998). In addition, Liang *et al.* (2010) have shown that *in vitro* activation of both group III mGluR and NMDAR induces an increase in expression of the neurotrophic factors nerve growth factor (NGF), BDNF and GDNF.

1.9 General hypothesis: Pharmacological agents targeting individual group III metabotropic glutamate receptors expressed in microglial cells are able to inhibit microglial activation and release of pro-inflammatory factors, thereby reducing microglia-driven neurotoxicity towards dopaminergic neurons. Furthermore, mGluR expression by microglia is comparable *in vitro* and *in vivo*.

1.10 Aims

The aim of this thesis is to elucidate the effects of activating individual group III microglial metabotropic glutamate receptors on microglial reactivity and establish whether these effects translate into neuroprotection towards dopaminergic neurons. In addition, the *in vivo* expression of group III mGluR on neurons and microglia will be mapped out in the rat substantia nigra and striatum. Achieving these aims will help to identify the most appropriate mGluR to develop as a viable therapeutic strategy against Parkinson's disease.

1.11 Objectives

The objectives of this thesis are as follows:

- Establish the microglial cell line N9 as a viable *in vitro* model for microglial activation
- Establish expression of individual group III mGluR in N9 and primary microglia, as well as in the rat substantia nigra and striatum
- Determine the effects of activating the receptors mGlu4, mGlu7 and mGlu8 on microglial reactivity and investigate their signalling mechanisms
- Investigate whether the effects of activating individual group III mGluR on microglial reactivity translates to neuroprotection *in vitro*

Chapter II

Materials and Methods

2.1 *In vitro* methods

All *in vitro* experiments were carried out in ventilated sterile hoods (u.v. light irradiated and cleaned with 70% ethanol) with autoclaved or ethanol-sterilized equipment and latex gloves. Care was taken to ensure sterile conditions were maintained throughout the experimentation period by maintaining appropriate hood screen height and ventilation level. Incubators were cleaned monthly with 70% ethanol and water reservoir replaced weekly with autoclaved water containing prothermal blue (Lucerna Chemicals, Switzerland).

2.1.1 *Reagents and consumables*

All sterile cell culture plates (6, 24 and 96 well plates), sterile cell culture flasks, sterile plastic scrapers, ethanol, serological pipettes, sterile pipette tips, sterile eppendorf tubes (2.5 mL and 500 μ L) and sterile Falcon tubes (50 mL and 15 mL) were purchased from VWR (Lutterworth, UK). Sodium dodecyl sulphate (SDS), glycine, Tris-base, L-glutamine, Dulbecco's modified Eagle medium (D-MEM), penicillin/streptomycin solution, fetal bovine serum, trypan blue, Ponceau-S red, trypsin, paraformaldehyde powder, Griess reagent (sulfonilamide plus 1-(naphthyl)ethylenediamine), NADPH, nitrate reductase and Bradford reagent were purchased from Sigma-Aldrich (Dorset, UK). Antibodies employed in this thesis, along with the concentration employed and origin, are shown in **table 2.1** and **2.2**.

Table 2.1 Antibodies employed for immunocytochemical and immunohistochemical labelling (antibody concentrations in parenthesis denote concentrations employed during immunohistochemical labelling).

Target	Antibody type	Antigen	Target species	[Antibody]	Company	2° antibody conjugate	Host species	[Antibody]	Company
mGlu 1a	Rb Polyclonal	C-terminal peptide	Mk, Rt, H	10 µg/mL	Millipore (Ab1551)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 5	Rb Polyclonal	C-terminal peptide	Rb, Rt, H	10 µg/mL	Abcam (Ab27190)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 2	Rb Polyclonal	Human recombinant protein	Ms, Rt, H	10 µg/mL	Abcam (Ab13192)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 3	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	10 µg/mL	Millipore (Ab9449)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 4	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	10 µg/mL (5 µg/mL)	Abcam (ab53088)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
mGlu 6	Rb Polyclonal	C-terminal peptide	Rt	10 µg/mL (5 µg/mL)	Abcam (ab10314)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
mGlu 7	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	(5 µg/mL)	Abcam (Ab53705)	Alexa-488	Gt	(2 µg/mL)	Invitrogen (A-11034)
	Gt Polyclonal	N-terminal peptide	Ms, Rt, H	10 µg/mL	SantaCruz (sc-47156)	Alexa-488	Rb	2 µg/mL	Invitrogen (A-21222)
mGlu 8	Rb Polyclonal	Synthetic human peptide	Ms, Rt, H	10 µg/mL (5 µg/mL)	Abcam (Ab53094)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
MHC-II (Ox-6)	Ms Monoclonal	Rat thymocyte membrane glycoprotein	Rt	(10 µg/mL)	Serotec (MCA46G)	Cy-3 red	Gt	(2 µg/mL)	Invitrogen (A10521)
CD11b (Ox-42)	Ms Monoclonal	Protein purified from rat peritoneal macrophages	Rt	4 µg/mL (4 µg/mL)	Serotec (MCA275G)	Cy-3 red	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A10521)

Rb, Mk, Rt, H, Gt, Ms = Rabbit, Monkey, Rat, Human, Goat, Mouse

Table 2.2 Antibodies employed during immunolabelling of cell lysate Western blots.

Target	Antibody type	Target species	[Antibody]	Company	2° antibody conjugate	Host species	[Antibody]	Company
mGlu 4	Rb Polyclonal	Ms, Rt	2 µg/mL	Millipore (Ab15097)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
mGlu 7	Rb Polyclonal	Ms, Rt, H	2 µg/mL	Abcam (Ab53705)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
mGlu 8	Rb Polyclonal	Ms, Rt, H	2 µg/mL	Abcam (Ab53094)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
iNOS	Rb Polyclonal	Ms	0.2 µg/mL	Cayman (160862)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
TH	Rb Polyclonal	Ms, Rt, H	90 ng/mL	PeIFreeze (P40101)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
β-Actin	Ms Monoclonal	Ms, Rt, H	50 ng/mL	Abcam (ab6276)	HRP	Gt	20 ng/mL	Vector labs (P-2000)

2.1.2 N9 cell line

The murine microglial cell line N9 was used for all microglial experiments not involving primary microglia.

The N9 cell line was first developed by Dr. Ricciardi-Castagnoli *et al.* (1989) through oncogenic retroviral transformation of embryonic day 13 mouse microglial cells. N9 microglia were originally shown to retain microglial phenotypic cell surface markers, be devoid of astroglial and neuronal cell surface markers, and were shown to increase pro-inflammatory cytokine production upon LPS treatment. N9 cells were given as a gift to Dr. Deanna Taylor and a frozen stock produced (see section 2.1.7) and kept in liquid nitrogen.

Using a 37°C water bath, frozen cell aliquots (containing dimethyl sulfoxide, DMSO) (see section 2.1.7) were thawed in the presence of 1 mL warm Dulbecco's Modified Eagle Medium (DMEM with phenol red) (Sigma, D5796) containing 5% filter-sterilized heat-inactivated foetal calf serum (FCS) (BioSera, F1000), 50 U/mL Penicillin and 50 µg/mL streptomycin (Sigma, P4333), and 8 mM L-glutamine (Sigma, G7513) (this preparation of medium was used throughout when handling N9 cells, henceforth termed 'complete microglial medium'). Thawed cells were centrifuged (300g,

5 mins) to remove DMSO, resuspended in warm complete microglial medium and transferred to a 75 cm³ cell culture flask (VWR, 734-1713) with 25 mL warm (37°C) complete microglial medium. The cells were kept in an incubator at 37°C with 5% CO₂ ventilation (these incubating conditions were used throughout the experiments). Every 2-3 days the cells were passaged to avoid confluency. Passaging involved washing the cells with fresh warm DMEM to remove dead cells, scraping cells down with a sterile plastic scraper (VWR, 734-0385), collecting the cells in 10 mL warm complete microglial medium and centrifuging (300g x 5 min) to pellet cells. The resulting pellet was then resuspended in 1 mL complete microglial medium, and 0.5-1 mL of cell suspension (depending on the size of the cell pellet) added to 25 mL warm complete microglial medium in a 75 cm³ cell culture flask and transferred to the incubator.

In order to ensure N9 cells retained a microglial phenotype following defrosting, cells were fixed with paraformaldehyde and immunolabelled with an antibody targeting the macrophage specific marker CD11b (see sections 2.1.20-22) (**fig. 2.1**). CD11b is a subunit of the membrane-bound complement receptor macrophage-1(Mac-1) antigen specifically expressed in macrophage/microglia cells (*e.g.* Ford *et al.*, 1995). Mac-1 functions to aid in cell adhesion as well as aiding uptake of complement-coated particles, thus forming a key component of the innate immune system.

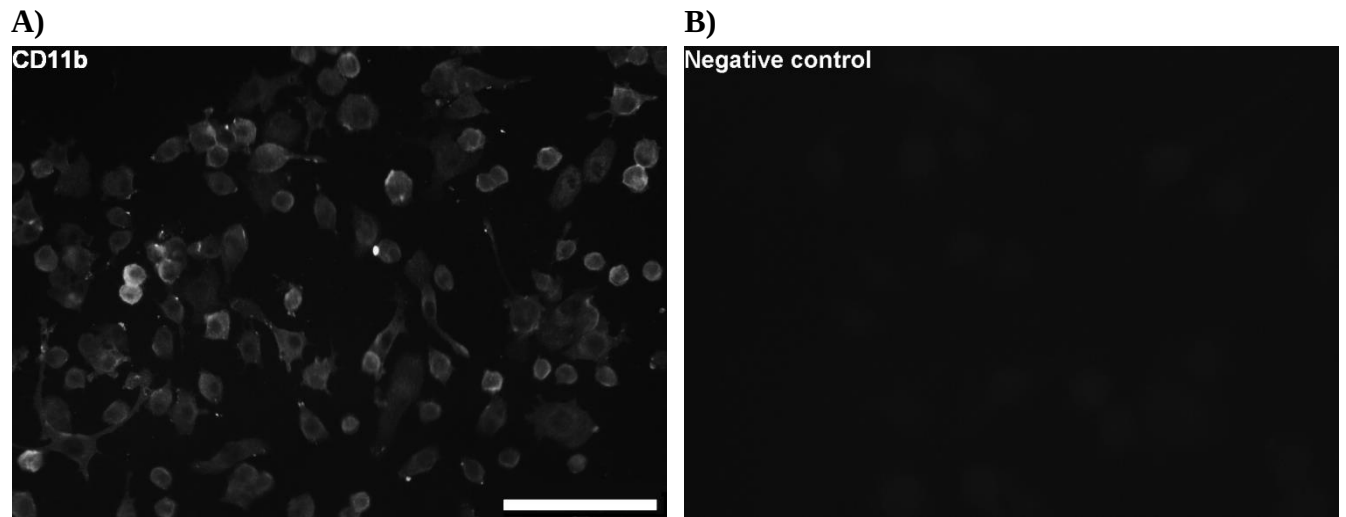


Figure 2.1 Retention of a microglial phenotype by defrosted N9 cells. The microglial phenotype of defrosted N9 cells was examined through immunolabelling fixed N9 microglia with an antibody targeting the monocyte/macrophage-specific cell membrane protein CD11b and tagged with a Cy3 red-conjugated secondary antibody (A). Negative control staining was obtained through omission of the primary antibody to rule out non-specific labelling (B). Scale bar = 250 μm .

2.1.3 Plating protocol for N9 microglia

In order to carry out experiments, N9 microglia were seeded onto sterile poly-L-lysine coated 6, 24 or 96 well plates. If plating on 24 well plates, a 13 mm-diameter autoclaved glass coverslip was placed inside the well onto which the cells were seeded. This was done to allow fluorescence microscopy analysis of paraformaldehyde-fixed immunolabelled cells.

Following removal of spent (old) medium and washing with fresh medium to remove dead cells, N9 microglia were detached from the 75 cm³ cell culture flask by scraping cells with a sterile plastic scraper and collected with warm complete microglial medium. Cells were pelleted by centrifugation (300g x 5 min) and resuspended in warm complete microglial medium. Cell number in the suspension was determined by mixing 10 µL of cell suspension with 10 µL of Trypan blue stain and placing 10 µL of the suspension/Trypan blue solution under a haemocytometer to manually count cells within a 0.1 µL volume. Only non-stained (*i.e.* viable) cells were counted (see section 2.1.12). Following cell counting, appropriate dilutions were made with warm complete microglial medium in order to plate out the necessary number of cells per well (c/w). N9 microglia were plated in 6, 24 or 96 well plates at a density of 600, 20 or 10 x 10³ c/w. Cells had to be plated at a high enough density to be able to obtain suitable concentrations of activation markers (*e.g.* cytokines). However, as cell density increases, microglia begin to retract their ramifications and adopt a non-resting morphology. Therefore, the chosen cell density was a compromise between a high cell density which would allow for detectable levels of markers within the cell culture medium and a cell density which would allow the majority of cells to retain a ramified morphology (increasing the concentration of markers in the culture medium by incubating microglia in a low volume of culture medium was not attempted to ensure culture medium remained fresh enough (*i.e.* low acidity and sufficient nutrients) through the experimental period to not affect the microglia). Microglia not used for plating were used to populate new 75 cm³ flasks at an appropriate density.

The seeded plates were transferred into the incubator for 30 minutes to allow cell adherence. Following this, the wells were washed with warm medium to remove any unadhered or dead cells and warm complete microglial medium added (2 mL, 500 μ L, or 100 μ L for 6, 24 or 96 well plates, respectively). Plates were incubated for 24 hrs before experimentation began to allow microglia to recover from the trauma of plating and readopt a resting morphology.

2.1.4 Primary microglial isolation by non-enzymatic method

Primary microglia were isolated from Wistar rat pups at post-natal day 7 (p7), purchased from Harlan laboratories (U.K.). Pups (6 per preparation) were sacrificed through decapitation with sterile scissors and brains immediately removed into ice cold 1x PBS (8 g/mL NaCl, 400 mg/mL KCl, 800 mg/mL NaH_2PO_4 , 3.56 g Na_2HPO_4 , 2 g/mL glucose and 0.2% bovine serum albumin, pH = 7.4). Following rinsing with 1x PBS to eliminate contaminating blood and removal of visible blood vessels and meninges, the cerebellum was removed and the brains were homogenized with a hand-held glass serrated pestle tissue grinder (Thomas Scientific, New Jersey, USA) in 1x PBS. The tissue homogenate (10 mL) was transferred to a sterile 50 mL Falcon tube, diluted in 1x PBS up to a volume of 50 mL and centrifuged at 450g at 20°C for 10 minutes. The resulting pellet was resuspended in 12 mL 66% Percoll (Sigma, P1644) (diluted in 1x PBS) and overlaid with 12 mL 34% Percoll and then 12 mL 1x PBS. Care was taken to overlay as slow as possible (~1 mL/minute) to ensure density layers formed as efficiently as possible with minimal mixing at the interface. To separate out the cells, the gradient was centrifuged at 1,800g at 4°C for 45 min (with centrifuge breaks off to avoid gradient disruption). The resulting myelin layer (**figure 2.2**) was discarded and the microglial layer in the 34/66% boundary collected with a Gilson P1000 pipette and transferred to a clean 50 mL Falcon tube containing 35 mL 1x PBS. The microglia suspension was centrifuged at 800g at room temperature (RT) for 10 minutes and the resulting pellet resuspended in 1 mL warm complete microglial medium and cell count measured as with N9 microglia (see section 2.1.2). On

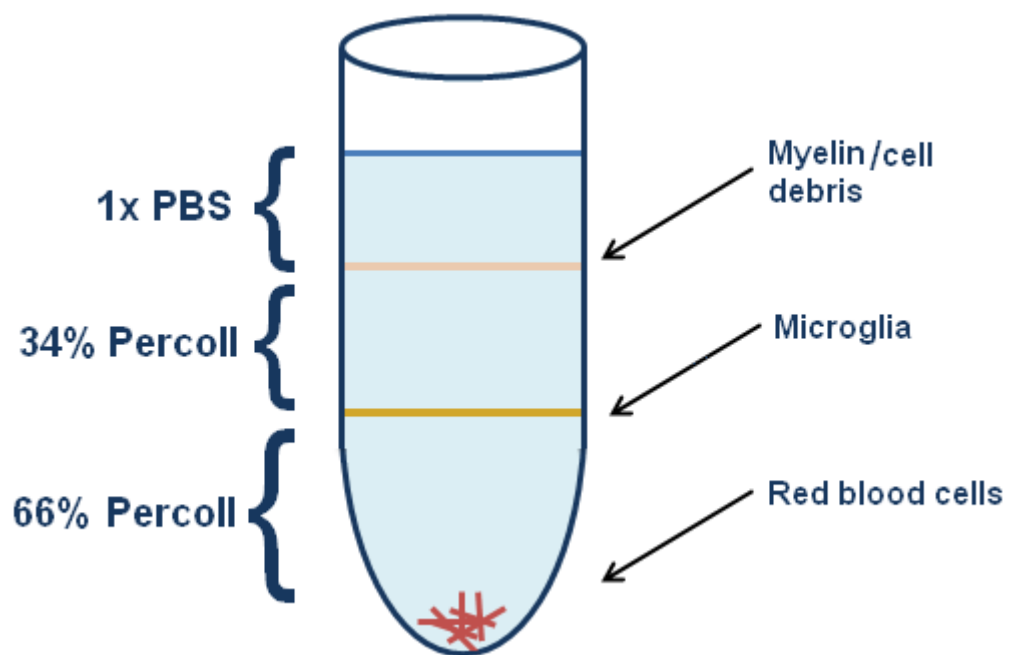


Figure 2.2 Isolation of rat primary microglia. Following homogenization of rat pup brains, the homogenates are subjected to a Percoll gradient centrifugation. Red blood cells sediment at the bottom of the falcon tube, while myelin debris and other cells separate into the PBS/35% boundary layer and microglial cells separate into the 35/70% boundary layer.

average, each rat brain yielded 500,000 cells. The separated microglia were seeded in 24 or 96 well plates at 90,000 or 10,000 c/w, respectively, and placed in the incubator (37°C, 5% CO₂) for 45 minutes to allow cell adherence (as with N9 cells, a sterile 13 mm diameter glass cover slip was placed inside the wells of 24 well plates and primary microglia plated onto it). The cells were finally washed with warm DMEM and placed in the incubator with fresh warm complete microglial medium (with 10% FCS instead of 5%). This time point was designated as *day in vitro 0* (DIV 0). The cells were allowed to recover for 24 hours before experimentation began. To confirm microglial phenotype, a sample of isolated cells were fixed with paraformaldehyde and immunolabelled with an antibody targeting the macrophage/microglia specific marker CD11b (see section 2.1.21-22). Isolated cultures were > 99% CD11b⁺, demonstrating the high microglial purity of isolated cells (**fig. 2.3**).

2.1.5 Neuronal cell lines

The neuronal cell lines SH-SY5Y and N27 were used to investigate neuroprotective effects of test compounds.

The SH-SY5Y cell line is an immortalized undifferentiated human neuroblastoma cell line capable of differentiating into a fully neuronal phenotype upon treatment with retinoic acid and BDNF (Encinas *et al.*, 2000). Differentiation induces expression of dopaminergic markers such as tyrosine hydroxylase (TH, the rate limiting enzyme in the synthesis of dopamine) and renders the cells more susceptible to 6-OHDA-induced death (Lopes *et al.*, 2010). The SH-SY5Y cell line was obtained from the Health Protection Agency Cell Culture Collections (cat # 94030304). The N27 cell line is a viral vector immortalized dopaminergic cell line derived from fetal rat mesencephalic cells. Unlike SH-SY5Y, N27 cells constitutively express dopaminergic markers, such as TH and dopamine transporter (DAT) without the need for further differentiation (Prasad *et al.*, 1998). The N27 cell line was a kind gift from Dr. Nabil Hajji, Imperial College London.

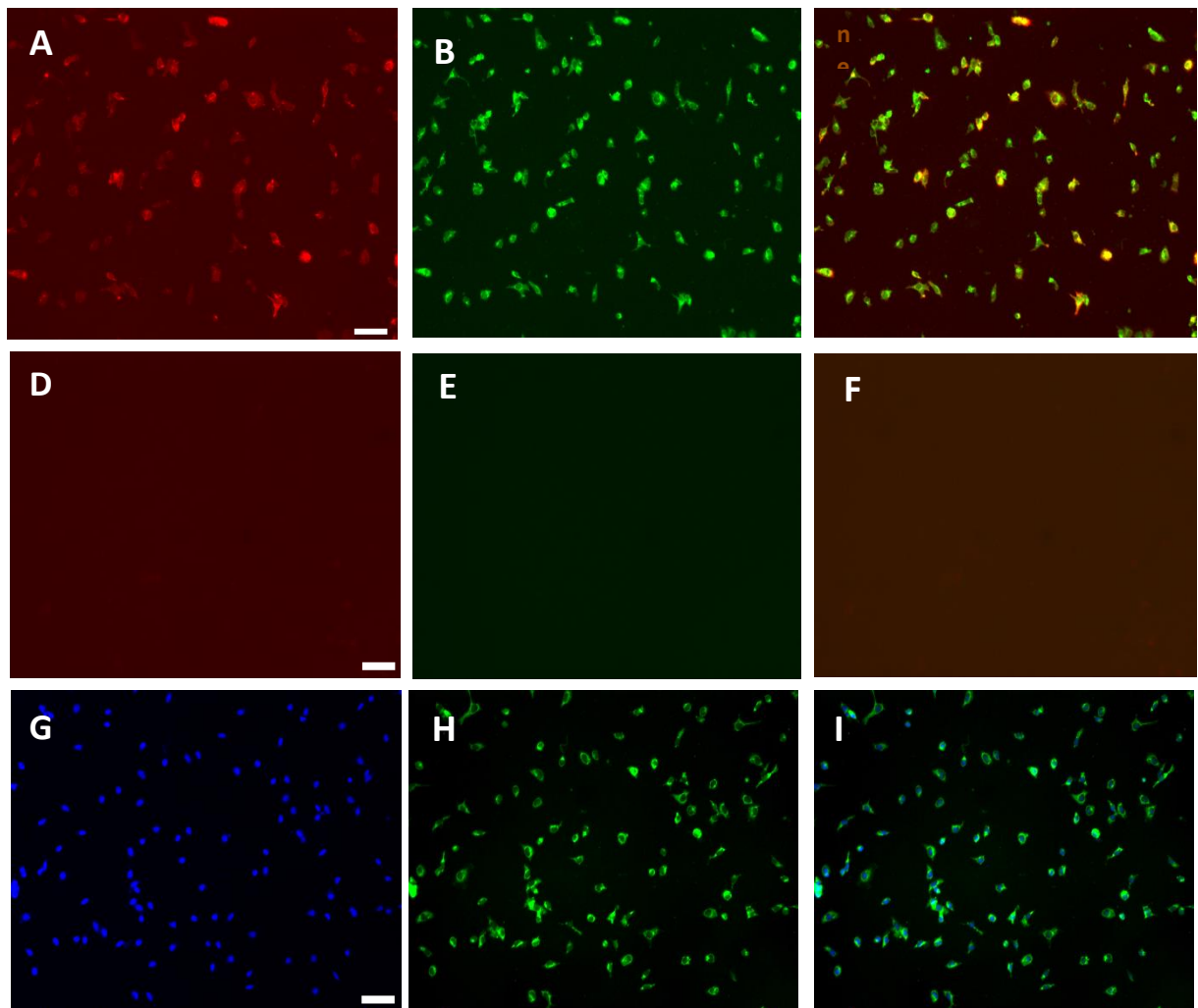


Figure 2.3 Primary microglia culture purity. Primary microglia extracted from neo-natal rat pups were plated onto glass coverslips and immunohistochemically labelled with the macrophage-specific antibody Ox-42. Primary microglia culture had virtually no contamination by non-microglia cells, as shown by colocalization of Ox-42 immunoreactivity (A) with mGlu3 immunoreactivity (B) (C, merged images). The specificity of the staining was confirmed by lack of cell staining upon omission of the primary antibody (negative control) (D-F). The ability of mGlu3 to label all present cells is demonstrated by the colocalization of mGlu3 immunoreactivity (H) with the nuclear stain DAPI (G) for all cells (I, merged images). Scale bar = 20 μ m.

SH-SY5Y cells were grown in a DMEM/Ham's F12 mixture (1:1 ratio) (Sigma, D5796 and N4888, respectively) supplemented with 1% non-essential amino acids (0.89 g/L L-alanine, 1.5 g/L L-asparagine, 1.33 g/L L-aspartic acid, 1.47 g/L L-glutamic acid, 0.75 g/L glycine, 1.15 g/L L-proline, 1.05 g/L L-serine) (Sigma, M7145), 10% heat-inactivated filter-sterilized FCS, 2 mM L-glutamine, 50 U/mL Penicillin and 50 µg/mL streptomycin (Sigma, P4333), henceforth termed complete SH-SY5Y medium. N27 neurons were grown in RPMI 1640 medium (Lonza, BE12-167F) supplemented with 10% heat-inactivated filter-sterilized FCS, 2 mM L-glutamine, 50 U/mL Penicillin and 50 µg/mL streptomycin (Sigma, P4333), henceforth termed complete N27 medium. Both neuronal cell lines were kept in 75 cm³ flasks incubated at 37°C with 5% CO₂ ventilation. Neuronal cells were passaged once confluency was reached (*i.e.* every 3-4 days). Unlike N9 cells, neuronal cells were passaged by trypsinization (see section 2.1.6).

In order to confirm that in our hands the N27 neurons adopted a dopaminergic phenotype without further differentiation, Western blots from N27 cell lysates were immunolabelled with an anti-TH antibody (see section 2.1.20). Cell lysates from N9 microglia were employed as negative controls. Immunolabelling revealed a prominent band at the predicted molecular weight for TH (~58 kDa) in all N27 lysates which was absent from all N9 lysates (**fig. 2.4**), confirming the dopaminergic phenotype of N27 neurons. To ensure equal protein loading, Western blots were double immunolabelled against β-actin.

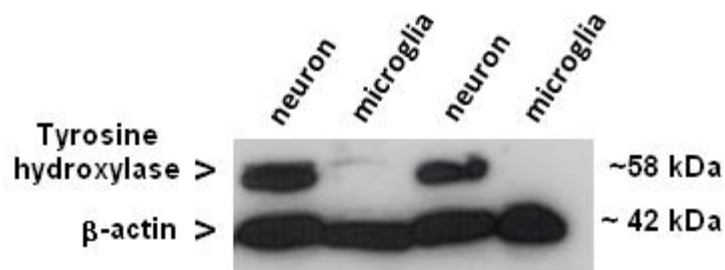


Figure 2.4 Tyrosine hydroxylase expression by the N27 neuronal cell line. Tyrosine hydroxylase (TH) expression was examined to determine dopaminergic phenotype of N27 neurons. TH expression was established by immunolabelling Western blots of N27 lysates with an anti-TH antibody. As negative control, lysates from N9 microglia were run in parallel. Immunolabelling revealed immunoreactivity at ~58 kDa, corresponding to TH (predicted size = 60 kDa), specifically on neuronal lysates, without any reactivity seen for microglial lysates. To ensure equal protein loading, Western blots were double immunolabelled against β-actin. Each lane represents an independent lysate.

2.1.6 Plating protocol for neurons

Unlike microglia, neurons had to be detached from the culture flask by trypsinization instead of scraping since the latter caused cell rupturing and lowered the number of viable cells. Therefore in order to plate neurons, old medium was removed from the flasks and the cells washed with sterile PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4) at room temperature in order to remove any trypsin inhibitors found in the FCS. Cells were detached from the flask by addition of 5 mL trypsin (Sigma, 59427C) diluted in sterile PBS. The neurons were incubated in trypsin for 2-3 minutes, after which time point the cell culture flask was tapped strongly twice to detach adhered neurons. The trypsin solution was collected and centrifuged at 200g x 5 minutes. The resulting pellet was resuspended in the appropriate warm neuronal complete medium and the number of viable cells counted as with N9 cells (see section 1.2). Appropriate dilutions were made with warm complete medium to obtain the desired cell density per well (*i.e.* 500,000 c/w for 6 well plates and 10,000 c/w for 96 well plates) and the cells incubated for 1 hour to allow cell adherence. After this, the cells were washed with warm medium, and the wells filled with the appropriate warm complete medium (2 mL and 100 µL for 6 and 96 well plates, respectively). Cells were allowed to recover for 24 hours before experimentation began.

2.1.7 Cell freezing and thawing protocol

In order to create a stock of cells of low passage number, both the N9 microglia and neuronal cell lines were aliquoted and frozen. The neuronal cells were allowed to reach confluency in the culture flask before freezing, while microglia were only allowed to reach 70% confluency. After appropriately detaching the cells from the culture flask and pelleting by centrifugation, the cells were resuspended in appropriate complete medium (5 mL) with 10% FCS in the absence of antibiotics. Sterile DMSO (Sigma, D2650) was then added drop-wise (10% v/v) with constant stirring of the cell solution to avoid osmotic shock. The cell suspension was aliquoted (500 µL) into freezing vials and

placed at -20°C for 1 hour before transfer into -80°C overnight. The vials were then transferred to a liquid nitrogen store for long-term storage.

Frozen cells were thawed by adding 200 µL of warm appropriate complete medium into the freezing vial and placing in a 37°C water bath. Once cells had thawed, the vial was vortexed and the cell suspension transferred to a 50 mL Falcon tube containing 10 mL warm appropriate complete medium and pelleted by centrifugation to remove DMSO. The cell pellet was resuspended in 2 mL appropriate warm complete medium and plated on a single well of a 6 well plate. Once cells had recuperated from the thawing procedure and had proliferated to appropriate confluency (70% and 100 % for microglial and neuronal cells, respectively), cells were appropriately detached from culture plate and transferred to a 75 cm³ flask with 25 mL warm appropriate complete medium.

2.1.8 *Microglia-activating compounds and test compounds*

2.1.8.1 *Microglia-activating compounds*

Activation of microglia in this thesis was achieved primarily through administration of the bacterial cell wall component LPS. LPS was employed due to its high potency to activate microglia. Furthermore, signalling pathways engaged by LPS, namely TLR-4 receptor ligation, NFκB activation and pro-inflammatory protein expression, are known to also be engaged by compounds relevant in neurodegenerative diseases such as amyloid-β (Jin *et al.*, 2008b; Walter *et al.*, 2007) and α-synuclein (Alvarez-Erviti *et al.*, 2011; Zhang *et al.*, 2005) or activated by components released from damaged neurons (Pais *et al.*, 2008; Chen and Nunez, 2010). Therefore, employing LPS would allow us to elicit a robust microglial activation through mechanisms relevant to neurodegenerative diseases.

The LPS compound employed in this thesis, rough (R)-form LPS derived from *Salmonella Minnesota* (Enzo Life Sciences, ALX-581-008-L002) activated TLR-4 receptor exclusively, as shown by lack of activity in TLR-4-deficient macrophages (**figure 2.5**). The stock LPS (1 mg/mL in

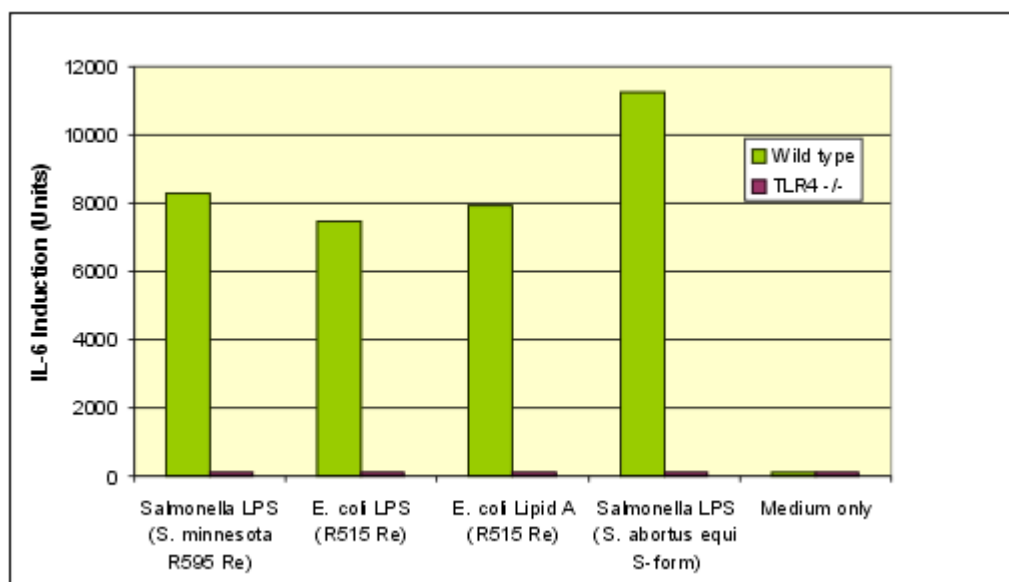


Figure 2.5 Specific activation of TLR-4 receptor by R-form LPS derived from Salmonella Minnesota. LPS employed in this thesis (R-form, derived from *Salmonella Minnesota*) induced a robust expression of IL-6 in macrophages derived from wild type mice. However, induction of IL-6 expression by the same form of LPS is absent in macrophages derived from TLR-4 deficient mice, demonstrating that R-form LPS acts specifically through TLR-4 receptors. (Graph adapted from Enzo Lifesciences' product information sheet).

sterile water) was kept in the dark at 4°C. Dilutions were made with de-ionized autoclaved water to achieve the desired concentrations in a constant administration volume.

In addition to LPS, microglial activation was attempted through administration of 6-OHDA, a dopamine analogue widely employed to model dopaminergic neuronal degeneration in animals.

6-OHDA is liable to autoxidation (Soto-Otero *et al.*, 2000), therefore 6-OHDA solutions were prepared under anti-oxidizing conditions with minimal lighting. Powder 6-OHDA (Sigma, 162957) was dissolved in ascorbic acid (Sigma, A5960) to give a 10 mM solution and 1 mL aliquots created. The aliquots were wrapped in aluminium foil, placed inside an air-lock plastic bag and kept at -80°C until used. The initial concentration of ascorbic acid used was 0.2% (dissolved in 0.9% saline). However, live/dead assays (see section 2.1.10) demonstrated this solution to be toxic to the cells, therefore the ascorbic acid concentration was decreased to 0.05% (**fig. 2.6**).

2.1.8.2 Test compounds

2.1.8.2.1 mGluR agonists

The group III-wide orthosteric agonist L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4) was purchased from Tocris Scientific (cat# 0103) and dissolved in autoclaved deionized water to 5 mM. The mGlu 7-specific allosteric agonist N,N'-Bis-(diphenylmethyl)-1,2-ethanediamine dihydrochloride (AMN082) was purchased from Ascent Scientific (cat# Asc-011) and dissolved in autoclaved deionized water to a concentration of 2.5 mM. The mGlu 4-specific positive allosteric modulator cis-2-[(3,5-Dichlorophenyl)amino] carbonyl]cyclohexanecarboxylic acid (VU0155041) was purchased from Ascent Scientific (cat# asc-248) and dissolved in autoclaved deionized water to 5 mM. The selective mGlu 8 orthosteric agonist (S)-3,4-dicarboxyphenylglycine (DCPG) was purchased from Tocris Scientific (cat# 1302) and dissolved in autoclaved deionized water to 50 mM. All mGluR ligands were aliquoted and stored at -20°C until use.

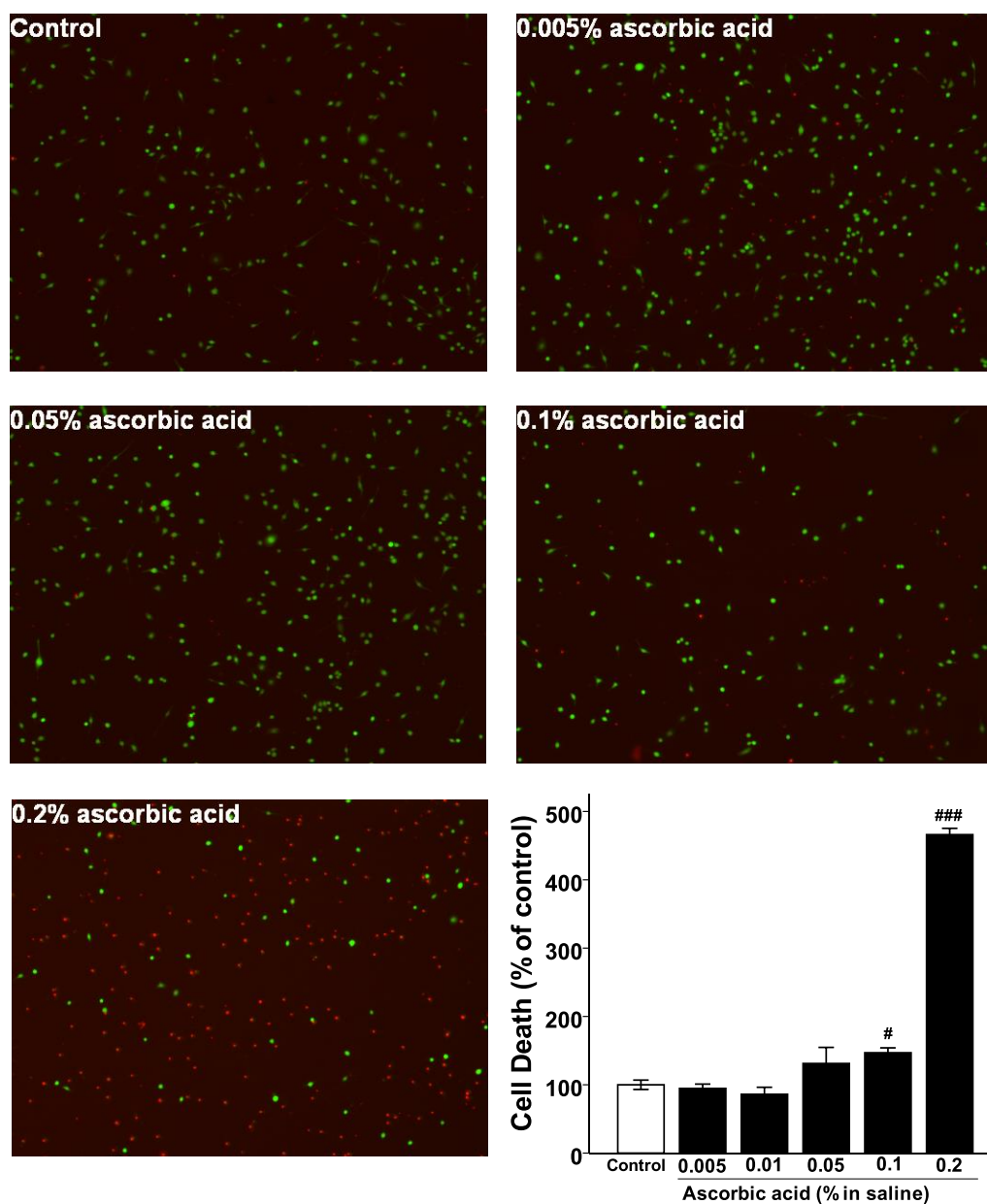


Figure 2.6 Toxicity of ascorbic acid solutions. Cell viability of microglia incubated in increasing concentrations of ascorbic acid (dissolved in saline) was examined through Live/Dead assays. Ascorbic acid concentrations higher than 0.05% significantly increased the percentage of cells incorporating ethidium bromide (red objects) compared to cells with active esterases (green objects), indicating increased cell death. Images are representative of three independent experiments. Results are displayed as mean $\pm$ SEM of three independent experiments. #, ### denote $p < 0.05$, 0.005 vs. control, respectively.

2.1.8.2.2 *mGluR antagonists*

The group III general orthosteric antagonist (RS)- α -Methylserine-O-phosphate (M-SOP) was purchased from Tocris Scientific (cat# 0803) and dissolved in autoclaved deionized water to 50 mM. The mGlu 7-specific allosteric antagonist 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)isoxazolo[4,5-c]pyridin-4(5H)-one (MMPiP) was purchased from Ascent Scientific (cat # asc-245) and dissolved in DMSO to 5 mM. All mGluR antagonists were aliquoted and stored at -20°C until use.

2.1.8.2.3 *NF κ B inhibitor*

The NF κ B inhibitor 1-pyrrolidine carbodithioic acid ammonium salt (APDC) was purchased from Sigma Aldrich (cat# P8765), dissolved in autoclaved deionized water to 1.25 mM and stored at 4°C until use.

2.1.9 *Microglia cell treatment*

2.1.9.1 *Microglial activation*

Microglial activation by LPS or 6-OHDA was carried out after a 24 hour resting period following plating to allow microglia to readopt a resting morphology (**fig 2.7**). When 6-OHDA was employed, work was carried out with lights off and plates were covered in aluminium to prevent 6-OHDA autoxidation. All LPS/6-OHDA concentrations were administered in a constant volume throughout the thesis. In order to maintain a constant volume within the culture well, the volume of LPS/6-OHDA to be administered was first removed from the culture medium. The experimental end-point was designated at 18 hours post-LPS/6-OHDA treatment, at which point microglial activation was assessed qualitatively through assessment of microglial morphology and quantitatively through measurement of pro-inflammatory cytokine release (IL-1 β , IL-6 and TNF α), pro-inflammatory enzyme expression (iNOS) and production of reactive molecular species (nitric

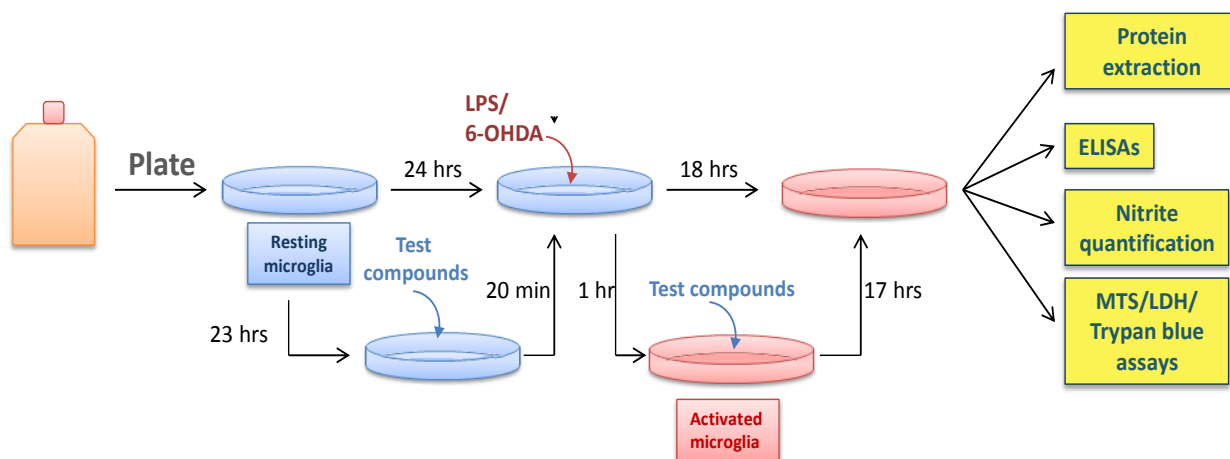


Figure 2.7 Schematic representation of protocols employed to assess microglial activation after treatment with LPS/6-OHDA with or without test compounds. After an initial 24 hours resting period following plating, the cells (primary cells or N9 cells) were treated with LPS or 6-OHDA. After a further 18 hours, activation was measured qualitatively through morphological shift or quantitatively through measurement of pro-inflammatory protein expression (cytokines and enzymes) and nitric oxide synthesis. The effect of test compounds on microglial activation was assessed by administering test compounds 20 minutes prior or one hour following LPS treatment and assessing microglial activation 18hrs following LPS treatment. In order to assess the role of glutamate on the effects of mGluR ligands, glutamate was removed from the culture medium by incubating the microglia with glutamine-free complete microglial medium for one hour before toxin treatment.

oxide). This time-point was chosen as a compromise between peak expression time-points of iNOS/NO, IL-6, IL-1 β and TNF α (Lin *et al.*, 2011; Byrnes *et al.*, 2009; Pinteaux-Jones *et al.*, 2008; Bryn *et al.*, 2006; Shigemoto-Mogami *et al.*, 2001; Zujovic *et al.*, 2000).

2.1.9.2 *Test compound treatment*

Following treatment with LPS, test compounds (mGluR ligands/APDC) were administered to microglia to investigate their potential to modulate microglial activation (**fig. 2.7**). mGluR agonists/PAMS and APDC were added directly to microglia culture wells either one hour after (post-treatment) or 20 minutes before (pre-treatment) LPS treatment. In antagonistic studies, antagonists were added 25 minutes before addition of agonists to ensure receptor occupancy. In order to keep a constant volume within the culture wells, any volume administered was first removed from the culture medium. The wells were gently stirred after toxin and test compound treatment to ensure an even distribution. The experimental end-point was designated at 18 hours post-toxin treatment, at which point microglial activation was quantified as above (see section 2.1.9.1).

In the case of test compounds diluted in DMSO, the above method of administration resulted in high cell death due to aggregation of DMSO into shard-like structures. Therefore, the administration method was modified as follows: the desired amount of test compound was placed in a 2 mL Eppendorf tube. After this, 1 mL of medium from the target well was aspirated and transferred to the Eppendorf tube, immediately followed by rigorous vortexing to ensure even compound distribution without aggregation of DMSO. The contents of the Eppendorf were then gently transferred to the target well, followed by incubation as above.

2.1.9.3 *Assessment of microglial neurotoxicity*

In order to assess whether activated N9 microglia were neurotoxic, and whether this phenotype could be modulated by mGluR ligand treatment, neurons were incubated with medium derived from microglia (*i.e.* microglial conditioned medium, MCM) under resting conditions or following LPS activation with or without mGluR ligand treatment (**fig. 2.8**). After an initial 24 hour resting period following neuronal plating, the neuronal medium was aspirated and replaced with 1.5 mL (6 well plate) or 100 μ L (96 well plate) of MCM from control or LPS-activated (with or without mGluR ligand treatment) (MCM was obtained from the microglial cultures at the microglial experimental end-point, see section 2.1.9.1). Before transfer to neurons, floating microglia were removed from the MCM by centrifugation (600g x 5 minutes) in order to avoid transfer of microglial cells to neuronal cultures. Neuronal cell viability was examined following 48 hour incubation with MCM. In order to assess the direct neuronal effect of mGluR ligands and LPS carried over in the MCM, mGluR ligands and LPS were incubated in complete microglial medium in the absence of microglia for 18hrs before transfer to neuronal cultures, and neuronal viability assessed 48 hours later.

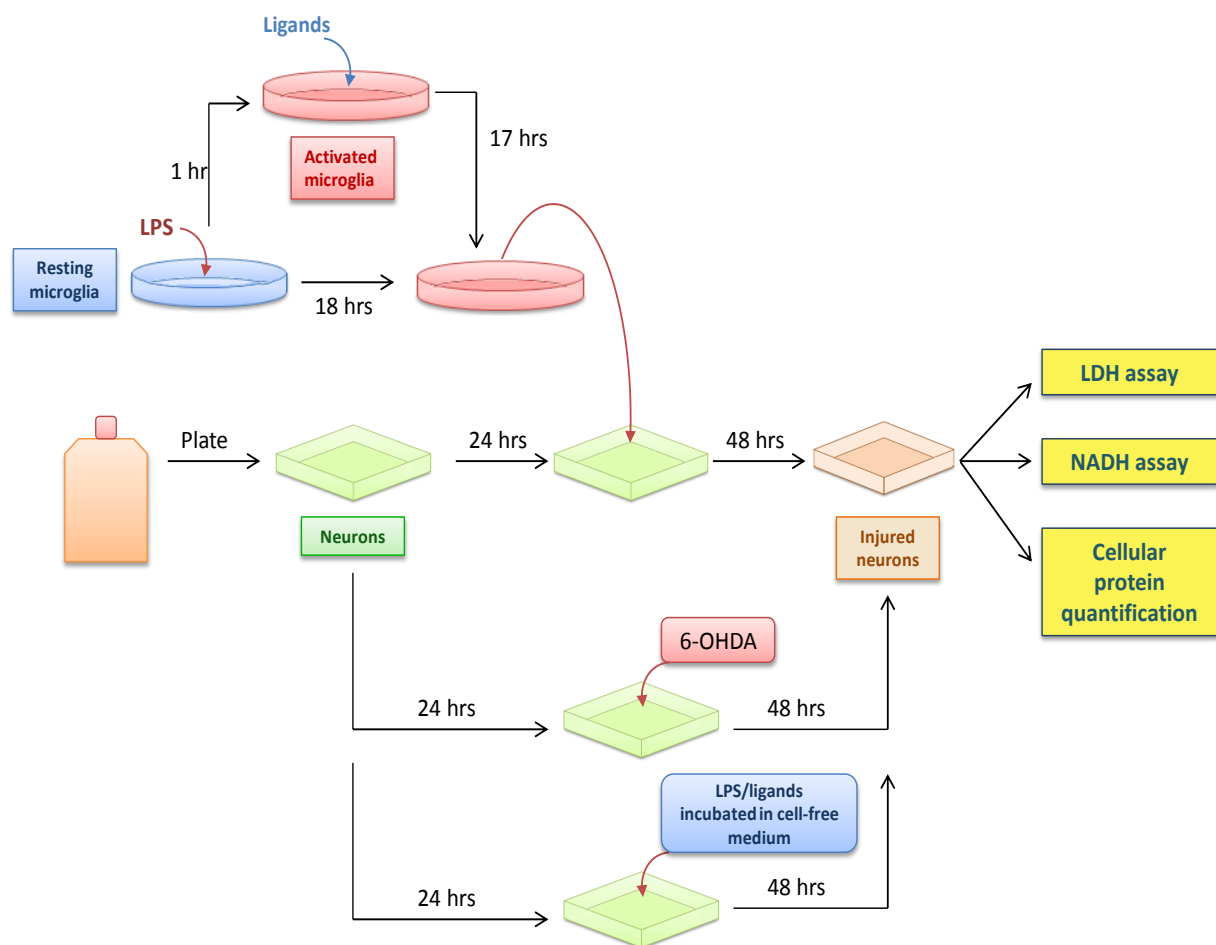


Figure 2.8 Schematic representation of protocols employed to assess neuronal viability following treatment with microglial conditioned medium or 6-OHDA. Following a 24 hour resting period, neurons were treated with microglial conditioned medium (MCM) derived from control or activated microglia with or without mGluR ligand treatment. To assess the neuronal effects of LPS/ligand carried over in the MCM, neurons were directly treated with LPS/ligands incubated in cell-free medium. Direct treatment of neurons with 6-OHDA served as positive control for neuronal cell death. Neuronal viability was assessed through NADH and protein quantification, as well as quantification of LDH release.

In order to obtain a positive control for neuronal cell death, neurons were treated with the toxin 6-OHDA (**fig. 2.8**). After the initial 24 hour resting period, 6-OHDA was directly added to neuronal cultures and the cells incubated for a further 48 hours before assessing cell viability. As with microglia, work with 6-OHDA was carried out with minimum lighting and treated wells wrapped in aluminium foil to avoid 6-OHDA autoxidation.

Neuronal viability was assessed through intracellular nicotinamide adenine dinucleotide (NADH) quantification by the MTS assay, cellular protein quantification by the Bradford assay, and assessment of cell membrane integrity by an LDH release assay (see sections 2.1.12-15).

2.1.10 Live/Dead assays

Live/Dead (L/D) assays were carried out in order to establish what concentrations of LPS/6-OHDA proved toxic to microglia. L/D assays use the fluorescent dye calcein and ethidium bromide to establish cell viability. Live cells incorporate the acetomethoxy derivative of calcein (calcein-AM) into their cytosol, where active esterases remove the acetomethoxy molecule, allowing calcein to chelate ions and fluoresce. As only live cells have active esterases, only live cells will fluoresce. Ethidium bromide, on the other hand diffuses through the damaged membrane of dead cells and binds to nucleic acids, where its red fluorescence is markedly increased due to proton transfer stabilization (Poole *et al.*, 1993, Olmstead *et al.*, 1977).

Cells were plated onto sterile glass coverslips (13mm diameter) (3 per condition) in 24 well plates at a density of 20,000 c/w. At the experimental end point, each well received 0.3 µl of 4 mM calcein-AM and 2 µl of 2 mM ethidium bromide and incubated for 15 minutes at 37°C. The plates were then placed on a fluorescent microscope, where images (3 per coverslip) were captured of live and dead cells and merged using OpenLab software. Each image captured an average of 200-400 cells. Image Pro-Plus software was then used to count the number of green and red objects within a

certain intensity range. The results were expressed as the percentage of dead cells within a population corrected for the percentage of dead cells within the control wells.

Although L/D assays are sensitive to cell death, the use of ethidium bromide and fluorescence microscopy proved too impractical for quick determination of cell viability due to ethidium bromide's carcinogenic properties and the time lag between photograph capture. Therefore trypan blue exclusion, MTS and LDH assays, and protein quantification were adopted as assays for cell viability (see following sections).

2.1.11 Trypan blue exclusion assay

Trypan Blue (TB) exclusion assays were carried out to determine toxicity of compounds. TB is a diazo dye which is excluded from viable cells with intact membranes, but is incorporated into dead or dying cells with compromised membranes, therefore it is a rapid method for identifying viable cells (see for example Vincent and Maiese, 2000).

Cells were plated at 600,000 c/w on 6 well plates. At the experimental end point, cells were scraped with a sterile plastic scraper and the medium containing both floating cells and scraped adherent cells collected into an Eppendorf tube. After vortexing, 10 μ L of medium were added to 10 μ L TB solution, incubated for 1 minute and a haemocytometer used to quantify the number of cells incorporating trypan blue within four 0.25 mm² segments equalling a volume of 0.1 μ L (average number of total counted cells per condition = 43.03). Viability was assessed as the number of dead cells as a percentage of total (live and dead) cells. The counting protocol was repeated twice per experiment for each condition.

2.1.12 MTS assay

Intracellular NADH levels are a reliable indicator of cell viability as the amount of NADH produced by cells is proportional to their metabolic activity (*e.g.* Wang *et al.*, 2010). Tetrazolium

salts have been widely used to quantify NADH, as they are readily converted to coloured formazan products by NADH derived from mitochondrial respiration (Bartrop *et al.*, 1991; Gieni *et al.*, 1995).

In order to quantify NADH, cells were seeded in 96 well plates at a density of 10,000 c/w (unless stated otherwise). At the experimental end point, the medium was replaced with fresh complete medium in order to avoid acidification of medium (which turned the medium yellow-orange due to presence of phenol red) from interfering with the assay and the plate incubated for 3 hours. Following this, 20 μ L of a tetrazolium (MTS salt) solution (Promega, Southampton) was added to the wells. The plate was finally incubated for a further hour and the optical density (OD) at 490 nm measured with a microplate reader. The OD of experimental conditions was expressed as a percentage of the OD of control conditions.

2.1.13 Lactic dehydrogenase assay

Cell viability was further assessed through quantification of release of the cytosolic enzyme lactic dehydrogenase (LDH) into the cell culture medium. A key determinant of cellular viability is cellular membrane integrity, which is maintained in an active energy-dependent manner. Hence, membrane integrity is lost in compromised or dead cells, resulting in an inability to maintain a functional barrier between their intracellular milieu and the extracellular environment and causing intracellular components (such as ions and proteins) to leak from the cell. Therefore, measurement of LDH levels released into the cell culture medium give an indirect, yet accurate, assessment of cellular membrane integrity and cell viability (*e.g.* Koh *et al.*, 1987; Mukhin *et al.*, 1997). Importantly, LDH levels can be used to determine both necrotic and apoptotic cell death (Lobner, 2000). In this thesis, LDH released from compromised cells into the cell culture medium was quantified with a non-radioactive cytotoxicity assay kit (Abcam, Cambridge). LDH catalyzes the conversion of lactate to pyruvate, with the concomitant production of NADH. Hence, monitoring the production of NADH during pyruvate synthesis allows for the enzymatic activity of LDH, which

is directly proportional to LDH concentration, to be quantified. NADH was quantified through reaction with a water soluble tetrazolium salt, giving rise to a yellow chromophore.

At the experimental end point, cells plated in 6 well plates were gently shaken to distribute released LDH evenly throughout the culture medium. Following this, medium was transferred to a 96 well plate (10 μ L in triplicate) and incubated with 100 μ L LDH reaction mixture (tetrazolium salt plus excess lactate) for 30 minutes. Care was taken to protect the reaction from light by wrapping the 96 well plate in aluminium foil. After 30 minutes incubation, the OD at 450nm was read with a microplate reader.

For microglial neurotoxicity assays, released neuronal LDH was calculated as the percentage of total LDH in the culture well (*i.e.* released plus intracellular LDH) in order to control for low LDH levels due to decreased cell number. This was achieved by adding 80 μ L of cell lysis buffer to the culture well and incubating for 5 minutes. Following this, medium was transferred to a 96 well plate (10 μ L in triplicate) and incubated with 100 μ L LDH reaction mixture for 5-10 minutes. The reaction time was adjusted in order to ensure colour development did not become saturated and be able to obtain an accurate reading of total LDH levels.

2.1.14 Protein quantification

Protein quantification through Bradford assays was carried out either to assess cell viability or to determine lysate volume required in SDS-PAGE experiments.

The Bradford assay relies on the dye coomassie brilliant blue interacting with and being stabilized by proteins in the sample, causing a shift in the dye colour from red/brown to blue which can be detected through optical density quantification at 595 nm wavelength. Therefore, the higher the concentration of protein in the sample, the greater the amount of stabilized dye will form, yielding a more intense blue colour.

After cell lysis (see section 2.1.18), lysate samples were placed in 96 well plates. Each condition was quantified in triplicate. Bradford reagent (Sigma, B6916) (250 μ L) was then added to each well and the plate allowed to stand for 5 minutes. Following careful shaking to ensure even protein/dye complex distribution, the OD was measured at 595 nm with a microplate reader. To translate OD values into protein concentration, a standard curve was prepared by placing 5 μ L of protein standards (0-1.4 mg/mL, bovine serum albumin dissolved in deionized water) in triplicate into the 96 well plate and adding 250 μ L Bradford reagent. Since the Bradford assay is only linear within a protein concentration range of 0.1-1.4 mg/mL (**fig. 2.9**), the lysate sample volume in the wells had to be adjusted to ensure protein concentration fell within the linear range. Appropriate corrections were then carried out to compensate for the dilution factor.

In order to employ protein quantification as a measure of cell viability, the assay's sensitivity to cell death had to be confirmed. To achieve this, dopaminergic neurons (N27 cells) were incubated with increasing concentrations of the known dopaminergic toxin 6-OHDA for 24 hours. As can be seen in **figure 2.10**, increasing 6-OHDA concentrations induced a steady decline in protein concentration, indicating the assay's suitability as a cell viability parameter.

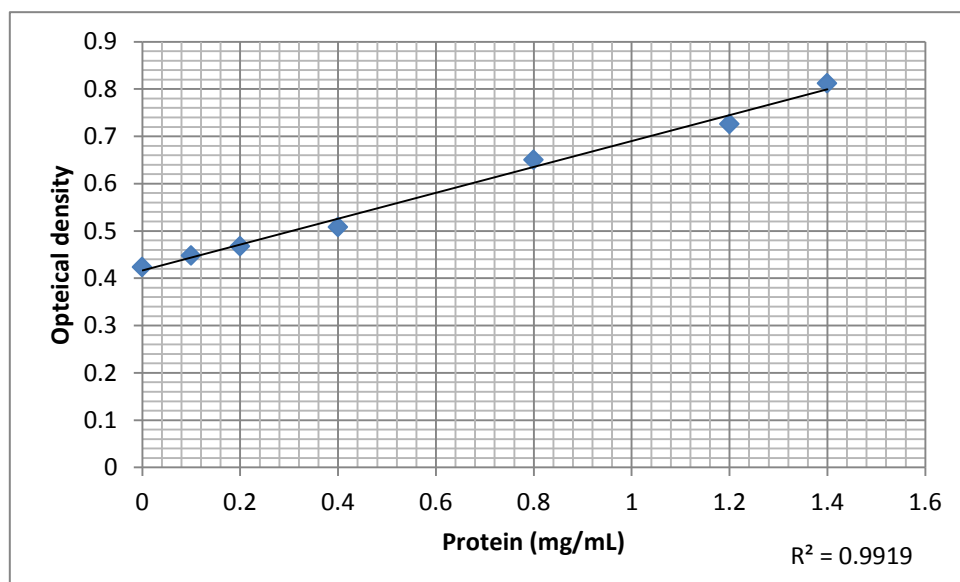


Figure 2.9 Protein standard curve employed during Bradford assay. A standard curve was constructed for each Bradford assay in order to translate optical density measurements into protein concentration. Protein standards (0-1.4 mg/mL) were prepared by serially diluting bovine serum albumin in deionized water. After addition of Bradford reagent, the optical density was measured at 595 nm. A linear regression was employed to set the line of best fit.

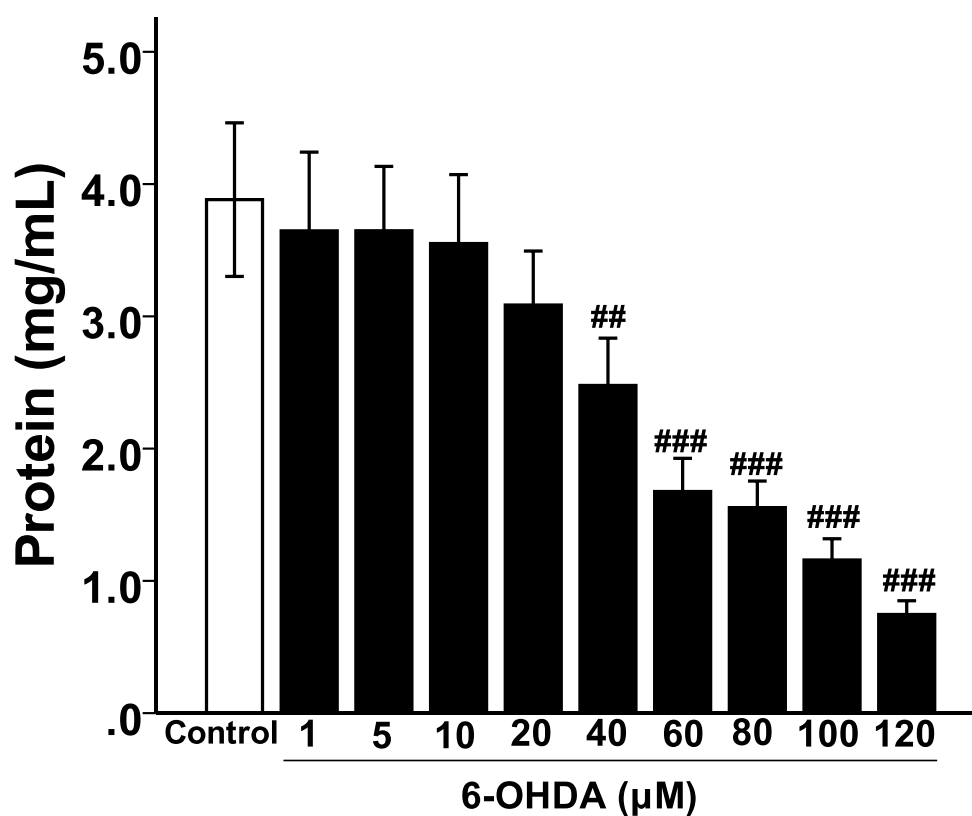
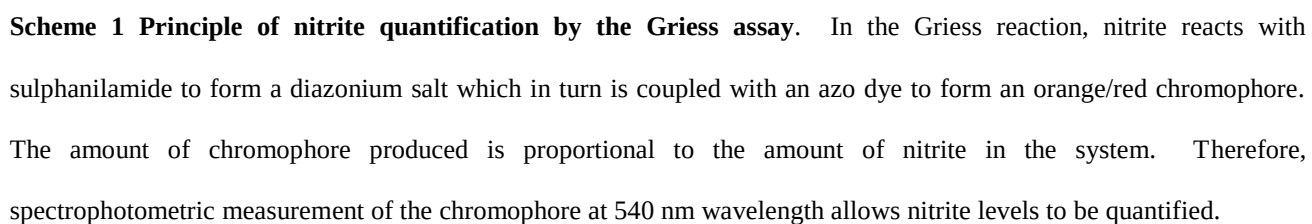


Figure 2.10 Reliable detection of cell death by protein quantification through the Bradford assay. In order to confirm the Bradford assay's sensitivity to cell death, dopaminergic N27 cells were treated with increasing concentrations of the known dopaminergic neuron toxin 6-OHDA. A steady decline in protein concentration was seen with increasing 6-OHDA concentrations, confirming protein quantification as a valid parameter of cellular viability. Bars represent mean $\pm$ SEM of 4 independent experiments measured in duplicate. ##, ### denote $p < 0.01$, 0.005 vs. control, respectively. $F = 26.53$.

2.1.15 Nitric Oxide quantification

Nitric oxide (NO) is a simple, yet versatile gas with a wide range of biological functions, from vasodilation to pathogen defence (reviewed by Bredt, 1999). Levels of NO can be measured through various methods, including mass spectrometry and liquid chromatography (Feelisch and Stamler, 1996). However, due to the short half-life of NO and relatively low levels in biological systems, a more practical method is to measure NO concentrations indirectly by quantification of its stable metabolites, nitrate (NO_3^-) and nitrite (NO_2^-). In this thesis, levels of NO were measured indirectly using the principle first described by the German chemist J.P. Griess, namely the Griess reaction. In the Griess reaction, diazotization of sulfonilamide by nitrite, followed by coupling with the azo dye 1-(naphthyl)ethylenediamine (**scheme 1**), results in the production of an orange/red diazonium product (chromophore) in proportion to the amount of nitrite in the system (Miranda *et al.*, 2001). Hence, measuring the production of the chromophore through spectrophotometry allows the nitrite levels to be quantified. However, a limitation of this method is its lack of reactivity with nitrate. Therefore, in order to accurately measure total NO levels, nitrate must first be reduced to nitrite using nitrate reductase and NADPH. However, because NADPH interferes with the reaction between sulfonilamide and nitrite, any surplus NADPH left from the nitrate reduction reaction must first be neutralized. This is achieved by the addition of lactate dehydrogenase and sodium pyruvate in order to generate lactate with the conversion of NADPH to NADP^+ .

In order to measure nitrite, a protocol was first devised where cells were seeded directly onto a 96 well plate (at 20,000 c/w) in order to carry out the Griess reaction directly on the wells containing cells. However, it was found that a slight evaporation of medium in the plate's outer wells during incubation at 37°C significantly affected the apparent nitrite concentration. Therefore the protocol had to be modified as described below.



In order to accurately quantify nitrite levels, cells were plated in 6 well plates at 600,000 c/w. At the experimental end point, 75 μ L of medium were transferred to 96 well plates (in triplicate for each condition) and incubated with 40 mU/mL nitrate reductase (Sigma, N7265) and 80 μ M NADPH (Sigma N1630) for 45 minutes at 37°C protected from light. The plate was then incubated with 88.9 U/mL lactate dehydrogenase (Sigma 59747) and 8.8 mg/mL sodium pyruvate (Sigma,P8574) for 20 minutes at 37°C protected from light. Finally, 75 μ L of Griess reagent (sulfonilamide plus 1-(naphthyl)ethylenediamine) (Sigma 03553) were added to each well and incubated for 20 minutes at room temperature in the dark. Levels of nitrite were determined by measuring the optical density at 540 nm. Optical density was converted to nitrite concentration with the use of a nitrite standard curve (0-60 μ M) (**fig. 2.11**) generated by incubating sodium nitrite standards (diluted in D-MEM) with Griess reagent. A separate standard curve was prepared for each plate to control for any variance in the spectrophotometer's sensitivity. Diluting the sodium nitrite standards with D-MEM allowed us to control for any background OD due to the medium's composition or colour. One possible aspect which might preclude the use of D-MEM in the Griess assay is the fact that the medium contains phenol red. Phenol red is a pH indicator, therefore its colouring varies depending on the pH of the medium, ranging from pink to yellow. Therefore, changes in pH resulting from different treatment protocols would potentially lead to inaccuracies in the Griess assay. However, additon of the Griess reagent, which is highly acidic, masked any change in medium pH, allowing for a precise reading of nitrite content. To demonstrate this, a 35 μ M sodium nitrite standard was constructed with D-MEM of varying acidity (acidified with HCl) and Griess reagent added to quantify nitrite. As can be seen from **figure 2.12**, increasing the medium's acidity did not affect the Griess assay's precision, thereby indicating culturing cells in D-MEM containing phenol-red is suitable for nitrite quantification by the Griess assay.

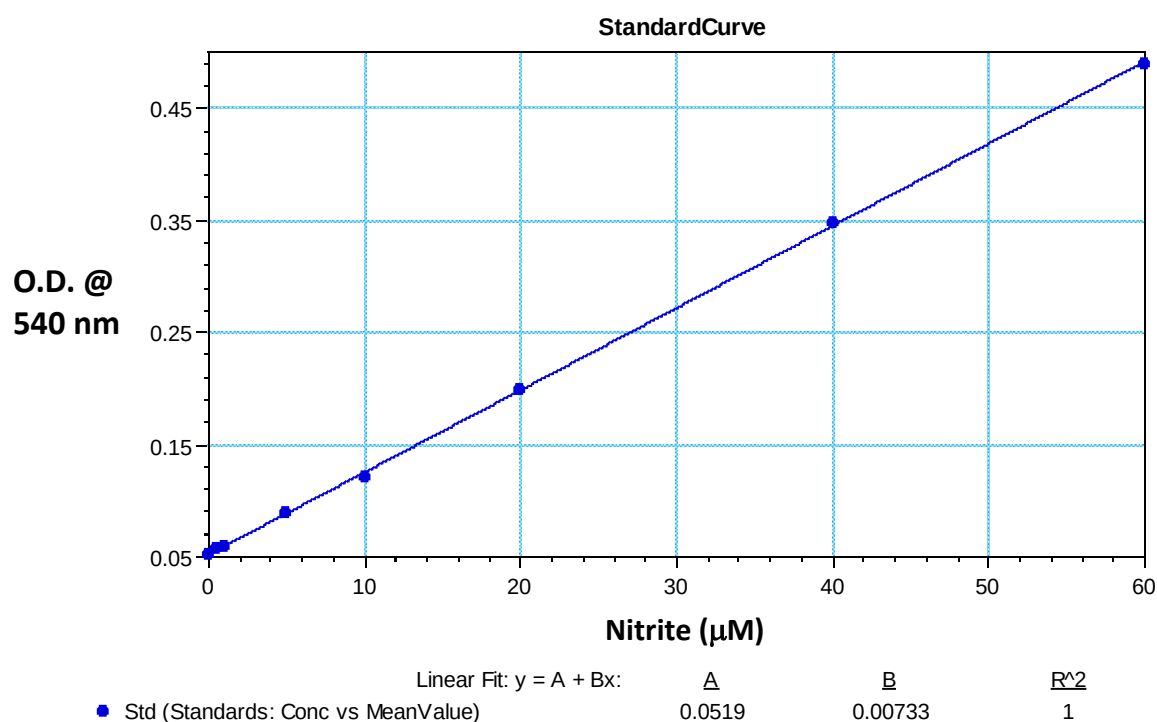


Figure 2.11 Nitrite standard curve. A nitrite standard curve was employed to translate optical density values to nitrite concentration during Griess assays. The standard curve was constructed by the reaction of sodium nitrite standards (0, 0.5, 1, 5, 10, 20, 40, 60 μM) (diluted in complete microglial medium) with Griess reagent and measurement of optical density at 540 nm. A new standard curve was constructed for each nitrite quantification in order to control for variance in the spectrophotometer's sensitivity. A linear regression was employed to establish line of best fit.

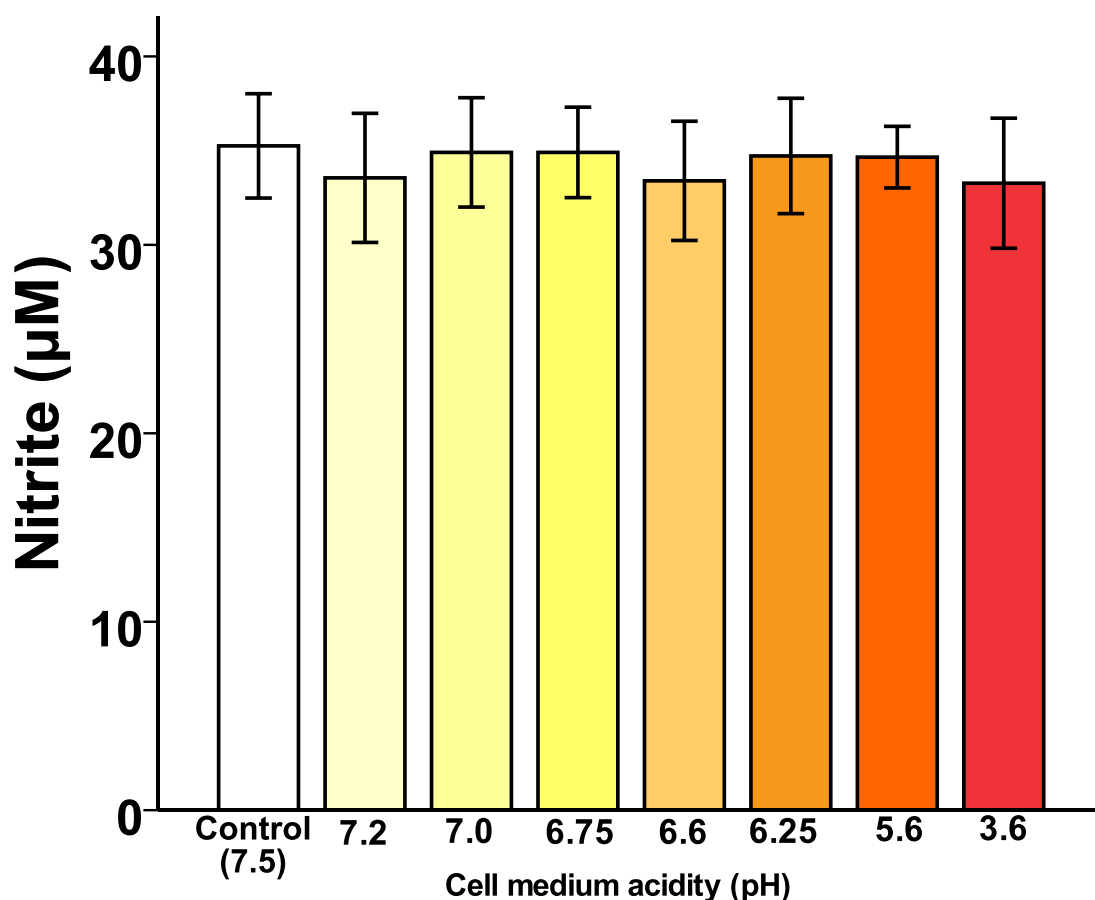


Figure 2.12 Effect of medium acidity on nitrite quantification by the Griess assay. The presence of phenol red caused the cell culture medium to change colour depending on the pH of the medium, leading to possible interference with the Griess reagent's red/orange colour development in the presence of nitrite. However, addition of the Griess reagent, which is highly acidic, masked any medium acidification due to cellular metabolism, allowing for a precise reading of nitrite content. This was demonstrated by constructing a 35 µM sodium nitrite standard in cell culture medium of varying pH (acidified with concentrated HCl). The Griess assay consistently and correctly measured 35 µM regardless of medium acidity, indicating the presence of phenol red did not preclude the use of cell culture medium for Griess assays. Results are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate. Statistical significance was assigned as $p < 0.05$, as determined by a one-way ANOVA with Tukey's post-hoc test. $F = 0.74$.

2.1.16 *TNF α* ELISA

The effect of different treatment conditions on the production of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF α) by microglia was assessed through an enzyme linked immunosorbent assay (ELISA). ELISAs exploit the specificity of antibodies for a particular protein to capture and detect the target protein. Amplification reaction steps allow for efficient detection of minute amounts (pg/mL) of protein of interest (**fig. 2.13**).

At the experimental end point, 1 mL of medium was collected from each condition and stored at -20°C. Once medium from at least three independent experiments had been collected, samples were thawed and subjected to ELISA analysis according to the manufacturer's instructions (eBioscience, #88-7324). Briefly, a 96 well plate was coated with 100 μ L/well of purified anti-mouse TNF α antibody (capture antibody), sealed and incubated overnight at 4°C. The wells were then washed five times with 250 μ L wash buffer (PBS/ 0.05% Tween-20), allowing for 1 minute soaking between washes. The plate was firmly tapped on a lint free paper towel after the last wash to ensure the plate was free of wash buffer. Following this, wells were blocked with 200 μ L of PBS/fetal bovine serum for 1 hour at room temperature. One hundred microlitres of standards (1000, 500, 250, 125, 62.5, 15.625, 7.81 pg/mL recombinant mouse TNF α in PBS/fetal bovine serum, stored at -80°C) or samples were then added in duplicate, the plate sealed and incubated at 4°C overnight. The following day, washing was repeated and 100 μ L of biotin-conjugated polyclonal anti-mouse TNF α added to each well and incubated for 1 hour at room temperature. After a further washing step, 100 μ L of avidin-horse radish peroxidase (HRP) solution were added to each well and incubated for 30 minutes at room temperature. As four avidin proteins bind to each biotin protein, this step served as a signal amplification step. A final washing step consisting of 7 washes was followed by the addition of 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) solution (substrate solution) to each well and incubated at room temperature for 15 minutes. The TMB reaction with the HRP produces a light blue chromogen which can be detected spectrophotometrically. Finally, 50 μ L of 1M H₂SO₄

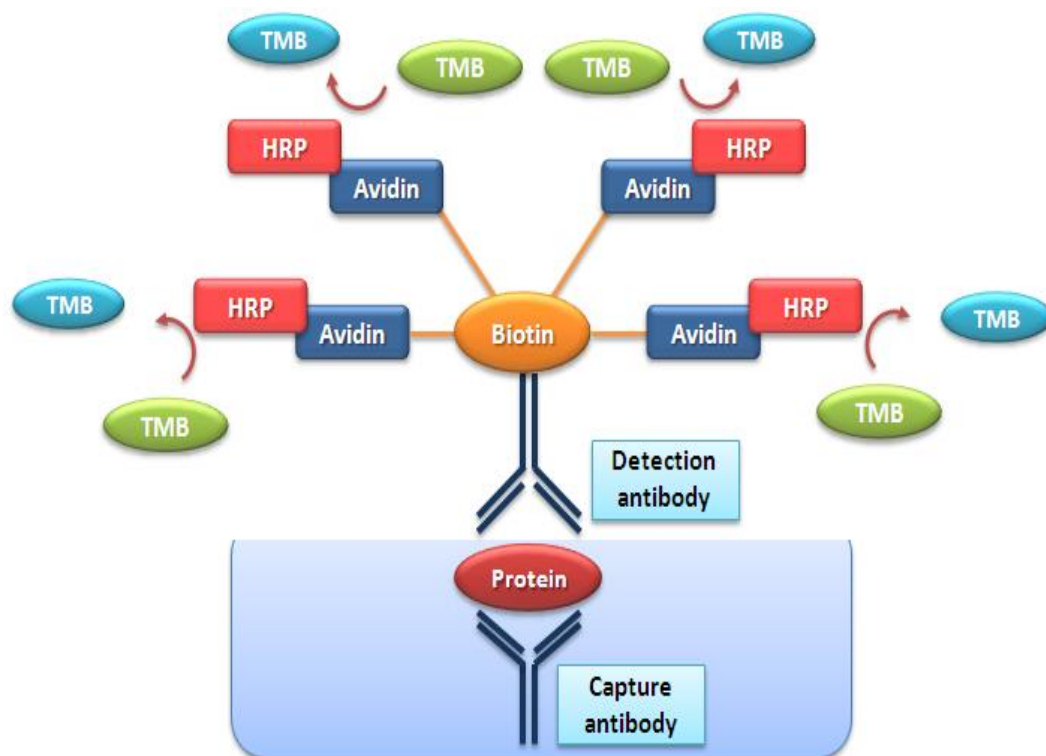


Figure 2.13 Schematic representation of ELISA method Enzyme-linked immunosorbent assays (ELISA) employ the binding specificity of antibodies to capture and detect a protein of interest. A capture antibody coated onto a plate well binds the protein of interest within a sample. The biotin-linked detection antibody serves to amplify the detection signal, as four avidin proteins bind to a single biotin protein. The protein of interest is detected through a chromogenic reaction between a substrate (tetramethylbenzidine, TMB) and a horse-radish peroxidase enzyme linked to the avidin protein. Therefore, as more protein of interest is present in the sample, more colour will be developed in the plate well.

were added to each well to stop the reaction and the optical density read at 450 nm on an automatic plate reader. Optical density was converted to TNF α concentration with the use of a standard curve (fig. 2.14).

In a preliminary ELISA, TNF α concentration in the samples was found to exceed the ELISA's standard curve maximal concentration, therefore samples were diluted (1:3) in cold D-MEM for subsequent TNF α ELISA.

2.1.17 IL-6 and IL-1 β ELISA

The effect of different treatment conditions on the production of the pro-inflammatory cytokines IL-6 and IL-1 β by the microglial cell line N9 was assessed through an ELISA. The ELISA principle for IL-6/IL-1 β was the same as for TNF α (fig. 2.13).

At the experimental end point, one millilitre of medium was collected from each condition and stored at -20°C. Once medium from at least three independent experiments had been collected, samples were thawed and IL-6 or IL-1 β quantified through an ELISA (eBioscience, #88-7064; Peprotech, 900-K47). Briefly, a 96 well plate was coated with 100 μ L/well of purified anti-mouse IL-6 or IL-1 β antibody (capture antibody), sealed and incubated overnight at 4°C. The wells were then washed five times with 250 μ L wash buffer (PBS/ 0.05% Tween-20), allowing for 1 minute soaking between washes. The plate was firmly tapped on a lint free paper towel after the last wash to ensure plate was free of wash buffer. Following this, wells were blocked with 200 μ L of PBS/fetal bovine serum for 1 hour at room temperature. One hundred microlitres of standards (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 0 pg/mL recombinant mouse IL-6/IL-1 β in PBS/fetal bovine serum) and samples were then added in duplicate, the plate sealed and incubated at 4°C overnight. The following day washing was repeated and 100 μ L of biotin-conjugated polyclonal anti-mouse IL-6/IL-1 β added to each well and incubated for 1 hour at room temperature. After a further washing step, 100 μ L of avidin-horse radish peroxidase solution were added to each well and

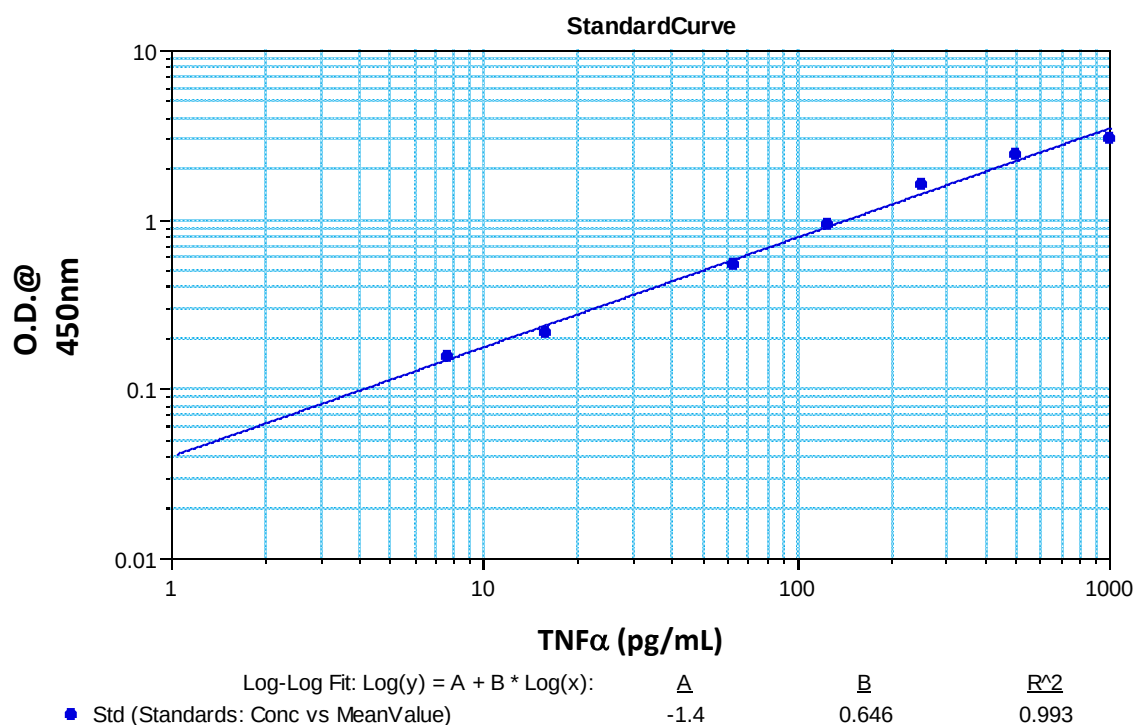


Figure 2.14 TNF α standard curve employed during TNF α ELISA. A TNF α standard curve (1000-7.81 pg/mL) was constructed for each ELISA experiment with the use of recombinant mouse TNF α protein. The standard curve allowed for the optical density values of samples to be converted into total TNF α protein concentration. In order to obtain reliable results, samples were diluted in D-MEM in order for the optical density to fall within the range of the standard curve. A logistic regression was employed to establish line of best fit.

incubated for 30 minutes at room temperature. A final washing step consisting of 7 washes was followed by the addition of 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) solution (substrate solution) to each well and incubated at room temperature for 15 minutes. The TMB reaction with the HRP produces a light blue chromogen which can be detected spectrophotometrically. Finally, 50 μL of 1M H_2SO_4 were added to each well to stop the reaction and the optical density read at 450nm on an automatic plate reader. Optical density was converted to concentration in pg/mL with the use of a standard curve (**fig. 2.15**).

2.1.18 cAMP ELISA

The ELISA protocol for cAMP detection differs from that of cytokine ELISAs in that the cAMP ELISA relies on a competitive binding reaction to quantify cAMP levels. Therefore, whereas colour development in the ELISA for the cytokines is directly proportional to the amount of protein in the samples, colour formation in the cAMP ELISA is inversely proportional to the amount of cAMP in the samples. Hence, wells coated with a polyclonal goat anti-rabbit antibody are incubated with a polyclonal rabbit anti-cAMP antibody, a cAMP conjugate linked to an alkaline phosphatase enzyme (which produces a chromophore upon reaction with its substrate) and either samples or cAMP standards. Once the anti-cAMP antibody attaches to the bound polyclonal goat antibody, it competitively binds either non-conjugated cAMP (from samples or standards) or the cAMP-alkaline phosphatase conjugate. Therefore, as the levels of non-conjugated cAMP in the sample/standards increase, amount of alkaline phosphatase-conjugated cAMP able to bind to the plate decreases, resulting in less colour development following addition of the enzyme's substrate (p-nitrophenyl phosphate) (**fig. 2.16**).

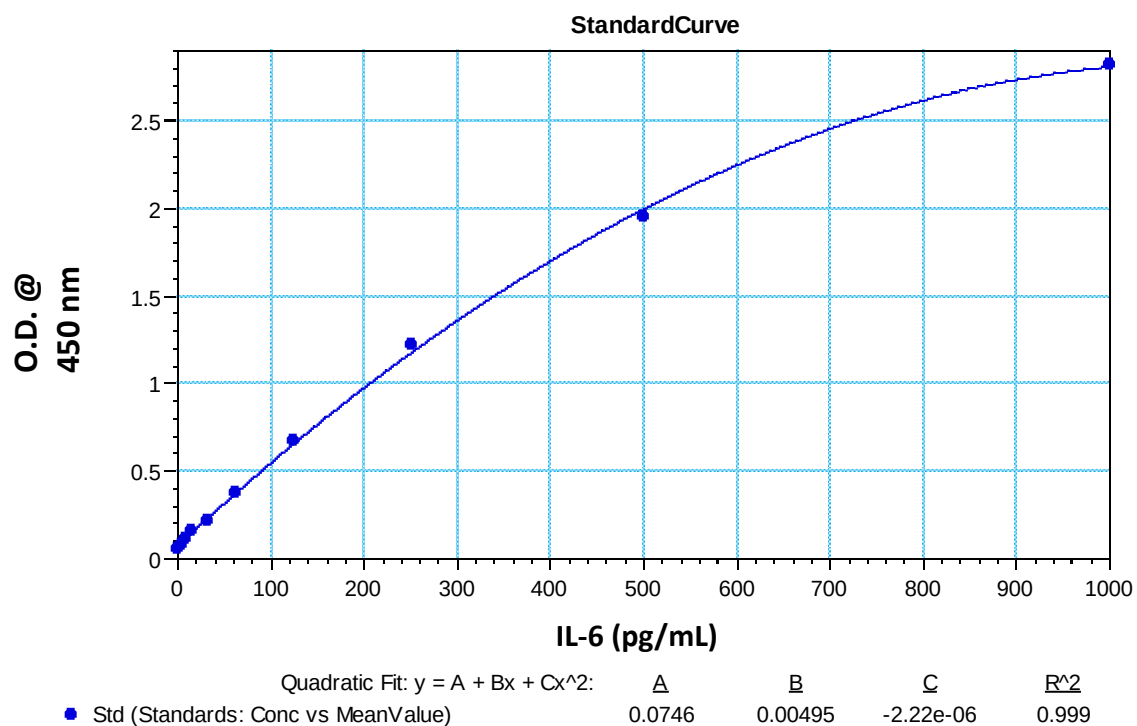


Figure 2.15 IL-6 standard curve. An IL-6 standard curve (1000-7.81 pg/mL) was constructed for each ELISA experiment with the use of recombinant mouse IL-6 protein. The standard curve allowed for the OD values of samples to be converted into total IL-6 protein concentration. The concentration of IL-6 in the samples fell within the standard curve's range, therefore no dilution steps were taken. A quadratic regression was employed to establish line of best fit.

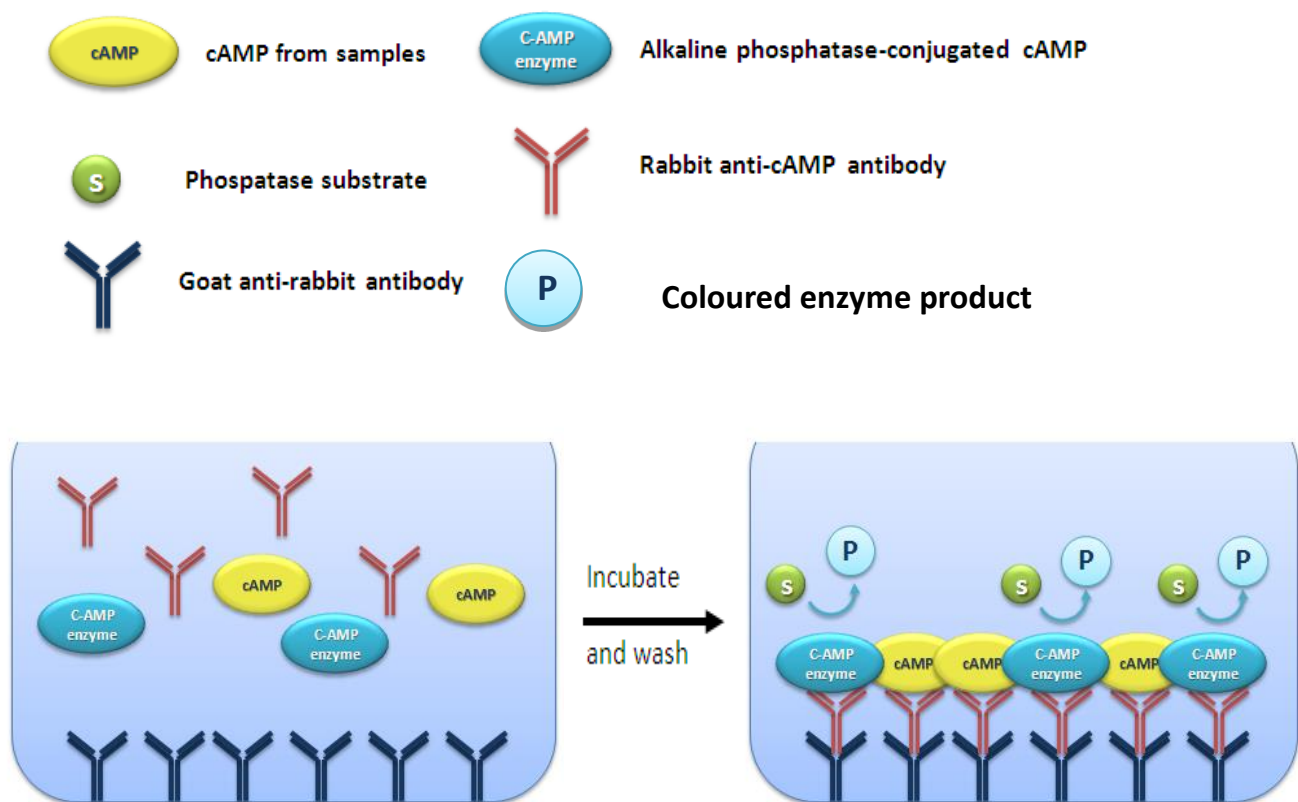


Figure 2.16 Schematic representation of cAMP ELISA method. Cyclic AMP was quantified in cell lysates through an enzyme-linked immunosorbent assay (ELISA). A goat anti-rabbit antibody-coated plate is incubated with a rabbit anti-cAMP polyclonal antibody, a cAMP-alkaline phosphatase conjugate and either samples or cAMP standards. As the rabbit anti-cAMP antibody binds to the immobilized goat anti-rabbit antibody, it binds either cAMP-phosphatase conjugate or cAMP derived from the samples/standards in a competitive manner. Therefore, as the cAMP concentration in the samples/standards increases, less cAMP-phosphatase conjugate binds to the antibody, resulting in less cAMP-phosphatase conjugate in the wells to react with the phosphatase substrate to produce a coloured solution. Hence, colour development is inversely proportional to cAMP concentration in the samples.

The cAMP ELISA protocol was employed to investigate the effect of mGluR ligands and/or forskolin on cAMP production. Microglia were plated on 6 well plates at 600×10^3 c/w. After the initial 24 hrs resting period, cells were incubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM in DMSO, Sigma, I7018) for 20 minutes to prevent cAMP degradation, followed by a 30 minute incubation with the adenylyl cyclase activator forskolin (10 μ M in DMSO, Sigma, F6886) with or without mGluR ligands. In receptor antagonism studies, the mGlu7-specific allosteric antagonist MMPIP was added 20 minutes before forskolin/mGluR ligand treatment to ensure receptor occupancy. In order for the DMSO to not aggregate in the cell culture wells and damage the cells, the phosphodiesterase inhibitor, forskolin or MMPIP were first placed in a 2 mL Eppendorf tube. One millilitre of medium from the target well was then transferred to the Eppendorf tube immediately followed by thorough vortexing. The medium was then gently replaced in the target well. Following the forskolin/mGluR incubation, the medium was aspirated from the culture well and microglia lysed with 250 μ L 0.1M HCl at room temperature for 30 minutes. The lysates were centrifuged (19,000 g x 4 minutes) to pellet cell debris and stored at -20°C. Once lysates from three independent experiments had been collected, a cAMP ELISA kit (Enzo Life Sciences, ADI-900-163) was used to quantify cAMP in the lysates. In order to increase the assay's sensitivity, the lysates were acetylated according to the manufacturer's instructions. Briefly, acetic anhydride and triethylamine were mixed in a 1:2 ratio to create the acetylating reagent. Five microlitres of the acetylating reagent were then added to 100 μ L of cell lysate, vortexed and kept in ice. The ELISA was carried out in a 96 well plate pre-coated with a goat anti-rabbit polyclonal antibody. Fifty microlitres of neutralizing buffer were added to each well to neutralize HCl. One hundred microlitres of acetylated standards (acetylation protocol was same as for samples) (40, 20, 5, 1.25, 0.312, 0.078 pmol/mL) or samples were pipetted into the plate in duplicate, followed by 50 μ L of cAMP conjugated to alkaline phosphatase and 50 μ L of rabbit polyclonal anti-cAMP. The plate was sealed and incubated at room temperature for 2 hours with gentle shaking. After this time, the plate was

washed three times with 400 μ L wash buffer (Tris buffered saline with detergents). The plate was firmly tapped on a lint free paper towel after the last wash to ensure plate was free of wash buffer. Two hundred microlitres of p-nitrophenyl phosphate (enzyme substrate) were added to each well and the plate incubated for a further hour at room temperature without shaking. To stop the reaction, 50 μ L of trisodium phosphate (stop solution) were added to each well and the optical density read at 405nm with an automatic plate reader. Optical density was converted to cAMP concentration with the use of a standard curve (**fig. 2.17**).

2.1.19 SDS-PAGE and Western blotting

Electrophoretic separation and Western blotting were employed to identify and quantify protein expression in cells. At the experimental end point, the medium was removed and wells washed with ice cold PBS. Cells were lysed with 70 μ L radio-immunoprecipitation assay (RIPA) buffer (Sigma, R0278) with 10% protease inhibitors (Sigma, P8340) over ice and collected by scraping with a plastic scraper. This RIPA buffer volume was adopted to ensure cell culture well surface area was completely covered by buffer without overdiluting released cellular protein. The collected RIPA solution was further incubated at 4°C for 45 minutes to ensure complete cell lysis. The lysate was then centrifuged (19,000 g x 25 minutes at 4°C) to remove cell debris and the supernatant collected and stored at -20°C until used. Lysate protein content was quantified through a Bradford assay (see section 2.1.13). Lysate proteins (75 μ g/sample) were separated through sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (gel percentage, voltage and time varied according to identity of protein to be detected) alongside pre-stained molecular weight markers (Sigma, SDS7B2) in glycine/Tris running buffer (3.02 g Tris base, 18.8 g glycine, 1 g SDS in 1L distilled water) and transferred to methanol-activated PVDF membranes (Millipore, IPVH00010) in glycine/Tris transfer buffer (2.9 g glycine, 5.8 g Tris base, 0.37 g SDS, 200 mL methanol in 1L distilled water). After blocking membranes in Tris-buffered saline (TBS)/0.1%

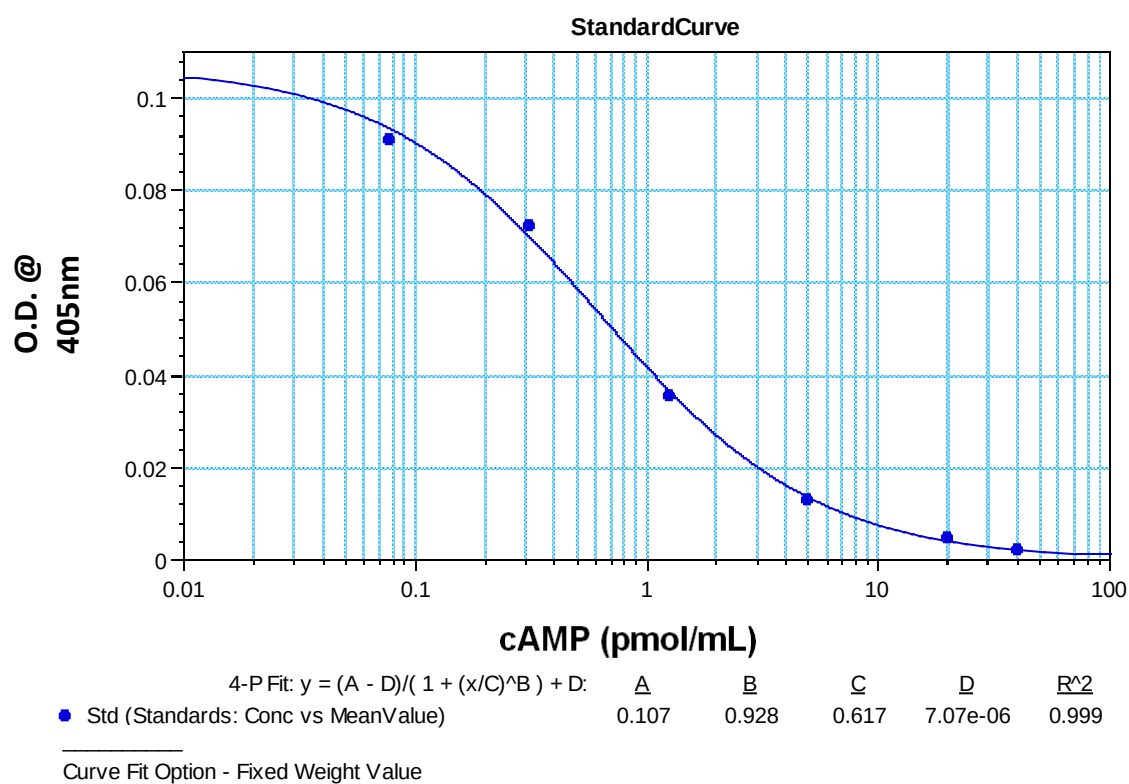


Figure 2.17 cAMP standard curve A cAMP standard curve (40pmol/mL-0.07.8 pmol/mL) was constructed for each ELISA experiment with the use of cAMP standards. The standard curve allowed for the OD values of samples to be converted into total cAMP concentration. The concentration of cAMP in the samples fell within the standard curve's range, therefore no dilution steps were taken. A 4-parameter non-linear regression was employed to establish line of best fit.

Tween-20/5% dried non-fat milk, they were labelled with appropriate primary antibodies (in Tris-buffered saline (TBS)/0.1% Tween-20/1% dried non-fat milk) over night at 4°C, tagged with horse radish peroxidase-conjugated secondary antibodies (Vector Labs, PI-1000 or PI-2000) and visualized with an enhanced chemiluminescence kit (BioRad, 170-5041).

2.1.20 Paraformaldehyde cell fixation

Primary microglia or N9 microglia were plated at 90,000 or 20,000 c/w, respectively, onto sterile glass cover slips (13 mm diameter) in 24 well plates. At the experimental end point, the medium was removed, cells washed twice with 250 µL ice cold PBS and the cells were fixed with 500 µL of ice cold paraformaldehyde (PFa) (4% in PBS: 137 mM NaCl, 2.7 mM KCl, 2 mM KH₂PO₄, 10 mM Na₂HPO₄) for 5 minutes at 4°C. After this time, PFa was replaced with PBS and the plates stored at 4°C.

2.1.21 Immunocytochemistry

Once fixed, cells were washed twice with 250 µL PBS/0.1% Triton-X and blocked with 250 µL 5% normal serum in PBS/0.1% Triton-X for one hour at room temperature with shaking. After washing with PBS/0.1% Triton-X, the cells were incubated with primary antibodies in PBS/0.1% Triton with 5% normal serum at 4°C overnight. The following day, after washing three times with 250 µL PBS/0.1% Triton-X, the cells were incubated with fluorescent dye-conjugated secondary antibodies in PBS/0.1% Triton with 5% normal serum for 1 hr at room temperature (with shaking) protected from light. After washing with PBS, the cells were incubated with the nuclear stain DAPI (1:1000 of 4 mg/mL stock solution) for 5 minutes, after which time it was replaced with PBS. The glass cover slips were mounted onto microscope slides using Vectashield mounting medium for fluorescence (Vector Laboratories, H-1000) and stored at 4°C protected from light. After 2-3 days to allow for drying, the glass cover slips were sealed using nail varnish and placed back in 4°C.

Negative control staining was performed to control for non-specific binding of secondary antibody to cellular or serum proteins. Negative control staining was carried out in the same manner as outlined above, but with omission of the primary antibody.

2.1.22 *Image analysis*

All fluorescent images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera. Images were analysed using Image-Pro Plus software.

2.1.23 *Data analysis*

Data presented represent three independent experiments, unless otherwise stated. Graphs were prepared using either SoftMax software, Microsoft Excel or SPSS (ver. 19) graphing tools and represent mean $\pm$ standard error mean (SEM). Results were analyzed for statistical significance using a Student's T-test (when comparing two groups) or one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test (when comparing three groups or more) using SPSS (ver. 19) statistical package. ANOVA with Tukey's *post-hoc* test was chosen when comparing multiple groups in order to correct for increase of type I error during multiple comparisons. A '*p*' value < 0.05 was considered statistically significant.

2.2 ***Ex vivo methods***

2.2.1 *Animals*

Male Sprague Dawley rats (Harlan, UK) weighting between 240-360g at time of death were used. On arrival, the animals were housed in groups of four on a 12hr light: dark cycle with *ad libitum* access to standard rat chow and water. Animals were allowed to acclimatize for 7 days in the Biological Services Unit prior to any experimental procedure.

2.2.2 *Tissue processing*

Animals were killed by CO₂ overdose followed by decapitation. The brain was quickly removed and placed in ice cold 4% paraformaldehyde (PFa) (average time lapse between death and PFa submersion = 5 min). The brains were submerged in PFa for 5 days to ensure fixing of whole brain and subsequently transferred into 30% sucrose for 2 days (or until brains sunk) for cryoprotection. Once cryoprotected, the brains were snap frozen by submerging them into isopentane cooled in dry ice (20 min). The frozen brains were wrapped in aluminium foil, placed inside an air-lock bag and kept at -80°C until cryostat sectioning.

However, this tissue processing method proved inadequate for detection of GDNF and certain mGluR, possibly due to excessive protein linkage by PFa which resulted in the epitopes becoming masked. Therefore, fixing of fresh dissected brain was replaced by transcardial perfusion with PFa. Animals were terminally anaesthetized with 1 mL pentobarbital (injected intraperitoneally) and transcardially perfused with 150 mL ice-cold 0.1M PBS (130 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) followed by 150 mL ice-cold 4% PFa (pH 7.6). After perfusion, the brains were dissected out and post-fixed in 4% PFa for 4 hrs. Once fixed, the brains were submerged in 30% sucrose for cryoprotection. Once brains stopped floating, they were snap frozen in cold isopentane (cooled in dry ice x 20 min), wrapped up in aluminium, and stored in -80°C in an air tight plastic bag.

In order to immunolabel tissue, it was sectioned into 20 µm thick slices using a cryostat. The brain to be cut was transferred into the cryostat (-20°C) 1 hour prior to cutting to allow it to adjust to the new temperature. Tissue slices from the striatum to the substantia nigra were serially collected into PBS/0.1% azide using a 24 well plate and kept at 4°C until used.

2.2.3 *Tissue immunostaining*

Sections were placed in new 24 well plates and washed with PBS (3 x 5 min). The sections were blocked against non-specific binding by incubation in PBS/0.1% Triton-X/5% normal serum for 1 hr

at room temperature with shaking. Once blocked, sections were incubated for 48 with primary antibodies (see table 2.1) in PBS/0.1% Triton-X/5% normal serum at 4°C with shaking. Sections were then washed with PBS/0.1% Triton-X and incubated with fluorescent dye-tagged secondary antibodies in PBS/0.1% Triton-X/ 5% normal serum for 1hr at room temperature in the dark (wrapped in aluminium foil) with shaking. After washing with PBS/0.1% Triton, the slices were mounted onto microscope slides and air dried overnight at 4°C in the dark. The slices were finally coverslipped using anti-fading medium with DAPI (Vectashield, H-1200) and stored in the dark at 4°C. After a few days, the cover slips were sealed with nail varnish.

Chapter III

Establishing microglial inflammation

3. Establishment and activation of primary murine microglia and the N9 mouse microglial cell line

3.1 Introduction

Microglial cells are the first line of defence against infection and injury in the CNS. Microglial cells form part of the innate immune system and are considered the resident macrophage of the CNS. Under normal conditions, microglia exist throughout the CNS in a highly ramified state with motile processes which scan their immediate surroundings in a time frame of tens of minutes (Nimmerjahn *et al.*, 2005). Upon detection of infection or injury, microglia retract their processes, adopting an amoeba-like morphology, and shift their phenotype towards one with a greater ability for pathogen clearance or injury repair (Kigerl *et al.*, 2009), a process broadly termed microglial activation. Activated microglia may adopt a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype, depending on the activating stimulus. Pathogen detection leads to a pro-inflammatory phenotype, characterized by an increase in expression of pro-inflammatory cytokines such as IL-6 and TNF α to combat infection by induction of apoptosis and enhancement of the inflammatory response. In addition, pro-inflammatory microglia increase the synthesis of the reactive nitrogen species NO in order to kill invading organisms. Alternatively, microglial activation may lead to an anti-inflammatory phenotype characterized by expression of anti-inflammatory cytokines (*e.g.* IL-10) and tissue-remodelling enzymes (*e.g.* arginase-1) (David and Kroner, 2011; Kigerl *et al.*, 2009).

There is increasing evidence that microglia are kept in their resting state not only by lack of injury/infection signals, but also by direct interaction with neighbouring non-microglial cells. Accordingly, if the microglial cytokine receptor CD200R is activated by its ligand CD200, found on the membrane of neurons, microglial activation is inhibited (Jenmalm *et al.*, 2006). In addition, microglia from single cell cultures have an enhanced inflammatory response to amyloid- β than microglia in neuron-glia co-cultures (Lyons *et al.*, 2007). Therefore, microglial activation is an

important physiological function which is tightly regulated by the microglia's surrounding environment.

Hence, *in vitro/ex vivo* microglial models may confer microglia with a phenotype dissimilar to that found *in vivo*. However, this does not preclude the use of *in vitro* models to study microglial biology. Cell lines, both primary and immortalized, have the advantage that, unlike *in vivo* models, they allow for the study of a compound's direct effects on microglia. Furthermore, though they may exist under a different phenotype to *in vivo* microglia, cultured microglia still retain the basic cellular mechanisms and signal transduction pathways found *in vivo*. Therefore, they are useful for studying specific aspects of microglial activation. However, care should be taken to characterize the resting phenotype and activation potential of any microglial cell line before employing them as a model for *in vivo* microglial activation.

In this thesis, the N9 immortalized mouse microglial cell line was employed as an *in vitro* model of microglial activation. This cell line was chosen in preference of primary microglial cells due to its high proliferative rate, which allows for a higher number of experiments to be carried out, as well as the methodological and financial drawbacks of using primary cells. However, for N9 microglia to be a valid substitute for primary cells, its resting state after plating and its potential for activation had to be comparable to primary microglia. To this end, the resting state of primary murine microglia and the N9 immortalized microglial cell line was assessed through quantification of NO and its synthesising enzyme iNOS, quantification of pro-inflammatory cytokine IL-6 and TNF α release into the culture medium, as well as through qualitative assessment of cell morphology. In addition, the ability of primary and N9 microglia to undergo a phenotypic shift towards pro-inflammatory activation was assessed by measuring the above parameters after treating the cells with either the bacterial endotoxin lipopolysaccharide (LPS) or the neurotoxic dopamine analogue 6-OHDA.

- **Hypothesis:** Primary microglia and N9 microglia retain a similar non-inflammatory phenotype following extraction and plating, which can be reliably shifted towards a pro-inflammatory phenotype by treatment with lipopolysaccharide.

3.2 Experimental design

Primary microglia derived from post-natal day 7 (p7) rats were extracted through a Percoll gradient separation of homogenized brains and allowed to recover for 24 hrs before treatment (see section 2.1.4). The N9 microglial cell line is an oncogenic transformed embryonic mouse microglial cell line first devised by Dr. Ricciardi-Castagnoli *et al.* (1989). Similarly to primary microglia, N9 microglia were incubated for 24 hrs after plating from a growing culture stock (see sections 2.1.1-3) before treatment began to allow them to recover their resting morphology. Primary microglia were plated in 24 well plates at 90×10^3 cells per well. N9 microglia were plated in 6 or 24 well plates at 600 or 20×10^3 cells per well, respectively.

After the initial 24 hrs recovery period, primary microglia or N9 microglia were treated with LPS or 6-OHDA. These toxins were selected due to either their established inflammatory potency (LPS) or their relevance to PD animal models and dopaminergic neuron degeneration (6-OHDA) (see sections 1.6.3.2, 2.1.8 and 2.1.9). Toxins were added directly into the cell culture medium and microglial activation assessed at 18 hrs post-treatment (*i.e.* experimental end-point, 42 hrs after plating) (see **fig. 2.2**). This experimental end-point was chosen as a compromise between the peak expression time-points of IL-6 and TNF α and the peak synthesis time-point of NO (Shigemoto-Mogami *et al.*, 2001; Lin *et al.*, 2011; Byrnes *et al.*, 2009).

Activation was assessed through quantification of five different parameters. Firstly, activation was assessed through indirect chemical quantification of the reactive nitrogen species NO using the Griess reaction, which measures the levels of nitrite, a stable metabolite of NO. However, NO is metabolised into nitrite (NO₂) and nitrate (NO₃), therefore in order to obtain an accurate

measurement of total NO, nitrate is first converted to nitrite before the Griess reaction is carried out (see section 2.1.15). The NO-synthesising enzyme iNOS is not constitutively expressed in resting microglia (*e.g.* Li *et al.*, 2005), therefore it is in itself a useful marker of pro-inflammatory microglial activation. Hence, expression of the NO-synthesising enzyme iNOS was assessed through antibody-labelling of Western blots from microglial lysates, as well as immunocytochemical labelling of mixed microglia (see sections 2.1.19-21). In addition, microglial activation was assessed by quantifying release of the pro-inflammatory cytokines TNF α and IL-6 into the culture medium through ELISA (see sections 2.1.16 and 2.1.17, respectively). Finally, resting and activated microglia were differentiated through qualitative morphological determination.

3.3 Results

3.3.1 Primary microglia retain a resting phenotype after extraction and are robustly activated towards a pro-inflammatory phenotype by LPS treatment

3.3.1.1 *Effect of LPS treatment on nitric oxide synthesis and TNF α release in primary rat microglia*

Forty two hours after extraction, primary microglia produced low levels of NO, as assessed by total nitrite concentration in the culture medium (**fig. 3.1A**). However, an 18hr LPS treatment 24 hrs after extraction stimulated a marked increase in nitrite synthesis. LPS-induced nitrite synthesis peaked with an LPS concentration of 500 ng/mL (336% above control), decreasing with higher concentrations (**fig. 3.1A**). Unlike LPS however, an 18hr 6-OHDA treatment 24 hrs after extraction did not induce a significant increase in nitrite production under all concentrations tested (**fig. 3.1A**). Similarly, while TNF α release by unstimulated microglia was negligible 42 hrs after extraction (**fig. 3.1B**), LPS treatment stimulated a robust increase in TNF α release. However, LPS stimulation of TNF α release appeared to be saturated at 100 ng/mL LPS, as higher concentrations of LPS (up to 5 μ g/mL) stimulated a comparable increase in TNF α release (**fig. 3.1B**). Unlike LPS, 6-OHDA treatment failed to induce an increase in TNF α release under all concentrations tested (**fig. 3.1B**).

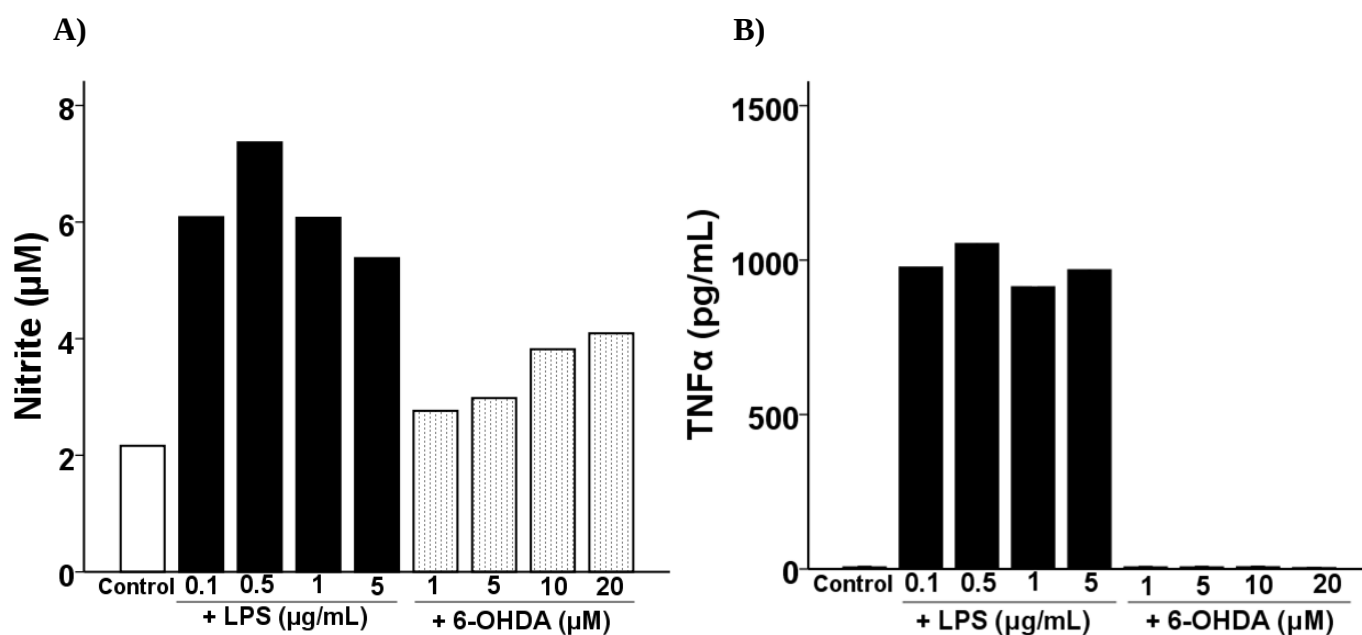


Figure 3.1 Nitrite production and TNF α release by resting and LPS- or 6-OHDA-treated primary rat microglia.

Primary rat microglia were treated or not with the bacterial component LPS or the toxin 6-OHDA for 18 hrs, after which time-point the cell culture medium was subjected to a Griess assay to quantify nitrite production (**A**) or an enzyme-linked immunosorbent assay to quantify TNF α release (**B**). LPS treatment led to a marked increase in nitrite production, with maximal effect achieved with 500 ng/mL. On the other hand, 6-OHDA treatment stimulated only a modest increase in nitrite production, with the highest concentration (20 μ M) stimulating the strongest effect. Similarly, while LPS treatment stimulated a robust increase in TNF α release with comparable TNF α release through all LPS concentrations examined, 6-OHDA failed to modulate TNF α release. Importantly, under control conditions, primary rat microglia produced low levels of nitrite and released a negligible amount of TNF α . Results are displayed the mean of two independent experiments, each measured in triplicate.

3.3.1.2 Effect of LPS treatment on primary microglial morphology

Primary microglia adopted a resting morphology 24 hrs after extraction characterized by a compact cell body with short protrusions, which persisted until the experimental end-point (42 hrs after plating) (**fig. 3.2A, B**). The majority of microglia were firmly attached to the culture plate surface, with only a small fraction (~10%) of cells floating in the medium or only weakly attached to the plate surface. An 18 hr LPS treatment (500 ng/mL) 24 hr following extraction stimulated a shift towards an activated morphology, characterized by retraction the microglial protrusions and cell body hypertrophy, ultimately adopting an amoeboid morphology (**fig. 3.2C, D**).

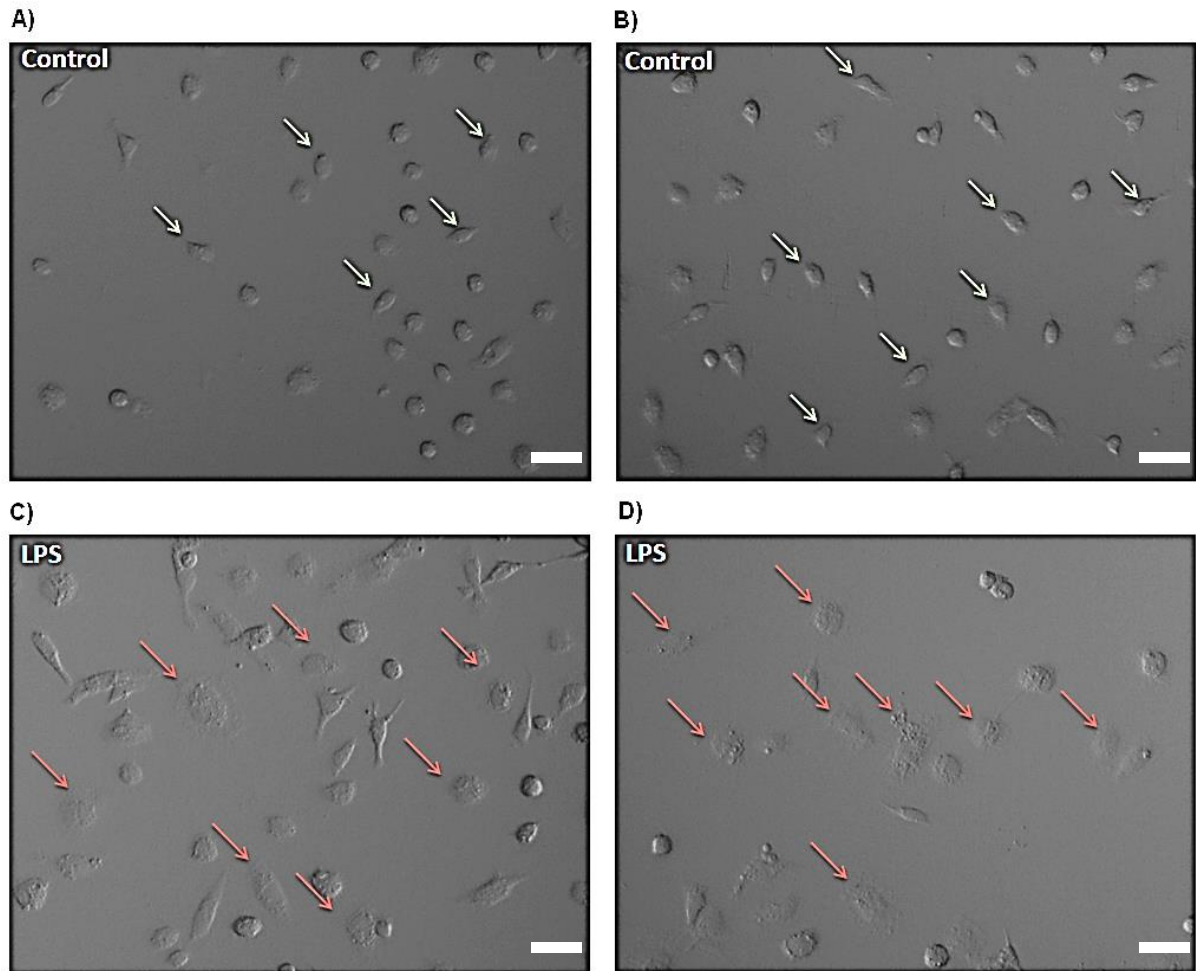


Figure 3.2 Resting and LPS-treated primary rat microglia cell morphology. Primary rat microglia were treated or not with the bacterial component LPS (500 ng/mL) for 18 hrs, after which time the microglial plates were transferred to a microscope equipped with a live-cell imaging chamber and images captured at a magnification of 200x. Control microglia (**A, B**) retained a resting morphology, characterized by compact cell bodies and short processes (green arrows). Conversely, LPS-treated microglia (**C, D**) adopted an activated morphology, characterized by retraction of their processes and expansion (hypertrophy) of their cell bodies (red arrows). Images are representative of two independent experiments. Scale bars = 30 μ m.

3.3.2 N9 microglia retain a resting phenotype after plating and are robustly activated towards a pro-inflammatory phenotype by LPS

3.3.2.1 Effect of LPS or 6-OHDA on nitric oxide production and iNOS synthesis in N9 microglia

Following plating, N9 microglia synthesised modest amounts of NO as measured by nitrite concentration in the culture medium. However, an 18 hr LPS-treatment 24 hrs following plating stimulated a marked increase in nitrite production, peaked at an LPS concentration of 500 ng/mL (348% of control) and declined with higher concentrations (**fig. 3.3A**). In accordance with nitrite quantification, antibody labelling of Western blots of lysates from untreated microglia failed to detect iNOS protein expression, while there was a clear and dose-dependent expression of iNOS in lysates from LPS-treated microglia (**fig. 3.3B**). Similarly, immunocytochemical labelling of fixed microglia (42 hrs after plating) with the same anti-iNOS antibody employed for Western blots showed only very weak labelling of untreated microglia (**fig. 3.3C**), while there was a marked increase in labelling in LPS (500 ng/mL) treated microglia (**fig. 3.3E**). Interestingly, LPS treatment did not induce iNOS immunoreactivity homogenously throughout the cell culture. Instead, iNOS reactivity was particularly elevated within certain cells, while reactivity was modest for others (**fig. 3.3E insert**). Double staining with the nuclear stain DAPI showed unfragmented nuclear morphology for both untreated and LPS treated cells, indicating healthy cell viability (**fig. 3.3 D, F**).

An 18 hr 6-OHDA treatment appeared to induce a slight increase in nitrite concentration within the cell culture medium (**fig. 3.4A**). However, this was not due to an effect of microglial activation, but rather 6-OHDA auto-oxidation (and hence brown/orange colouration of the culture medium) interfering with the Griess assay, as evidenced by increased nitrite detection in microglia-free medium incubated with increasing 6-OHDA concentrations (**fig. 3.4B**). However, 6-OHDA did induce marked cell death, as evidenced by an increase in ethidium bromide incorporation (**fig. 3.4C**) and a decline in cellular protein concentration (**fig. 3.4D**) in cells treated with increasing 6-OHDA concentrations, indicating the 6-OHDA solution employed was active.

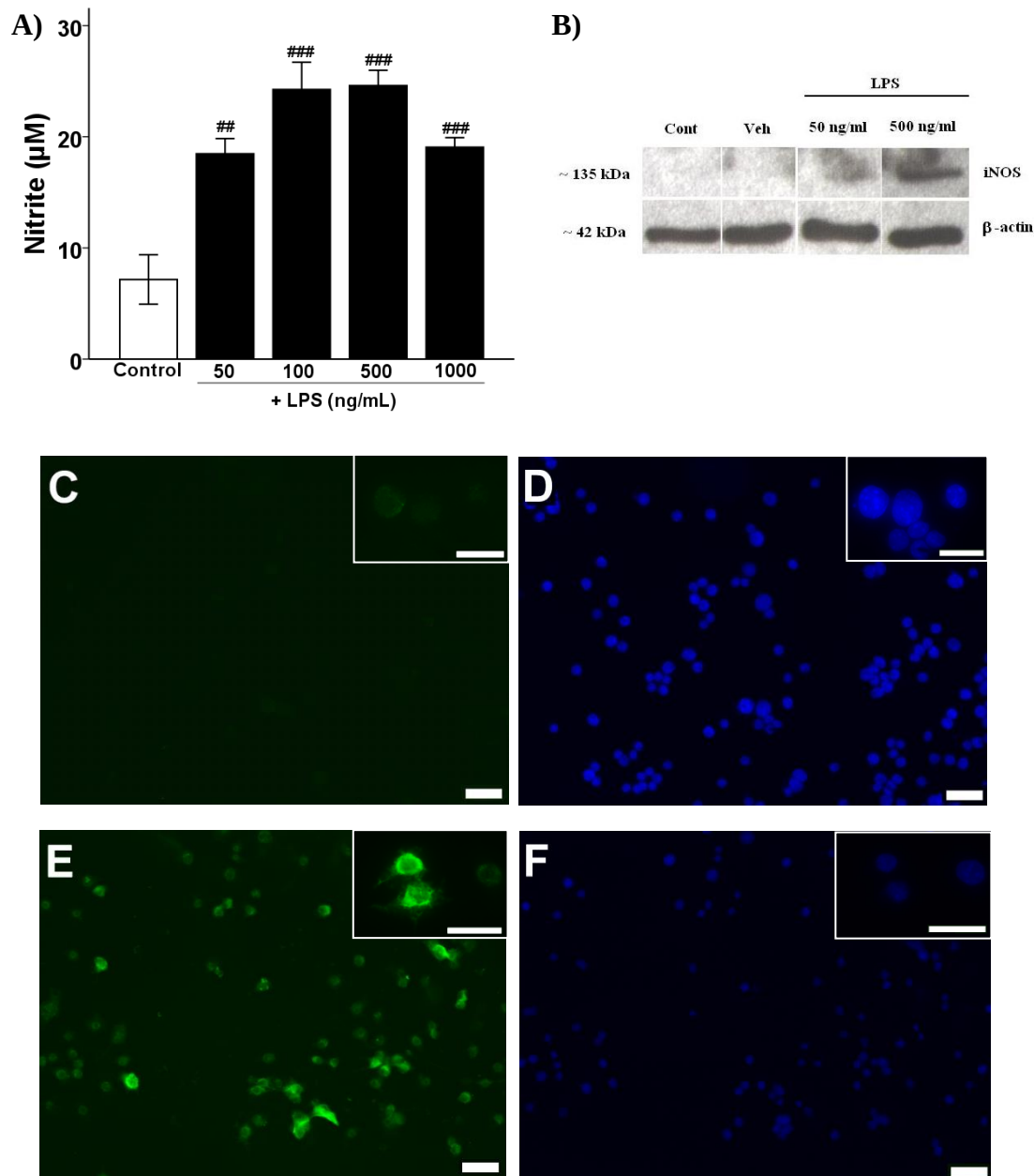


Figure 3.3 Nitrite production and iNOS expression by resting and LPS-treated N9 microglia. Following an 18hrs treatment of N9 microglia with the bacterial cell wall component LPS, nitrite production and iNOS expression were quantified through a Griess assay, and Western blotting or immunocytochemistry, respectively. While under control conditions N9 microglia produced low levels of nitrite, LPS treatment stimulated a robust increase in nitrite production, with maximal effect achieved with 500 ng/mL (A). Similarly, while iNOS expression and immunoreactivity were absent in control conditions, LPS treatment led to a marked increase in iNOS protein expression (B) and iNOS immunoreactivity in fixed N9 cells (C-F). Interestingly, iNOS immunoreactivity was not homogenous throughout the cell population, but was particularly strong within certain cells and weak in others (insert E) (panels C,D display control microglia; panels E, F display LPS-treated microglia; panels C,E display iNOS stained microglia; panels D,F display DAPI stained microglia). Results in panel (A) are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate. ##, ### denote $p < 0.01$, 0.005, respectively vs. control as determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 16.102$. Panels B-F are representative images of three independent experiments. C-F magnification = 10x (40x for inserts). Scale bars = 50 μm (30 μm for inserts).

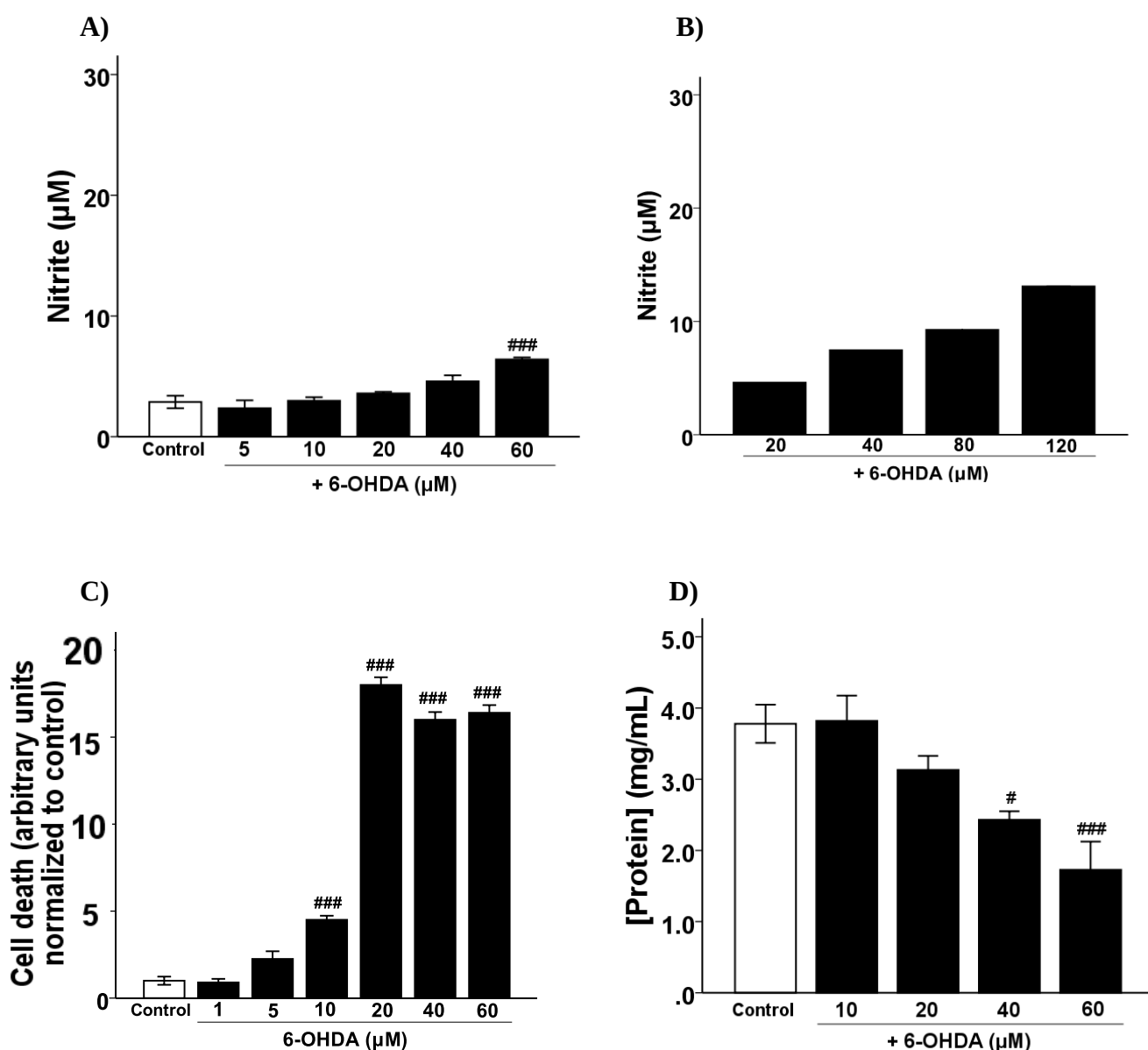


Figure 3.4 Nitrite production and cell viability in 6-OHDA-treated N9 microglia. An 18 hrs treatment of N9 microglia with the toxin 6-OHDA led to a modest yet significant increase in nitrite levels in the cell culture medium, as determined by a Griess assay (A). However, this effect was shown to be a false positive effect caused by the auto-oxidation of 6-OHDA interfering with the Griess assay, as indicated by increasing nitrite detection by the Griess assay in microglia-free medium incubated for 18 hrs with increasing concentrations of 6-OHDA (B). Despite the lack of effect of 6-OHDA on nitrite production by N9 microglia, an 18 hrs 6-OHDA treatment strongly reduced N9 microglia viability, as indicated by an increase in the percentage of cells incorporating ethidium bromide (*i.e.* Live/Dead assays) (C), and a decrease in cellular protein content (quantified through a Bradford assay) (D). Results are displayed as mean $\pm$ SEM of three independent experiments (with the exception of (B) which represents a single experiment), measured in triplicate (A-C) or duplicate (D). #, ### denote $p < 0.05$ or 0.005 vs. control, respectively, as determined by a one-way ANOVA with Tukey's *post-hoc* test. $F = 9.72$ (A), 9.91 (D).

3.3.2.2 *Effect of LPS treatment on IL-6 and TNF α release in N9 microglia*

Forty two hours after plating, unstimulated N9 microglia released almost undetectable levels of IL-6 into the cell culture medium (**fig. 3.5A**). However, an 18 hrs LPS treatment 24 hrs after plating stimulated a robust and dose-dependent increase in IL-6 release by N9 microglia (**fig. 3.5A**). Similarly, 42 hrs after plating, unstimulated N9 microglia released low levels of TNF α into the cell culture medium, bordering the detection limit of the ELISA kit (**fig. 3.5B**). However, an 18 hrs LPS treatment 24 hrs after plating stimulated a robust increase in TNF α release by N9 microglia. Unlike IL-6, TNF α release stimulation was comparable for all LPS concentrations examined (**fig. 3.5B**), indicating stimulation of TNF α release became saturated with the lowest LPS concentration tested (50 ng/mL).

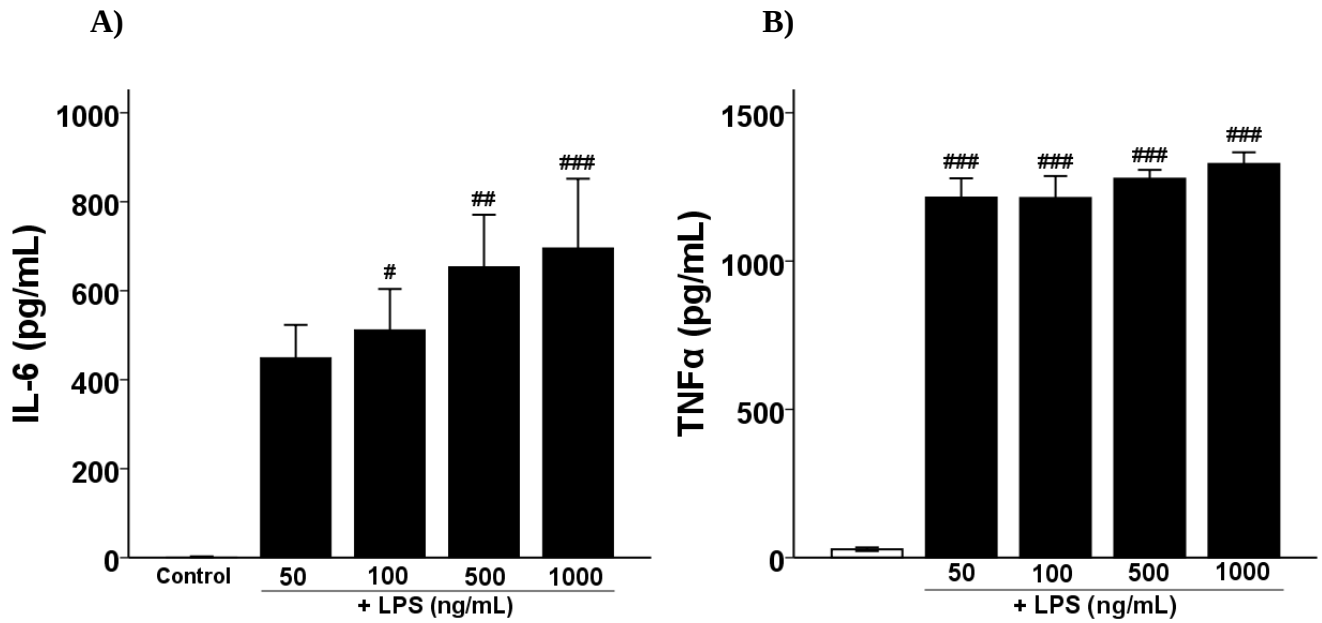


Figure 3.5 IL-6 and TNF α release by control and LPS-treated N9 microglia. Following an 18 hrs treatment of N9 microglia with the bacterial cell wall component LPS, the levels of IL-6 (**A**) and TNF α (**B**) release into the cell culture medium were quantified through an enzyme-linked immunosorbent assay. LPS treatment led to a robust increase in release of both cytokines. However, while LPS had a dose-dependent effect on IL-6 release, its effect on TNF α release appeared to become saturated at the lowest dose examined (50 ng/mL), with higher doses (up to 1000 ng/mL) eliciting a comparable response. Importantly, release of both cytokines under control conditions was almost negligible. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denotes $p < 0.05$, 0.01, 0.005 vs. control, respectively, as determined by a one-way ANOVA with Tukey's *post-hoc* test. $F = 7.4$ (A), 124.3 (B).

3.3.2.3 Effect of LPS treatment on N9 microglial morphology

N9 microglia recovered a resting morphology 24 hrs following plating, which persisted until the experimental end-point (42 hrs after plating), similarly to primary microglia. N9 microglia had a compact cell body from which a number of ramified processes extended (**fig. 3.6A, B**). The resting N9 morphology resembled that of resting microglia *in vivo* (**fig. 3.6E**), though the N9 processes were not as elongated or branched. However, in areas with high cell density, N9 microglia lost their ramifications but kept their compact cell body. Although most cells were firmly attached to the plate surface, a small fraction (~10%) of cells was either floating in the medium or weakly attached to the plate surface. LPS treatment (500 ng/mL x 18 hrs at 24 hrs following plating) stimulated N9 microglia to retract their ramified processes and caused hypertrophy of their cell body, ultimately adopting an amoeboid morphology (**fig. 3.6 C, D**).

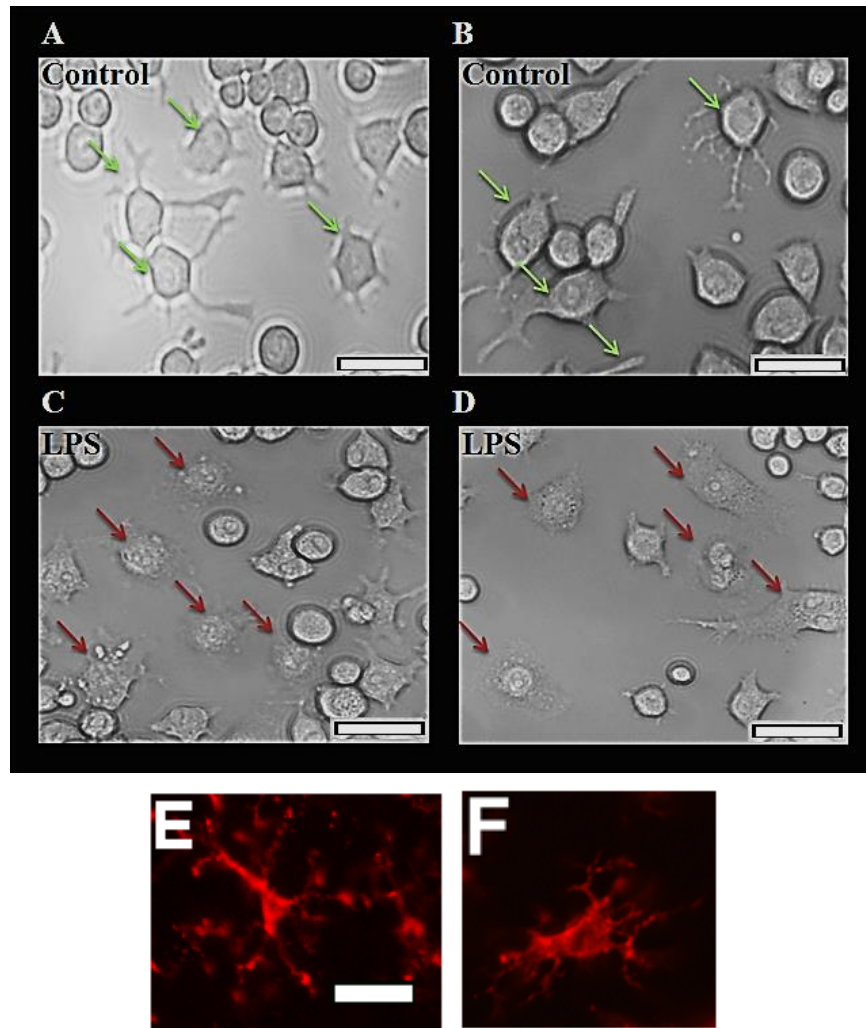


Figure 3.6 Morphology of resting and LPS-treated N9 microglia *in vitro* and resting and activated microglia *in vivo*. N9 microglia were treated or not with the bacterial component LPS (500 ng/mL) for 18 hrs. Microglial plates were then transferred to a microscope equipped with a live-cell imaging chamber and images captured at a magnification of 200x. Control microglia (**A, B**) retained a resting morphology, characterized by a compact cell body and long, ramified processes (green arrows). LPS treated microglia (**C, D**) adopted an activated morphology characterized by retraction of their processes and cell body hypertrophy (red arrows). Panels (**E**) and (**F**) show fixed mid-brain tissue from naïve and 6-OHDA-treated (intranigral injection) rats, respectively, labelled with an anti-CD11b (Ox-42) antibody or an anti-MHC-II (Ox-6) antibody, respectively. Note the compact cell body and ramified processes of the resting microglia in panel (E), reminiscent of resting N9 microglia *in vitro*. Similarly, note the shortened processes and expanded cell body of the activated microglia in panel (F), reminiscent of activated N9 microglia *in vitro*. Magnification = 20x (**A-D**), 40x (**E, F**). Scale bar = 20 μ m (**A-D**) or 10 μ m (**E-F**).

3.4 Discussion

Primary microglia were shown to retain a resting phenotype which persisted until 42 hrs following extraction, as assessed by low levels of nitrite production and negligible TNF α release into the culture medium. Furthermore, primary microglia retained a resting morphology characterized by a compact cell body from which extended a small number of processes. Importantly, LPS-treatment shifted primary resting microglia towards a pro-inflammatory phenotype characterized by a robust increase in nitrite synthesis and TNF α release, and a morphological shift towards an amoeboid morphology. Although dose-dependence was not particularly marked, nitrite synthesis appeared to be most strongly stimulated by an LPS concentration of 500 ng/mL, with higher concentrations resulting in lower stimulation. On the other hand, TNF α release was stimulated to an equal level by all concentrations examined, indicating primary microglia are more sensitive to LPS treatment with respect to TNF α release than to nitrite synthesis, and the TNF α response becomes saturated even at low LPS concentrations. Interestingly, 6-OHDA treatment failed to induce a shift towards a pro-inflammatory phenotype in primary microglia, as assessed by lack of increase in nitrite synthesis or TNF α release by all 6-OHDA concentrations tested. This was not due to a lack of activity by 6-OHDA, as the same solution caused a marked cell death in N9 microglia (see below).

Similarly to primary microglia, unstimulated N9 microglia were shown to retain a resting phenotype 42 hrs after plating, as assessed by low levels of nitrite production and undetectable iNOS expression, as well as negligible IL-6 and TNF α release into the culture medium. In addition, N9 microglia morphology resembled that seen in resting microglia *in vivo*, characterized by a compact cell body from which extended several ramified branches (though these branches were less extensive than those seen *in vivo*). Interestingly, N9 microglia acquired a more ramified resting morphology than primary microglia, suggesting N9 microglia rely less on neuronal or astrocytic cues to adopt a resting phenotype than primary microglia do. Importantly, LPS treatment stimulated a shift towards

a pro-inflammatory phenotype in N9 microglia, comparable to primary microglia. Accordingly, LPS-treatment stimulated a marked increase in nitrite production, which was accompanied by an increase in iNOS protein expression, indicating that the levels of nitrite increased due to an inflammatory response-related increase in NO synthesis, and not to an increase in breakdown of existing NO. The fact that a small amount of nitrite was detected even under resting conditions points to the involvement of other nitric oxide synthase enzymes, namely neuronal NOS (nNOS) and epithelial NOS (eNOS) within microglia *in vitro*. nNOS and eNOS are constitutively expressed in neurons and epithelia, respectively. These enzymes are primarily controlled by calcium levels and are involved in production of low levels of NO to act as a neurotransmitter or vasodilator. Both of these enzymes however have also been shown to be expressed in murine microglia, even under resting conditions (Kopec and Carroll, 2000).

Furthermore, LPS stimulated a robust increase in IL-6 and TNF α release, further confirming a pro-inflammatory N9 microglial phenotype. Importantly, the nitrite synthesis and TNF α release response to LPS in N9 microglia had a similar profile to the primary microglia response, with nitrite synthesis peaking with an LPS concentration of 500 ng/mL and declining with higher concentrations, and a more LPS-sensitive TNF α response which became saturated even at low LPS concentrations. In addition, LPS had a similar effect on cellular morphology on N9 microglia as with primary microglia, stimulating N9 microglia to completely retract their ramified processes and causing hypertrophy of their cell body, ultimately adopting an amoeboid morphology reminiscent of activated microglia *in vivo*.

Similarly to primary microglia, treatment of N9 microglia with 6-OHDA failed to stimulate an increase in NO synthesis. Although there was a modest increase in nitrite detection by the Griess assay, this was not due to an effect of 6-OHDA on N9 microglia as incubating 6-OHDA in culture medium in the absence of microglia replicated this increase in nitrite detection. This false positive nitrite synthesis increase was most probably due to 6-OHDA causing a marked brown colouring of

the culture medium which interfered with the optical density analysis of the Griess assay. This colouring was possibly due to an autoxidative reaction (Hanrott *et al.*, 2005), which increased in intensity with increasing 6-OHDA concentration. Surprisingly, though 6-OHDA-treatment failed to stimulate a pro-inflammatory phenotype, it caused marked microglial toxicity, as assessed by a significant increase in the number of cells incorporating ethidium bromide, as well as a sharp decrease in protein concentration within the culture well. 6-OHDA is believed to cause cellular damage to dopaminergic neurons specifically through selective cellular uptake by dopamine transporters. Once within the cell, it is thought to bind to mitochondrial membrane proteins and inhibit respiration (Blum *et al.*, 2001). However, extracellular 6-OHDA may cause cellular damage through an auto-oxidative reaction, which may produce reactive species such as quinones capable of damaging the cell membrane (Blum *et al.*, 2001). Since microglia cells lack dopamine transporters, autoxidation seems the most probable mechanism responsible for microglial cell death. The lack of an inflammatory response to 6-OHDA by primary microglia and N9 microglia indicates that induction of a microglial pro-inflammatory phenotype is a tightly regulated process which requires the activation of specific signalling pathways, and will not result from cellular damage *per se*.

Taken together, these results indicate that primary microglia are able to readopt a resting phenotype 24 hrs after extraction which can be robustly activated towards a pro-inflammatory phenotype by LPS-stimulation. Importantly, N9 microglia mimicked primary microglia in adopting a resting phenotype after plating which could be similarly activated towards a pro-inflammatory phenotype, validating the use of N9 microglia as a substitute for primary microglia. An LPS concentration of 500 ng/mL stimulated robust IL-6 and TNF α release, as well as a peak increase in nitrite synthesis in both primary and N9 microglia, therefore this concentration of LPS will be adopted for subsequent activation experiments.

3.5 Conclusion

The present results demonstrate that, as hypothesized, cultured N9 microglia retain a non-inflammatory phenotype and can be robustly activated towards a pro-inflammatory phenotype by treatment with LPS, but not 6-OHDA. Similarly, primary microglia retained a resting phenotype after extraction and could be activated towards a pro-inflammatory phenotype by LPS, but not 6-OHDA. Therefore, these results demonstrate the validity of N9 microglia as a substitute model of primary microglia.

Furthermore, an LPS concentration of 500 ng/mL proved to be the most efficient dose to induce microglial pro-inflammatory activation in both primary and N9 microglia, as assessed by a robust increase in IL-6 and TNF α release and iNOS expression, and a maximal stimulation of nitrite production. Therefore, a concentration of 500 ng/mL LPS was adopted for subsequent activation experiments.

Chapter IV

Metabotropic glutamate receptor expression *in vitro*
and *in vivo*

4.1 Introduction

Along with ionotropic glutamate receptors, metabotropic glutamate receptors transduce glutamatergic signalling into the cell (reviewed by Featherstone, 2010). Metabotropic glutamate receptors (mGluR) are heptahelical transmembrane receptors intracellularly coupled to G proteins. The eight receptors which have been identified to date can be divided into three different groups, depending on their amino acid sequence and signal transduction mechanism (see section 1.8.2) (reviewed by Conn and Pin, 1997).

Group I mGluR comprise two receptors, namely mGlu1 and mGlu5. These receptors are coupled to a $G_{q/s}$ protein, therefore promote activation of phospholipase C and production of diacylglycerol (DAG) and inositol trisphosphate (IP3) from phosphoinositides upon glutamate binding. Group I receptors are mainly found post-synaptically on neurons, where their activation leads to increased neuronal firing. However, expression of mGlu1 and mGlu5 has also been identified in non-neuronal cells, such as microglia (Byrnes *et al.*, 2009). Various splice variants for group I mGluR exist. Five splice variants have been found for mGlu1 (mGlu1a-e), and two splice variants for mGlu5 (mGlu5a, b) (reviewed by Niswender and Conn, 2010).

Group II mGluR comprise the receptors mGlu2 and mGlu3, which are believed to couple to a G_i protein. Therefore, receptor activation leads to inhibition of adenylyl cyclase, thereby decreasing production of the second messenger cyclic adenosine monophosphate (cAMP) (Conn and Pin, 1997). Group II mGluR have been identified both pre- and post-synaptically in neurons, where their activation leads to a decrease in neuronal excitation and neurotransmitter release, respectively. However, *in vitro* studies have shown microglia are also able to express mGlu2 and mGlu3 (Taylor *et al.*, 2002). Splice variants have also been identified for group II mGluR, though these correspond only to mGlu3. The most abundant mGlu3 splice variant (missing exon 4) results in a truncated receptor lacking a heptahelical transmembrane domain. Interestingly however, the truncated splice

variant still appears membrane bound, possibly through interaction with full length mGluR (Sartorius *et al.*, 2006), therefore may elicit intracellular signalling upon ligand binding.

Similarly to group II receptors, group III receptors are believed to couple to a G_i protein, and hence their activation generally leads to decreased cAMP production. Group III mGluR comprise mGlu4, mGlu6, mGlu7 and mGlu8, all of which have been found in the brain parenchyma, with the exception of mGlu6, which is thought to be primarily expressed in the retina (Hartveit *et al.*, 1995; Nakajima *et al.*, 1993). However, *in vitro* studies have suggested microglia derived from rat brains express mRNA and protein for all group III mGluR, with the exception of mGlu7 whose expression (both mRNA and protein) was not detected (Taylor *et al.*, 2003).

Two isoforms for mGlu4 have been convincingly identified: the full length receptor (mGlu4a) and an N-terminal truncated version only expressed in the taste buds (taste mGlu4) (Chauhari *et al.*, 2000).

Three mGlu6 splice variants have been identified in the rat and human retina (mGlu6a-c) (Valerio *et al.*, 2001). Interestingly, mGlu6b and mGlu6c appear to be truncated proteins consisting of only the extracellular N-terminal. Therefore, their expression has been suggested to lead to the generation of soluble mGlu6 receptors which might act as dummy glutamate receptors.

Of the four group III receptors, mGlu7 seems to undergo the most varied splicing, with 5 splice variants having been identified to date (mGlu7a-e) (Schulz *et al.*, 2002). As with group I mGluR, all mGlu7 splice variants differ in their intracellular C-terminal, therefore may confer different protein interaction characteristics to mGlu7. However, the exact biological role of mGlu7 isoforms has not been established.

Three splice variants have been identified for mGlu8, one of which (mGlu8c) translates only into the N-terminal domain. Therefore, as with other N-terminal truncated mGluR, mGlu8c has been suggested to act as a soluble glutamate receptor and hence decrease glutamatergic signalling (reviewed by Niswender and Conn, 2010).

In order to demonstrate that N9 microglia were a suitable cell line to study mGluR signalling, this chapter sets out to establish the expression of mGluR by N9 microglia. Furthermore, this chapter will compare mGluR expression in N9 microglia with primary microglia mGluR expression to corroborate the suitability of the N9 cell line. Finally, the *in vivo* expression of group III mGluR will be examined to establish receptor expression in two key brain regions involved in PD, namely the substantia nigra pars compacta (SNc) and the striatum.

- **Hypothesis:** Primary and N9 microglia express metabotropic glutamate receptors *in vitro*, and their expression pattern is replicated *in vivo*.

4.2 Experimental design

Metabotropic glutamate receptor expression was established through immunocytochemical (ICC) staining of fixed N9 and primary microglia, immunolabelling of Western blots of microglial lysates, and immunohistochemical (IHC) staining of fixed rat brain tissue.

To carry out ICC labelling, N9 and primary microglia were plated onto sterile glass cover slips in 24 well plates (20 or 90 x 10³ c/w, respectively). After an initial 24 hour recovery period to allow microglia to readopt a resting morphology, cells were fixed with 4% PFA, incubated with primary anti-mGluR antibodies and tagged with fluorescent dye-tagged secondary antibodies (see **table 4.1** and sections 2.1.20 and 2.1.21). Finally, the cover slips were placed on glass microscope slides and stored in the dark at 4°C. Negative control staining for unspecific secondary antibody binding was performed by omitting primary antibody incubation from the staining protocol. Stained cells were visualized under a fluorescent microscope and images captured at the same exposure (except detailed 40x images which were captured at varying exposures) using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera. Images are representative of

three or two independent experiments for N9 microglia and primary microglia immunolabelling, respectively.

Western blots of N9 microglia were employed to corroborate group III mGluR expression detected by ICC. Twenty four hours following plating, N9 microglia were lysed with RIPA buffer (containing protease inhibitors) on ice and whole cell lysates (60 µg protein for all experiments) resolved through SDS-PAGE. After transferring resolved proteins onto a PVDF membrane (see section 2.1.19), the membrane was stained with anti-mGluR antibodies (see **table 4.2**) and the labelled protein visualized by enhanced chemilumescence detection. As a positive control, rat brain lysates were ran in parallel.

Table 4.1 Antibodies employed during immunocytochemical labelling of N9 and primary microglia and immunohistochemical labelling of rat brain tissue. (Gt, goat; Rb, rabbit; Rt, rat; Ms, mouse; Mk, monkey; H, human)
(Concentrations employed for immunohistochemistry are shown in parentheses).

Target	Antibody type	Antigen	Target species	[Antibody]	Company	2° antibody conjugate	Host species	[Antibody]	Company
mGlu 1a	Rb Polyclonal	C-terminal peptide	Mk, Rt, H	10 µg/mL	Millipore (Ab1551)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 5	Rb Polyclonal	C-terminal peptide	Rb, Rt, H	10 µg/mL	Abcam (Ab27190)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 2	Rb Polyclonal	Human recombinant protein	Ms, Rt, H	10 µg/mL	Abcam (Ab13192)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 3	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	10 µg/mL	Millipore (Ab9449)	Alexa-488	Gt	2 µg/mL	Invitrogen (A-11034)
mGlu 4	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	10 µg/mL (5 µg/mL)	Abcam (ab53088)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
mGlu 6	Rb Polyclonal	C-terminal peptide	Rt	10 µg/mL (5 µg/mL)	Abcam (ab10314)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
mGlu 7	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	(5 µg/mL)	Abcam (Ab53705)	Alexa-488	Gt	(2 µg/mL)	Invitrogen (A-11034)
	Gt Polyclonal	N-terminal peptide	Ms, Rt, H	10 µg/mL	SantaCruz (sc-47156)	Alexa-488	Rb	2 µg/mL	Invitrogen (A-21222)
mGlu 8	Rb Polyclonal	Synthetic human peptide	Ms, Rt, H	10 µg/mL (5 µg/mL)	Abcam (Ab53094)	Alexa-488	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A-11034)
CD11b (Ox-42)	Ms Monoclonal	Protein purified from rat peritoneal macrophages	Rt	4 µg/mL (4 µg/mL)	Serotec (MCA275G)	Cy-3 red	Gt	2 µg/mL (2 µg/mL)	Invitrogen (A10521)
TH	Ms Monoclonal	Purified rat protein	Ms, Rt, H	(1:250)	Millipore (MAB318)	Cy-3 red	Gt	2 µg/mL	Invitrogen (A10521)

In vivo mGluR expression was examined through IHC staining of fixed adult rat tissue (see section 2.2.2 and 2.2.3). Terminally anaesthetized rats were transcardially perfused with 4% PFA, and the brain dissected out and post-fixed (4 hours with 4% PFA). After cryoprotection, the brains were snap frozen in isopentane and stored at -80°C. Using a cryostat, brains were sectioned into 20 µm thick slices and sections spanning the SNc or the striatum were serially collected into ice cold PBS in 24 well plates. The slices were stained with primary antibodies and fluorescent dye-tagged secondary antibodies (see **table 4.1**) and mounted onto glass microscope slides protected from light. Labelled tissue was visualized under a fluorescence microscope and images captured with a Q-imaging Q-CAM camera. Images are representative of immunolabelled tissue obtained from four rats.

Table 4.2 Antibodies employed during immunolabelling of Western blots of N9 cell lysate. (Gt, goat; Rb, rabbit; Rt, rat; Ms, mouse; H, Human)

Target	Antibody type	Antigen	Target species	[Antibody]	Company	2° antibody conjugate	Host species	[Antibody]	Company
mGlu 4	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	2 µg/mL	Abcam (Ab53088)	Horse radish peroxidase(HRP)	Gt	0.2 µg/mL	Vector labs (P-1000)
mGlu 7	Rb Polyclonal	C-terminal peptide	Ms, Rt, H	2 µg/mL	Abcam (Ab53705)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)
mGlu 8	Rb Polyclonal	Synthetic human peptide	Ms, Rt, H	2 µg/mL	Abcam (Ab53094)	HRP	Gt	0.2 µg/mL	Vector labs (P-1000)

4.3 Results

4.3.1 *In vitro* metabotropic glutamate receptor expression by microglia

4.3.1.1 *Expression of group I, II and III mGluR by N9 microglia*

Immunolabelling of fixed N9 microglia revealed positive reactivity against mGlu1 (**fig. 4.1A**). High magnification images revealed mGlu1 immunoreactivity was present throughout the cell. However, interestingly, staining was not homogenous, but had a grainy quality (**fig. 4.1B**). Similarly, immuno-labelling fixed N9 microglia revealed positive reactivity for mGlu5 (**fig. 4.1C**), with mGlu5 immunoreactivity not homogeneously distributed, but having a punctate distribution pattern more pronounced than mGlu1 staining (**fig. 4.1D**).

Immunostaining revealed positive reactivity for mGlu2 (**fig. 4.2A**) and mGlu3 (**fig. 4.2C**) receptors, which had moderate intensity for both receptors. High magnification images revealed both mGlu2 and mGlu3 receptors were homogeneously distributed throughout the cell membrane (**fig. 4.2B, D, respectively**).

Immunolabelling revealed N9 microglia express detectable levels of most group III mGluR. Hence, N9 microglia were shown to express modest levels of mGlu4 (**fig. 4.3A**). Similarly to group II receptors, high magnification images revealed mGlu4 reactivity to be homogeneously distributed through the cell membrane (**fig. 4.3B**). Interestingly, immunolabelling against mGlu6 revealed significant level of mGlu6 expression in N9 microglia (**fig. 4.3C**), which was similarly homogeneously distributed through the cell membrane (**fig. 4.3D**). N9 microglia were also immunopositive for mGlu7 (**Fig. 4.4A**). Intriguingly however, mGlu7 immunoreactivity appeared to be restricted to the nucleus, with only faint staining of the cell body (**fig. 4.4B**). The apparent nuclear localization of mGlu7 was further supported by the close co-localization with the nuclear stain DAPI (**fig. 4.4D**). While immunolabelling of N9 microglia revealed positive reactivity against mGlu8 (**fig. 4.4E**), this was only marginally above negative control reactivity (**fig. 4.4G**).

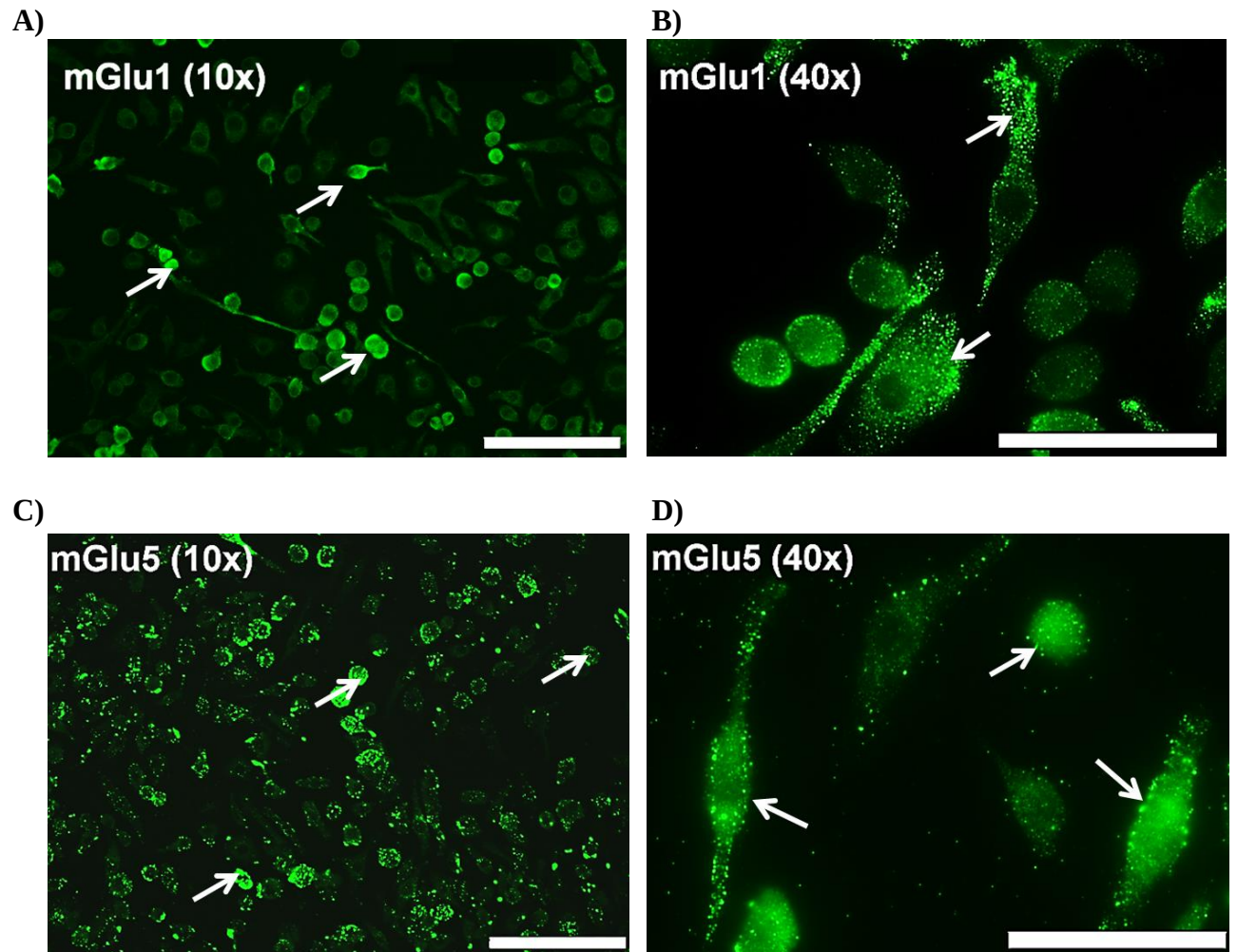


Figure 4.1 Group I mGluR immunoreactivity in N9 microglia. N9 microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were incubated overnight with primary anti-mGlu1 (A, B) or anti-mGlu5 (C, D) primary antibodies and tagged with secondary Alexa 488-conjugated antibodies. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of three independent experiments. Scale bars = 250 μm (A, C) or 100 μm (B, D). Arrows in A, C indicate N9 microglia cells with positive mGluR immunoreactivity. Arrows in B, D indicate uneven granular distribution of mGluR immunoreactivity in N9 microglia.

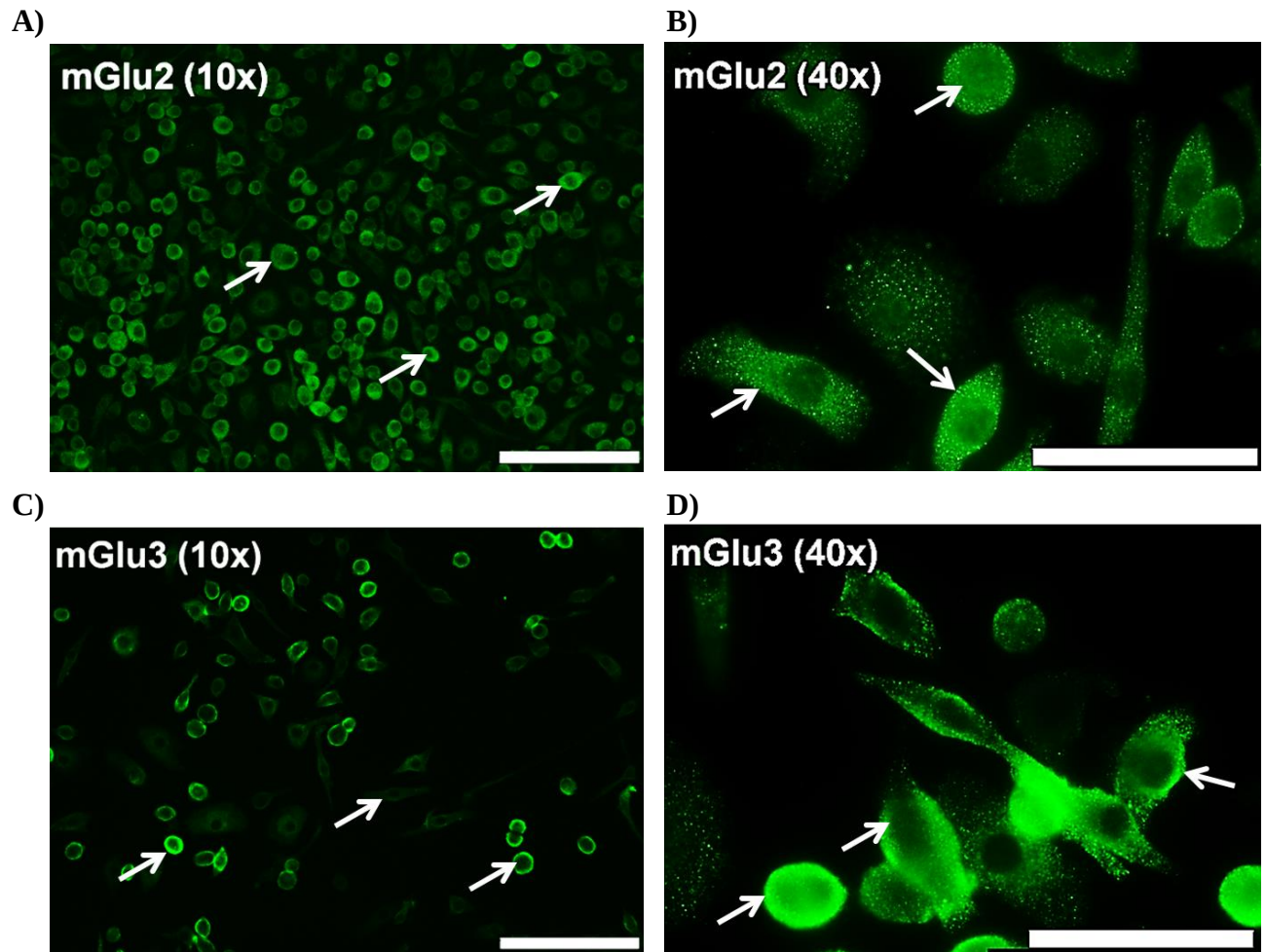


Figure 4.2 Group II mGluR immunoreactivity in N9 microglia. N9 microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were incubated overnight with anti-mGlu2 (**A**, **B**) or anti-mGlu3 (**C**, **D**) primary antibodies and tagged with secondary Alexa 488-conjugated antibodies. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of three independent experiments. Scale bars = 250 μm (**A**, **C**) or 100 μm (**B**, **D**). Arrows in A, C indicate N9 microglia cells with positive mGluR immunoreactivity. Arrows in B, D indicate homogenous distribution of mGluR immunoreactivity in N9 microglia.

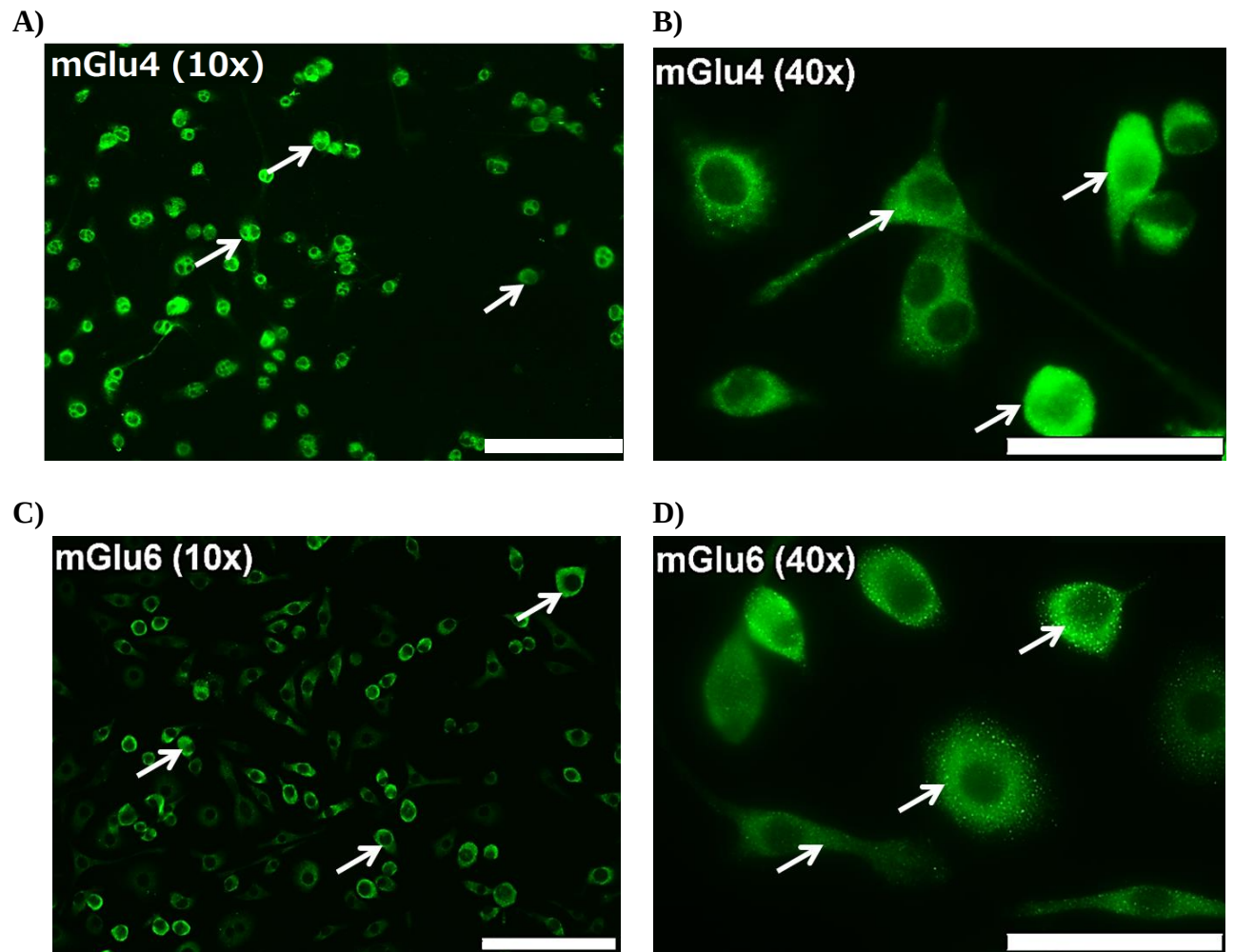


Figure 4.3 mGlu4 and mGlu6 immunoreactivity in N9 microglia N9 microglia were plated onto glass coverslips (20,000 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with anti-mGlu4 (A, B) or anti-mGlu6 (C, D) primary antibodies and tagged with secondary Alexa 488-conjugated antibodies. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of three independent experiments. Scale bars = 250 μm (A,C) or 100 μm (B, D). Arrows in A, C indicate N9 microglia cells with positive mGluR immunoreactivity. Arrows in B, D indicate homogenous distribution of mGluR immunoreactivity in N9 microglia

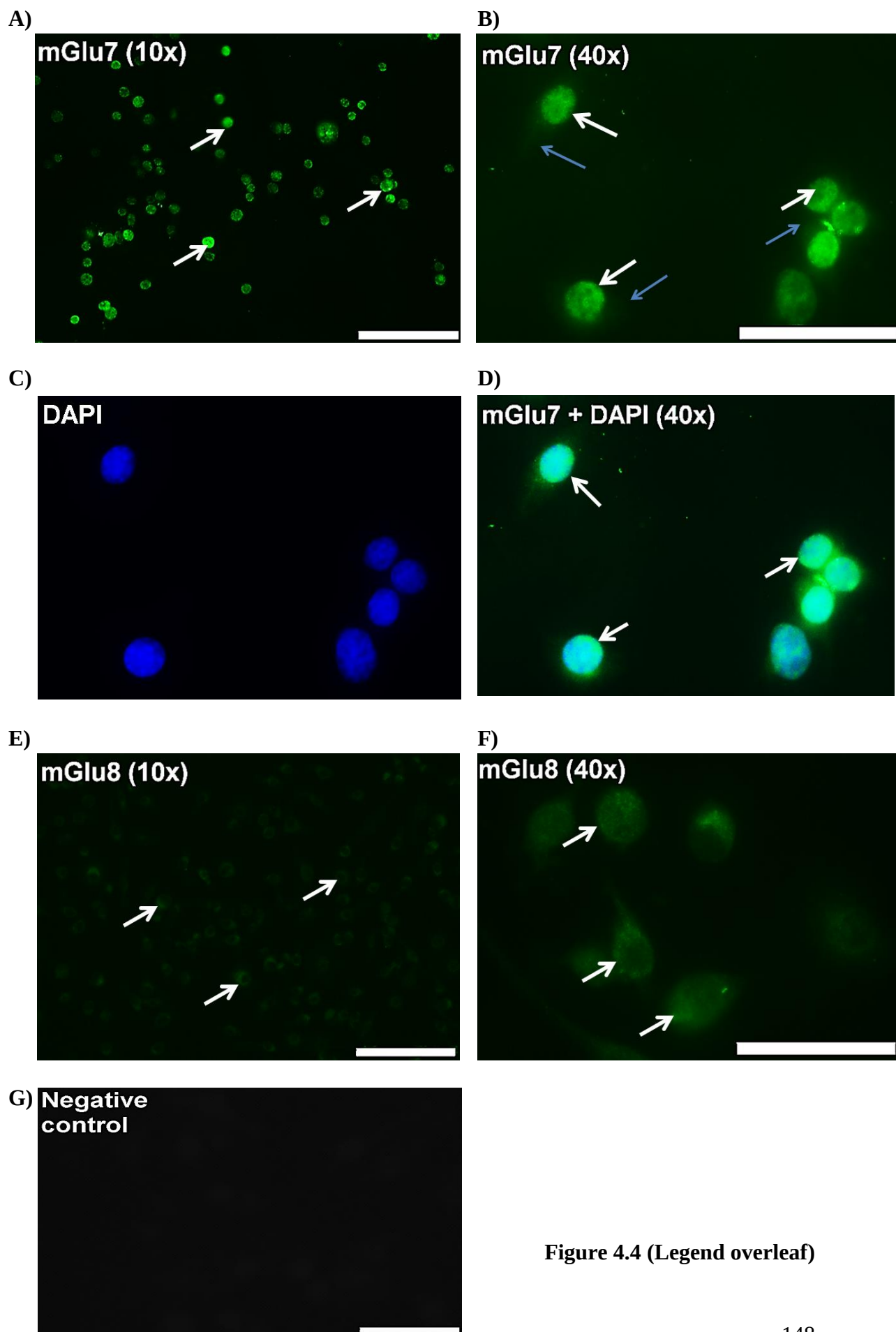


Figure 4.4 (Legend overleaf)

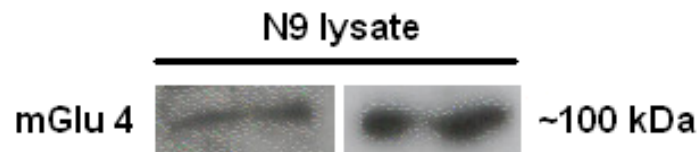
Immunocytochemical detection of group III mGluR expression by N9 microglia was corroborated by immunolabelling of Western blots from N9 microglia lysates. Incubation of Western blots with an anti-mGlu4 antibody revealed a prominent band at the predicted molecular weight (100 kDa) (**fig. 4.5A**). Similarly, a prominent band was detected at 100 kDa when employing an anti-mGlu7 antibody (**fig. 4.5B**). In addition, rat brain lysates resolved in parallel revealed a prominent band at the same molecular weight when labelled with the same anti-mGlu7 antibody (**fig. 4.5B**). However, no band was detected in Western blots incubated with anti-mGlu8 antibodies (**fig. 4.5C**).

4.3.1.2 Expression of mGluR by primary microglia

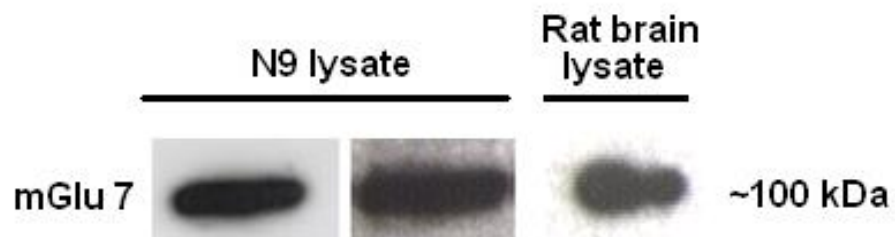
Expression mGluR by N9 microglia was confirmed in primary microglia through immunocytochemistry. Similarly to N9 microglia, immunolabelling of fixed primary microglia revealed positive reactivity for both mGlu1 and mGlu5 (**fig. 4.6A and 4.7A, respectively**). Immunolabelling intensity was comparable between the two receptors. High magnification images demonstrated mGlu1 and mGlu5 expression had a heterogenous distribution through the cell membrane (**fig. 4.6B and 4.7B**), though this was not as pronounced as in N9 microglia. Double staining of primary microglia with CD11b demonstrated the microglial purity of the cell culture to be close to 100% (**fig. 4.6C and 4.7C**), with close co-localization of mGluR immunoreactivity and CD11b immunoreactivity.

Figure 4.4 mGlu7 and mGlu8 immunoreactivity in N9 microglia N9 microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with anti-mGlu7 (**A-D**) or anti-mGlu8 (**E, F**) primary antibodies and tagged with secondary Alexa 488-conjugated antibodies. In addition, cells were labelled with DAPI to visualize nuclei. Negative controls were acquired through omission of primary body incubation (**G**). Blue arrows in (**B**) indicate cell body reactivity. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of three independent experiments. Scale bars = 250 μm (**A, E, G**) or 100 μm (**B, F**). White arrows in A, B indicate nuclear localization of mGlu7 immunoreactivity in N9 microglia. Blue arrows indicate mGlu7 immunoreactivity in the cell body. White arrows in D indicate colocalization of mGlu7 immunoreactivity and DAPI signal. White arrows in E, F indicate mGlu8 immunoreactivity.

A)



B)



C)



Figure 4.5 Immunodetection of mGluR in Western blots from N9 microglia lysates. N9 microglia whole cell lysates were resolved through SDS-PAGE and Western blotted onto PVDF membranes. Membranes were incubated overnight with primary antibodies targeting mGlu4 (**A**), mGlu7 (**B**), or mGlu8 (**C**) and tagged with horse radish peroxidase-tagged secondary antibodies. Immunoreactivity was determined through enhanced chemiluminescence. Rat brain lysates resolved in parallel served as positive control for mGlu7 expression. Images are from two independent experiments.

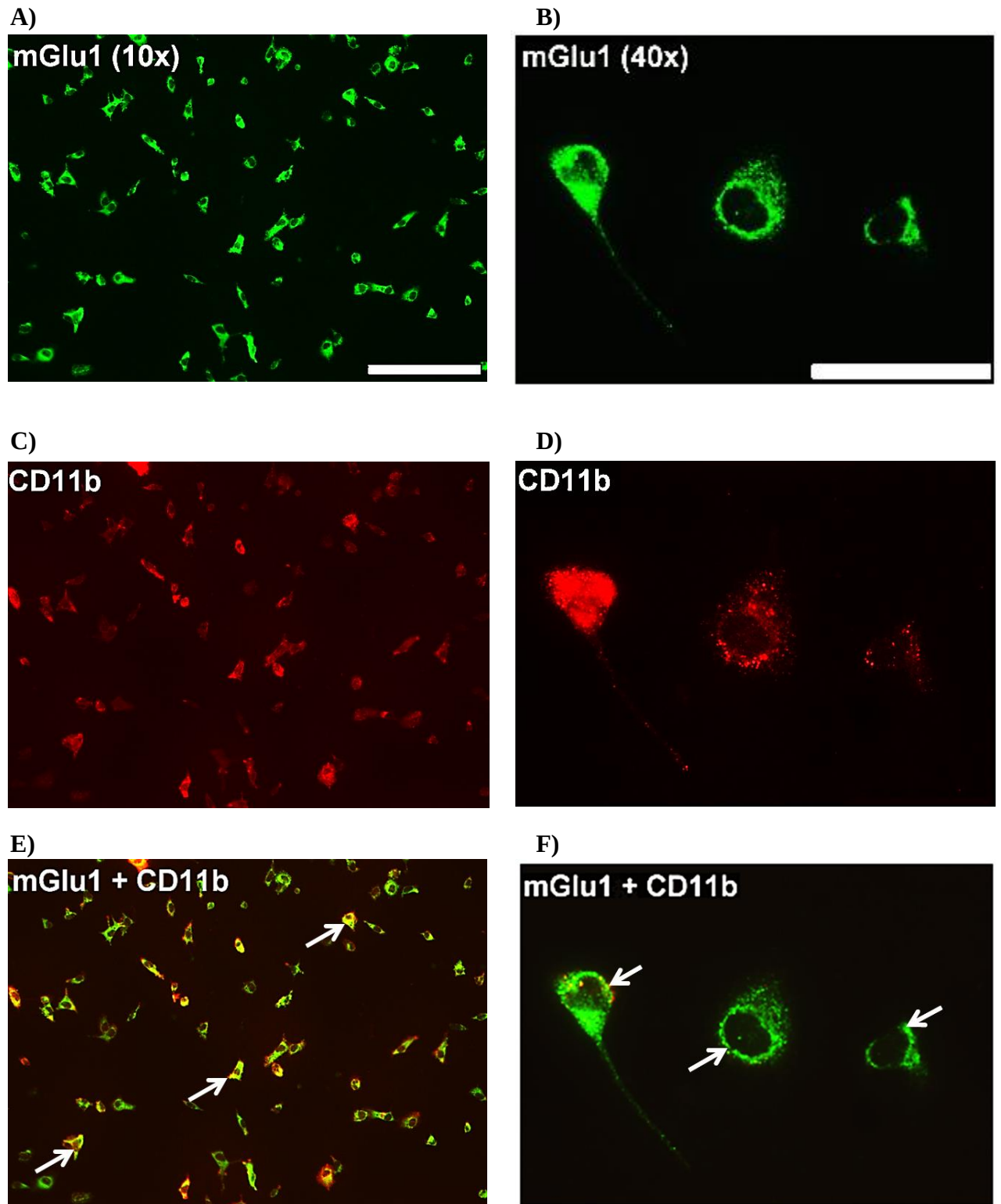


Figure 4.6 mGlu1 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were incubated overnight with primary anti-mGlu1 (A, B) and anti-CD11b (C, D) antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = $250 \mu\text{m}$ (A) or $100 \mu\text{m}$ (B). White arrows indicate mGluR and CD11b colocalization.

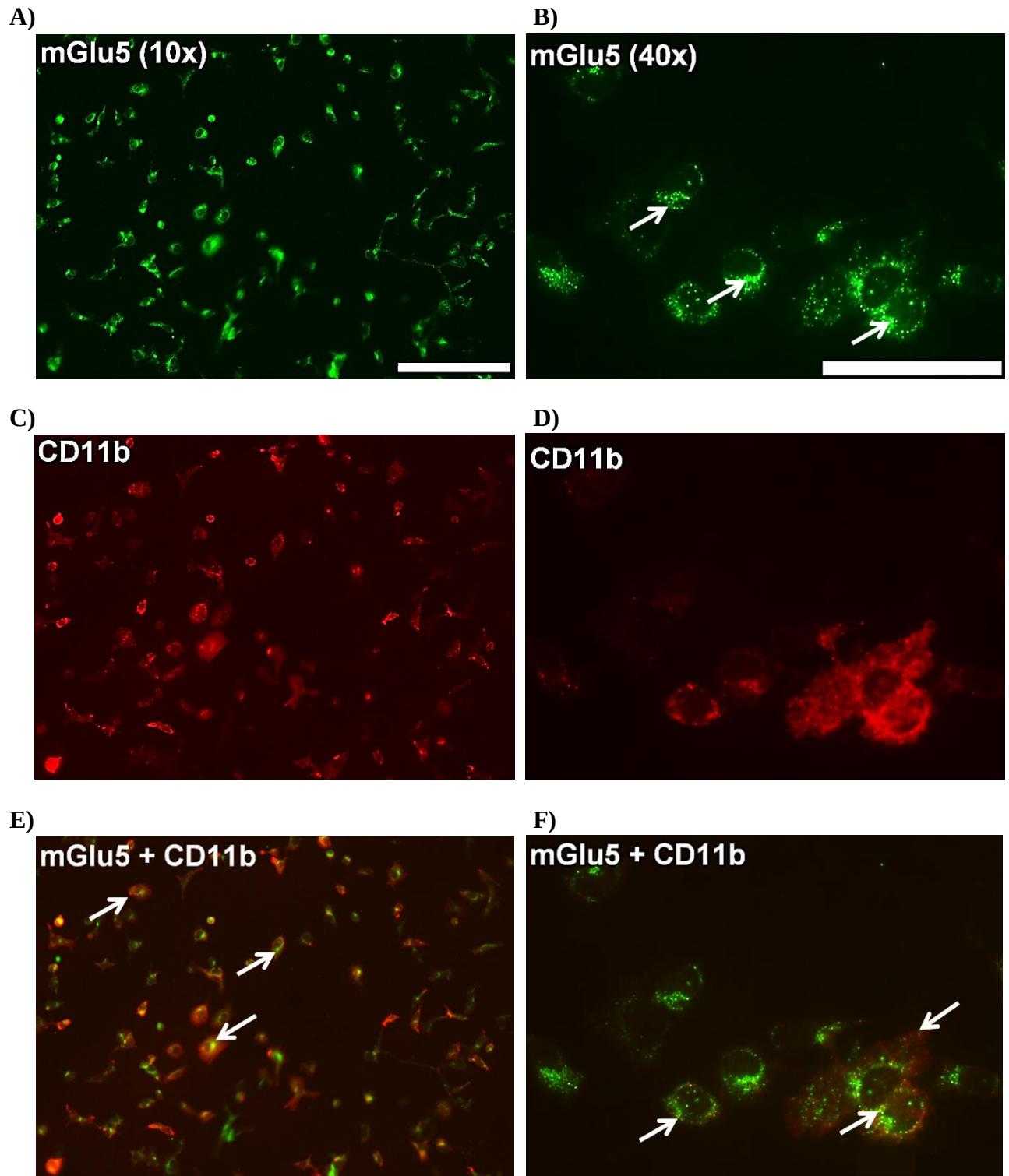


Figure 4.7 mGlu5 and CD11b expression in primary microglia Primary microglia were plated onto glass coverslips (20,000 cells per well) and fixed with 4% PFA. Cells were incubated overnight with primary anti-mGlu5 (**A, B**) and anti-CD11b (**C, D**) antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (**A**) or 100 μm (**B**). White arrows in **B** indicate uneven punctate distribution of mGluR immunoreactivity. White arrows in **E, F** indicate mGluR and CD11b colocalization.

Immunolabelling demonstrated primary microglia also express protein for mGlu2 (**fig. 4.8A**) and mGlu3 (**fig. 4.9A**). Unlike N9 microglia, mGlu3 immunoreactivity in primary microglia was significantly stronger compared to mGlu2. In addition, high magnification images revealed that, while mGlu2 and mGlu3 expression was not as evenly distributed throughout the cell, their distribution pattern was not as punctate as mGlu5 (**fig. 4.8B and 4.9B, respectively**). Double staining with CD11b revealed all mGlu2 or mGlu3 positive cells also expressed CD11b (**fig. 4.8E and 4.9E, respectively**).

Expression of group III mGluR was also corroborated in primary microglia. Immunolabelling revealed primary microglia expressed protein for mGlu4 (**fig. 4.10A, B**). Although mGlu4 immunoreactivity was low, it was still above negative control immunoreactivity (**fig.4.13A**). Similarly, primary microglia were shown to be positively immunoreactive for mGlu6 protein (**fig. 4.11A, B**), comparable to that seen in N9 microglia. Interestingly, mGlu7 immunoreactivity in primary microglia was strikingly intense (**fig. 4.12A**), comparable to that seen for mGlu3. However, unlike N9 microglia, mGlu7 immunoreactivity in primary microglia was not restricted to the nucleus, but was unevenly distributed throughout the cell (**fig. 4.12B**). Although very faint immunoreactivity against mGlu8 was seen in primary microglia, it was still above negative control immunoreactivity (**Appendix 1**). All mGlu4, mGlu6 or mGlu7-positive cells were also positively immunolabelled with an antibody targeting CD11b (**fig. 4.10E, 4.11E, 4.12E, respectively**).

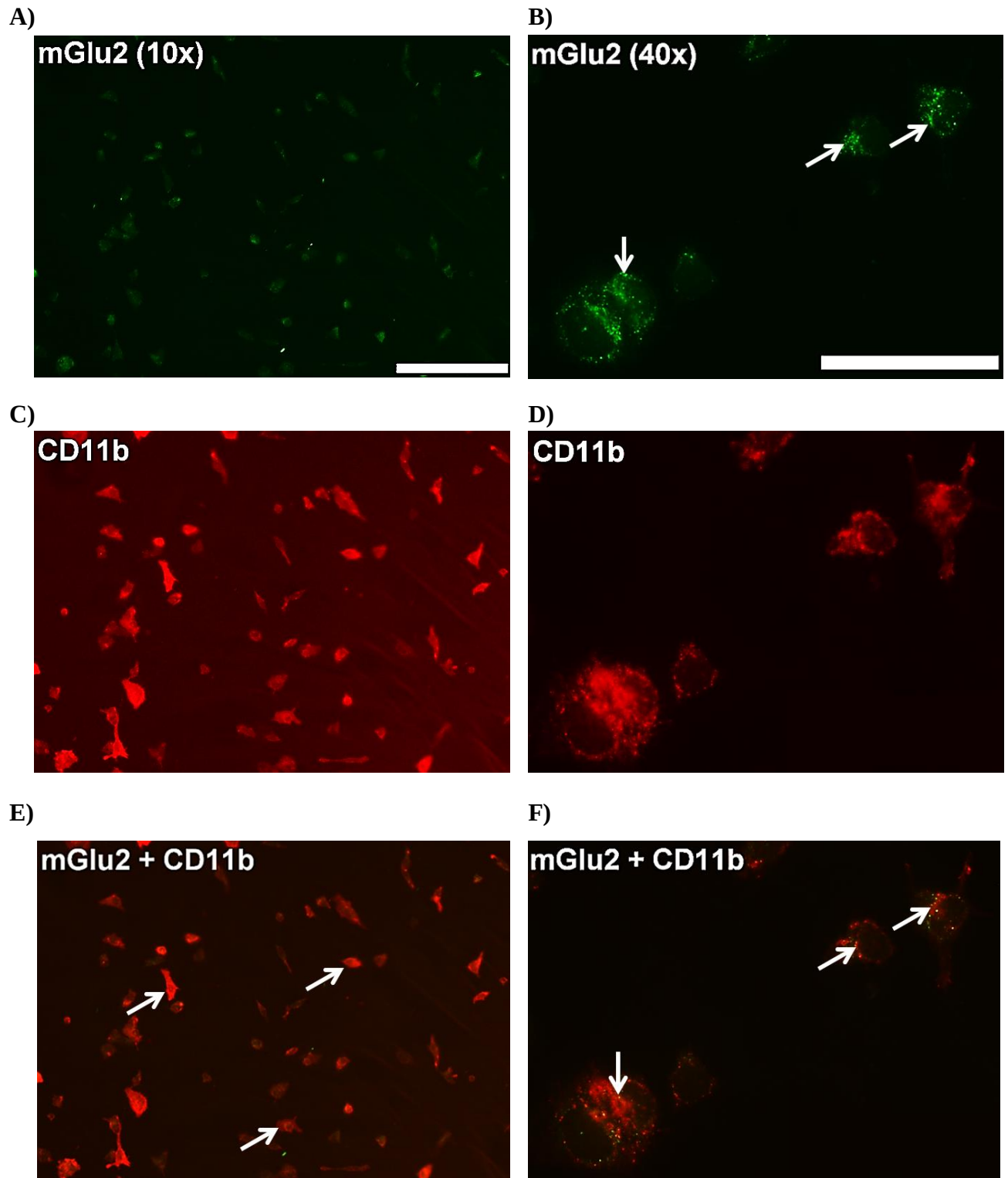


Figure 4.8 mGlu2 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with primary anti-mGlu2 (**A, B**) and anti-CD11b (**C, D**) antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μ m (**A**) or 100 μ m (**B**). White arrows in B indicate uneven distribution of mGluR immunoreactivity. White arrows in E, F indicate mGluR and CD11b colocalization.

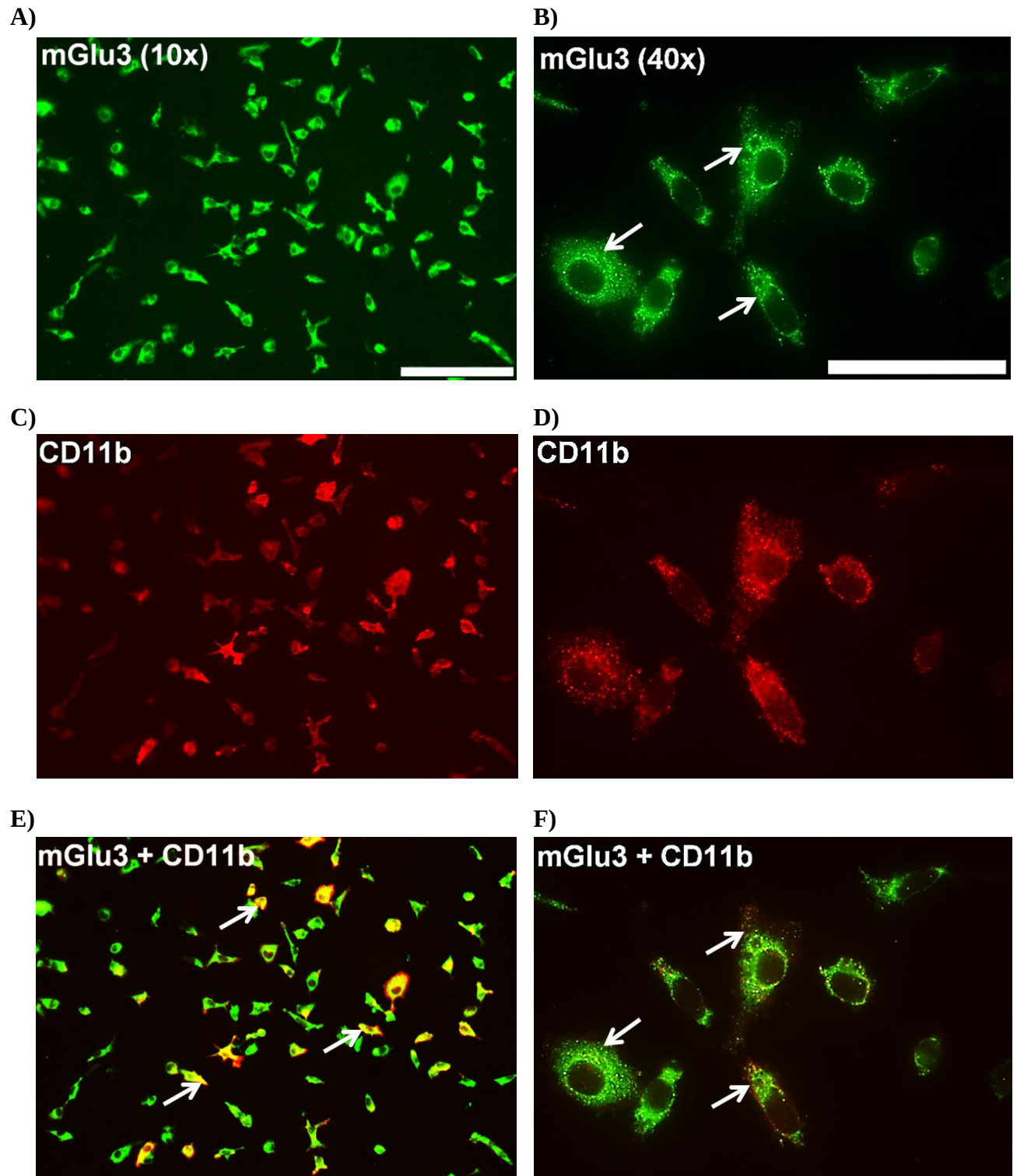


Figure 4.9 mGlu3 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with primary anti-mGlu3 (**A, B**) and anti-CD11b (**C, D**) antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (**A**) or 100 μm (**B**). White arrows in B indicate uneven distribution of mGluR immunoreactivity. White arrows in E, F indicate mGluR and CD11b colocalization.

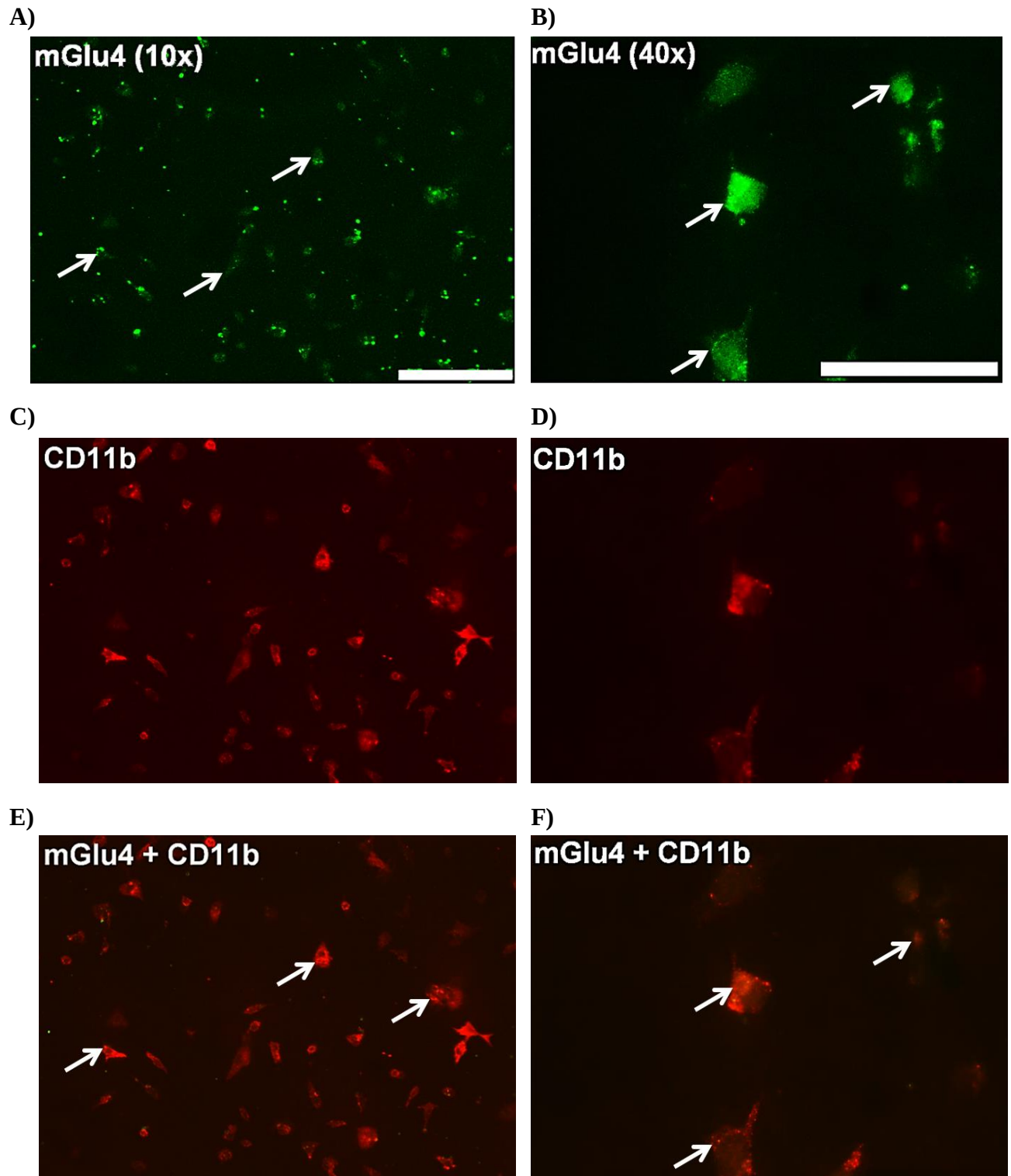


Figure 4.10 mGlu4 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with anti-mGlu4 (**A**, **B**) and anti-CD11b (**C**, **D**) primary antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (**A**) or 100 μm (**B**). White arrows in **A**, **B** indicate mGlu4 immunoreactivity. White arrows in **E**, **F** indicate colocalization of mGluR and CD11b immunoreactivity.

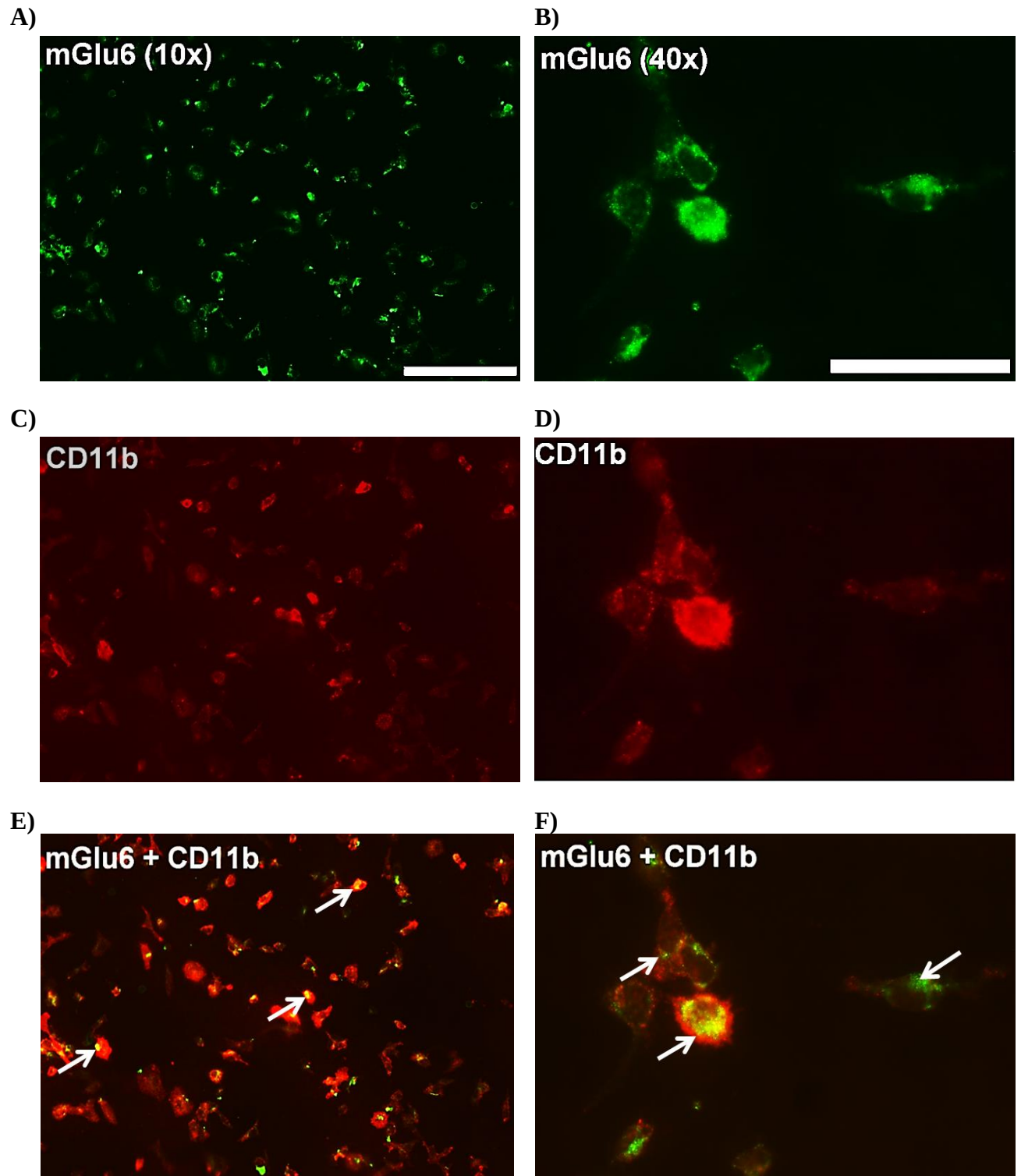


Figure 4.11 mGlu6 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with anti-mGlu6 (**A, B**) and anti-CD11b (**C, D**) primary antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (**A**) or 100 μm (**B**). White arrows indicate colocalization of mGluR and CD11b immunoreactivity.

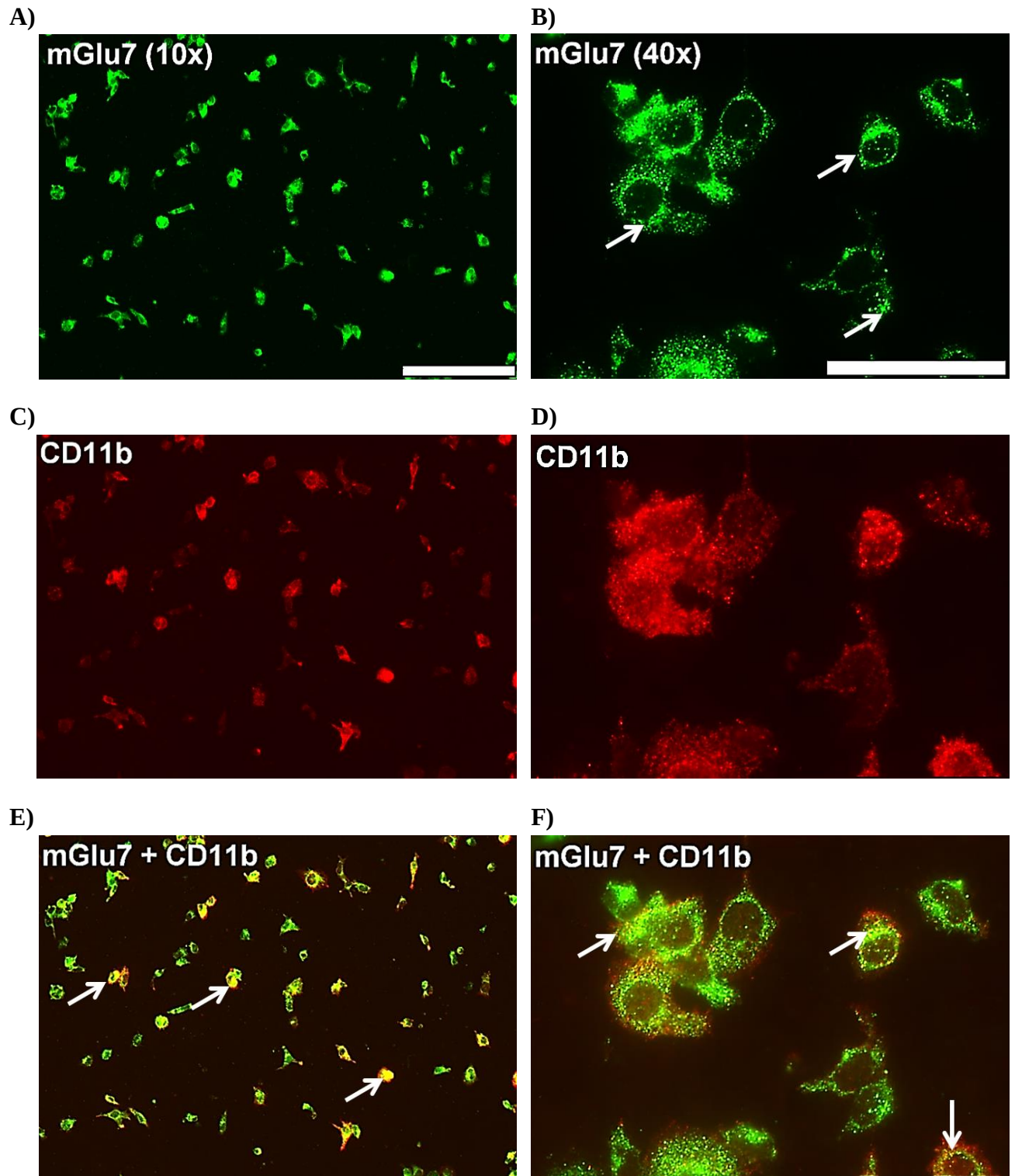


Figure 4.12 mGlu7 and CD11b expression in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with anti-mGlu7 (**A, B**) and anti-CD11b (**C, D**) primary antibodies and tagged with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies, respectively. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (**A**) or 100 μm (**B**). White arrows in B indicate uneven mGlu7 immunoreactivity through the cell body. White arrows in E, F indicate colocalization of mGlu7 and CD11b immunoreactivity.



Figure 4.13 Negative control staining in primary microglia. Primary microglia were plated onto glass coverslips (20×10^3 cells per well) and fixed with 4% PFA. Cells were then incubated with secondary Alexa 488-conjugated and Cy3-red-conjugated antibodies. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments. Scale bars = 250 μm (A) or 100 μm (B).

4.3.2 *In vivo* expression of group III mGluR

Immunolabelling of transcardially perfused rat brain tissue revealed positive reactivity for mGlu4 in the soma of cells within the SNc (**fig. 4.14A, B**). In addition, around half of the mGlu4-positive cells were also immunoreactive for tyrosine hydroxylase (TH), indicating a dopaminergic phenotype (**fig. 4.14E, F**). However, similar immunolabelling of the striatum (**fig. 4.15A**) failed to reveal immunoreactivity for mGlu4 above that seen in negative controls (**fig. 4.15B**).

Similarly, labelling with an anti-mGlu6 antibody revealed faint but discreet labelling of cell bodies within the SNc (**fig. 4.16A, B**), of which a proportion were also immunoreactive for TH (**fig. 4.16E, F**). Interestingly, though no cell bodies within the striatum were immunoreactive for mGlu6, there was intense immunoreactivity for mGlu6 on fibres surrounding striosomes (**fig. 4.17A, B**).

Immunolabelling of SNc tissue also revealed strong immunoreactivity for mGlu7 (**fig. 4.18A, B**). As with mGlu4, around half of the mGlu7-labelled cells were also immunoreactive for TH (**fig. 4.18E**). Interestingly, while TH reactivity covered the whole cell body, mGlu7 reactivity appeared to be restricted to the nucleus (**fig. 4.18F**). In addition to the SNc, striatal tissue also displayed strong mGlu7 immunoreactivity (**fig. 4.19A**), whose distribution was limited to cells surrounding striosomes. As with the SNc, mGlu7 immunoreactivity in the striatum appeared to be restricted to the nucleus, as assessed by the tight co-localization with reactivity for the nuclear stain DAPI (**fig. 4.19C**).

Immunoreactivity for mGlu8 was also seen in the SNc (**fig. 4.20A, B**). However, mGlu8 labelling was primarily seen in fibres crossing the SNc, with only faint and scarce labelling of cell bodies. Intriguingly, mGlu8-positive fibres appeared to cross the SNc only at places without TH immunoreactivity (**fig. 4.20E, F**). Immunoreactivity for mGlu8 was also detected in striatal

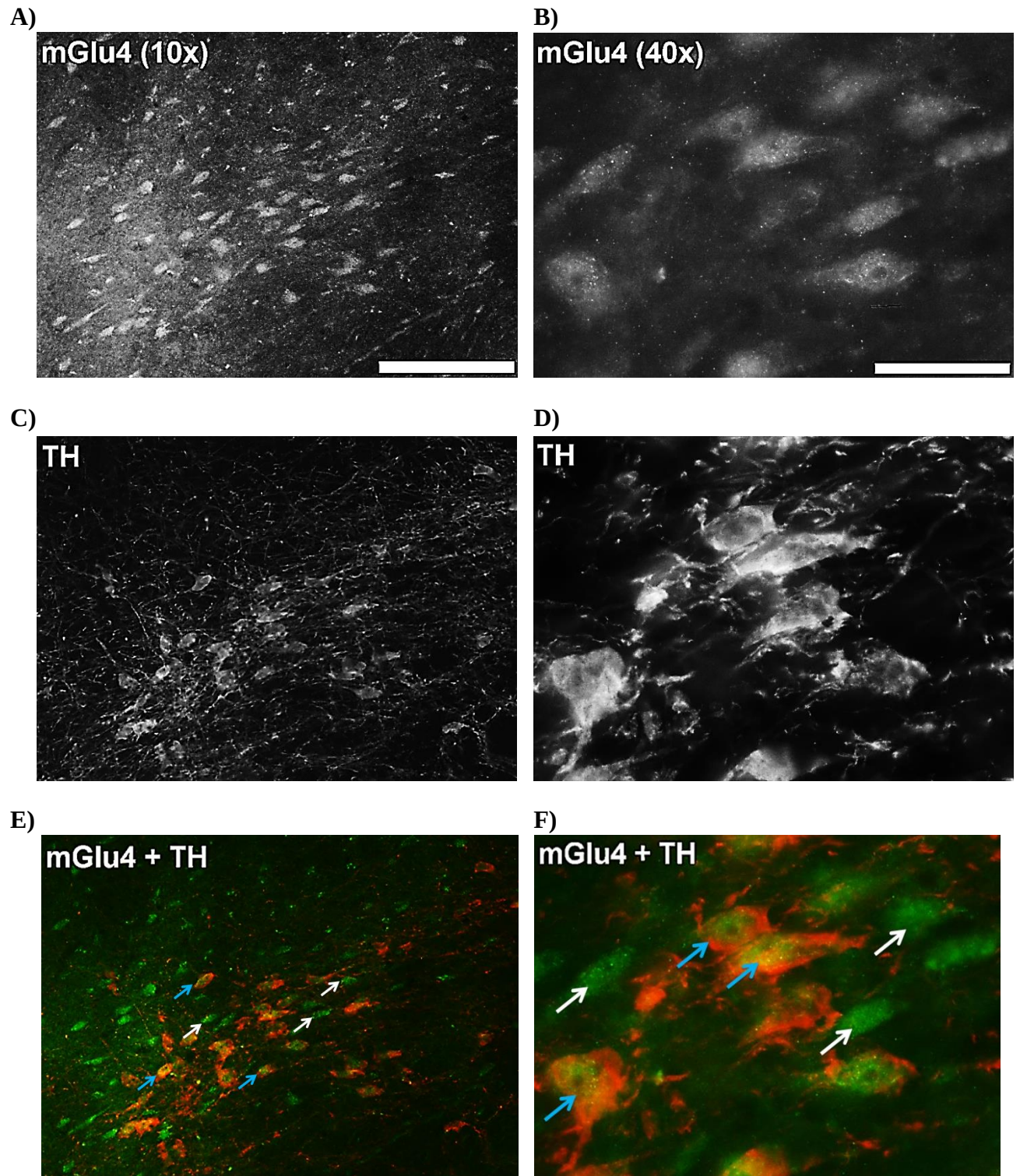


Figure 4.14 Identification of mGlu4 and TH expression in the rat substantia nigra. Fixed rat SNc slices (20 μm) were immunolabelled for mGlu4 (**A, B**) (green staining in **E, F**) and tyrosine hydroxylase (TH) (**C, D**) (red staining in **E, F**). Images were captured at 10x (**A, C, E**) or 40x (**B, D, F**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μm (10x) or 75 μm (40x). White arrows indicate cells with single mGlu4 immunoreactivity. Blue arrows indicate cellular colocalization of mGlu4 and TH immunoreactivity.

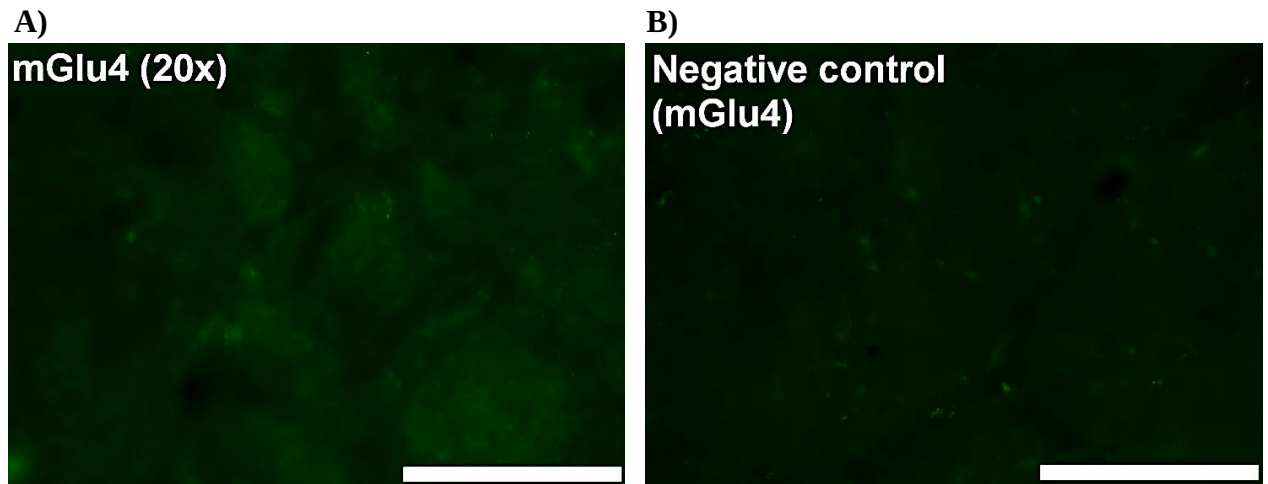


Figure 4.15 Identification of mGlu4 expression in the rat striatum. Fixed rat striatal slices (20 μm) were immunolabelled with a primary antibody targeting mGlu4 and tagged with an Alexa-488 conjugated secondary antibody (**A**). Negative control staining was obtained through omission of primary antibody incubation (**B**). No mGlu4 immunoreactivity was seen in the striatum above negative control. Images were captured at 20x magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 200 μm .

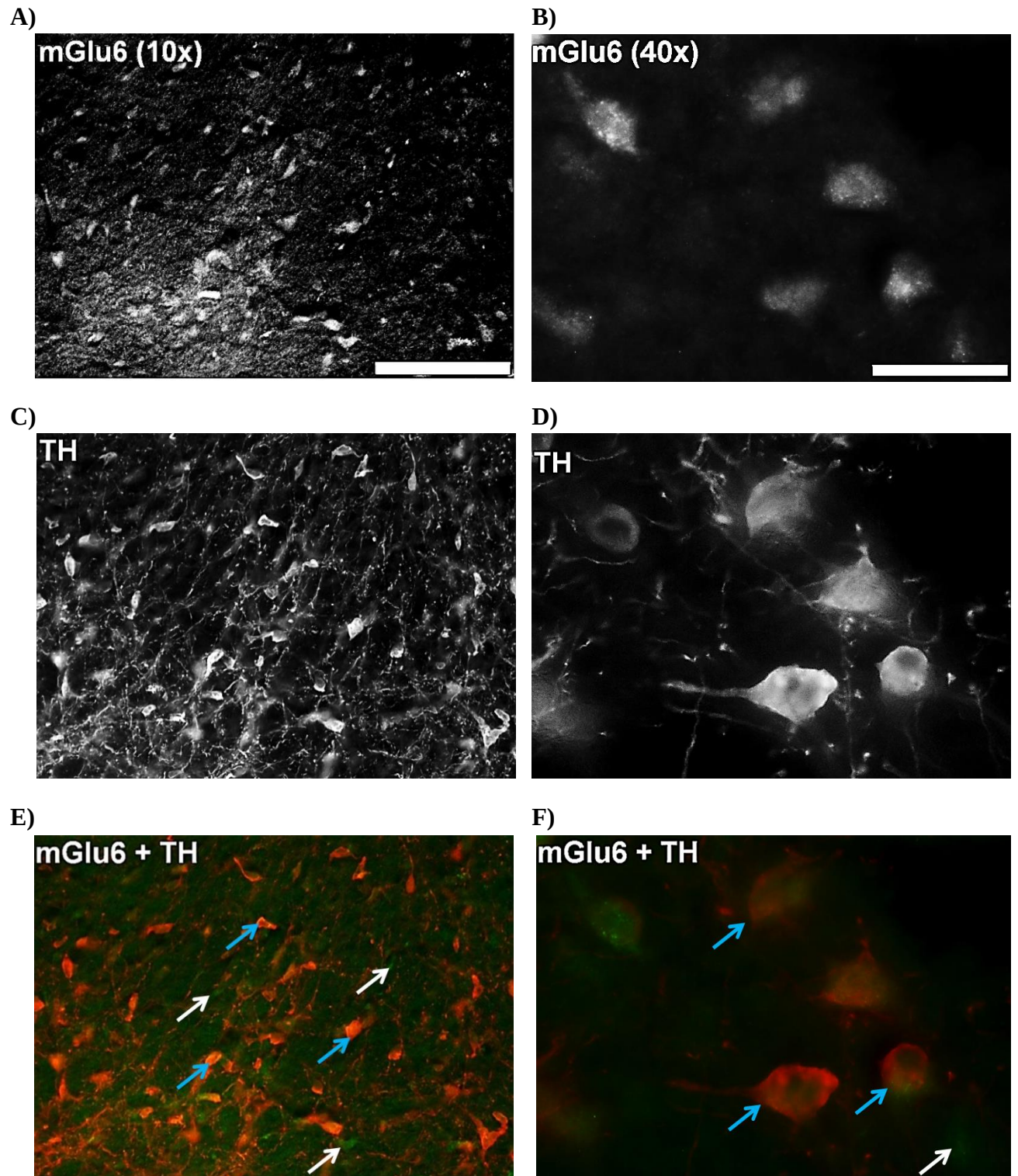


Figure 4.16 Identification of mGlu6 and TH expression in the rat substantia nigra. Fixed rat SNc slices (20 μ m) were immunolabelled for mGlu6 (**A, B**) (green staining in **E, F**) and tyrosine hydroxylase (**C, D**) (red staining in **E, F**). Images were captured at 10x (**A, C, E**) or 40x (**B, D, F**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μ m (10x) or 75 μ m (40x). White arrows indicate cells with single mGlu6 immunoreactivity. Blue arrows indicate cellular colocalization of mGlu6 and TH immunoreactivity.

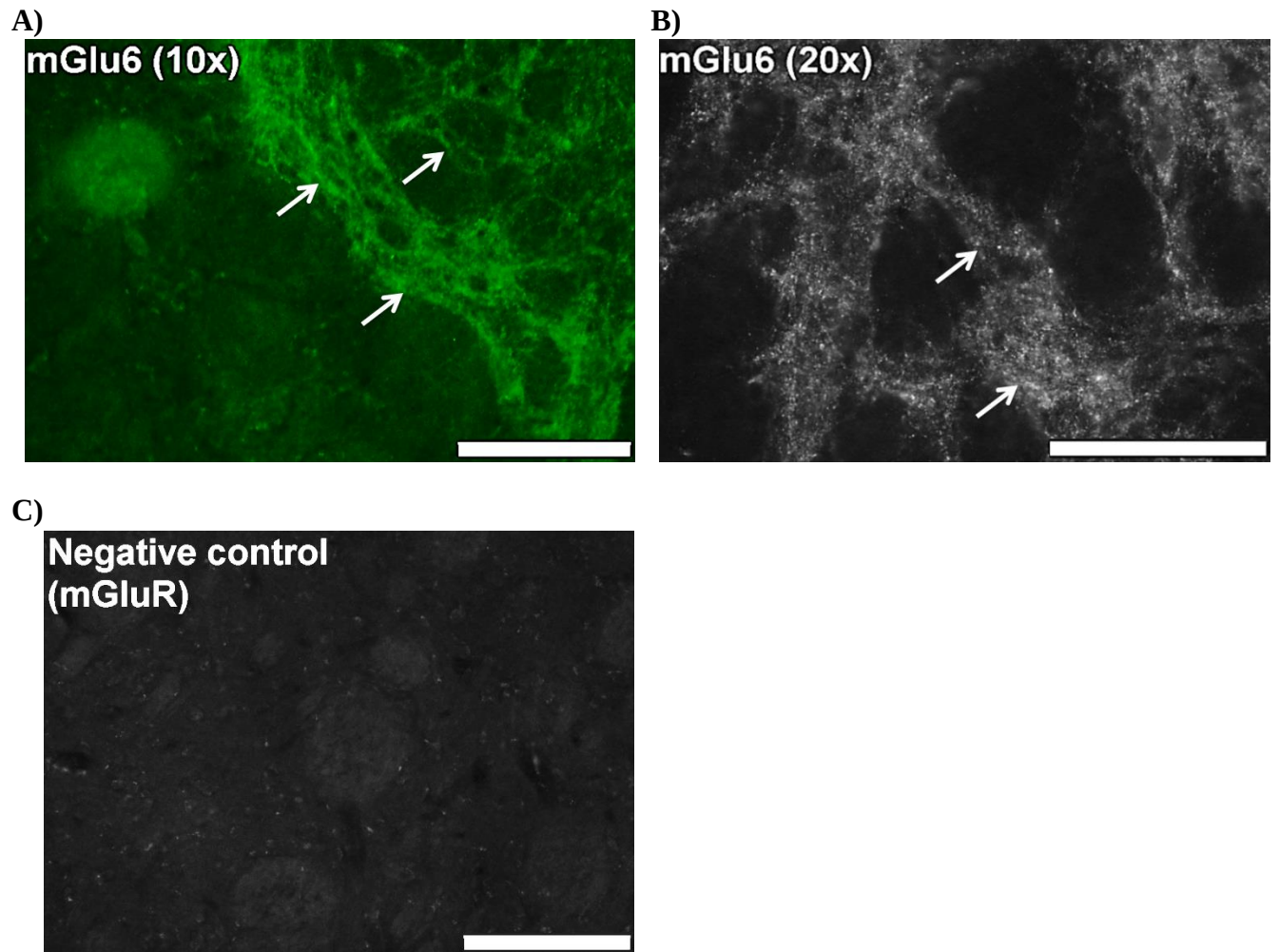


Figure 4.17 Identification of mGlu6 expression in the rat striatum. Fixed rat striatal slices (20 μm) were immunolabelled with a primary antibody targeting mGlu6 and tagged with an Alexa-488 conjugated secondary antibody (**A, B**). Negative control staining was obtained through omission of primary antibody incubation (**C**). Images were captured at 10x (**A, C**) or 20x (**B**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μm (10x) or 200 μm (20x). White arrows indicate mGlu6 immunoreactivity in striatal fibres surrounding striosomes.

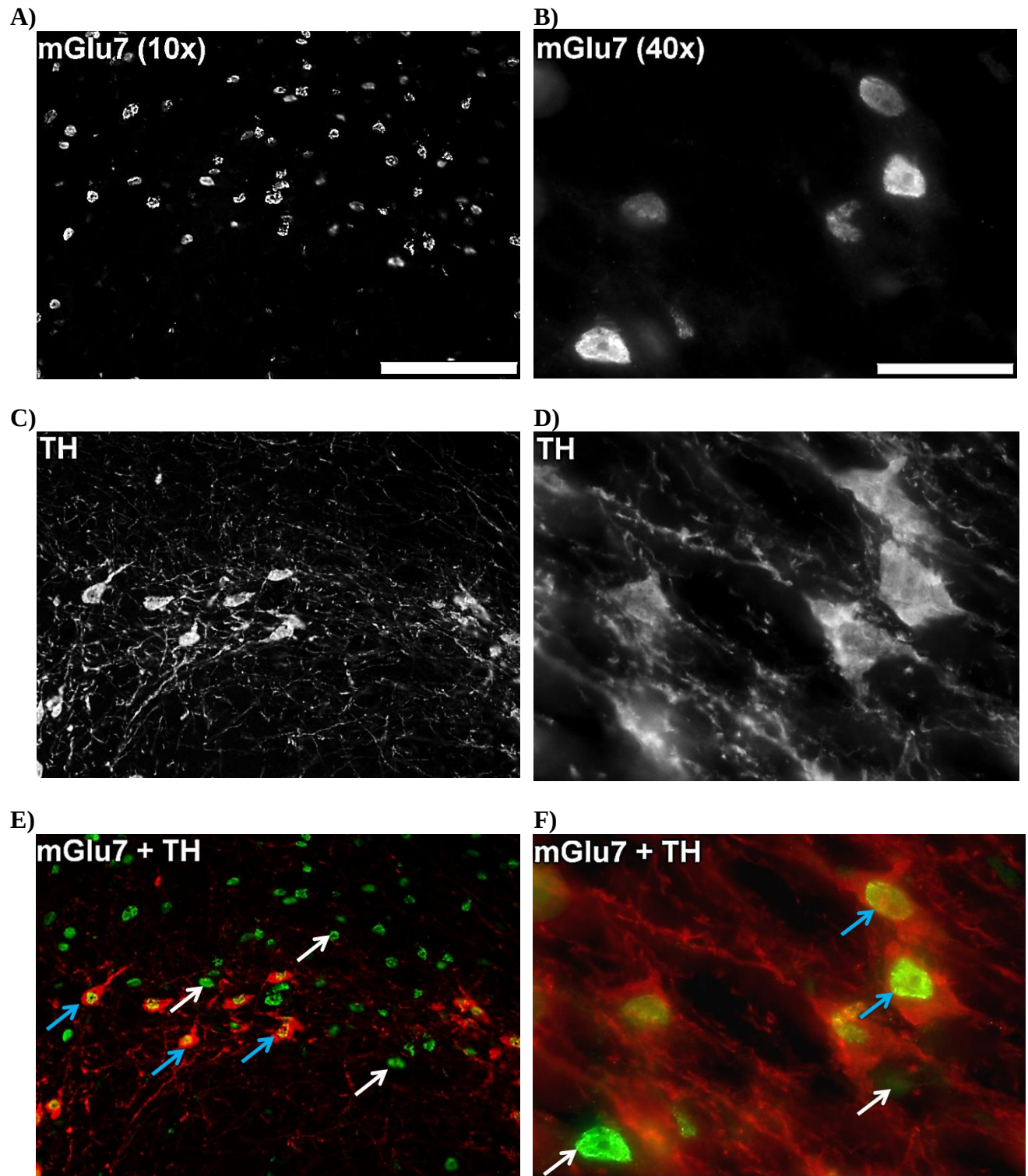


Figure 4.18 Identification of mGlu7 and TH expression in the rat substantia nigra. Fixed rat SNc slices (20 μm) were immunolabelled for mGlu7 (**A**, **B**) (green staining in **E**, **F**) and tyrosine hydroxylase (**C**, **D**) (red staining in **E**, **F**). Images were captured at 10x (**A**, **C**, **E**) or 40x (**B**, **D**, **F**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μm (10x) or 75 μm (40x). White arrows indicate cells with single mGlu7 immunoreactivity. Blue arrows indicate cellular colocalization of mGlu7 and TH immunoreactivity.

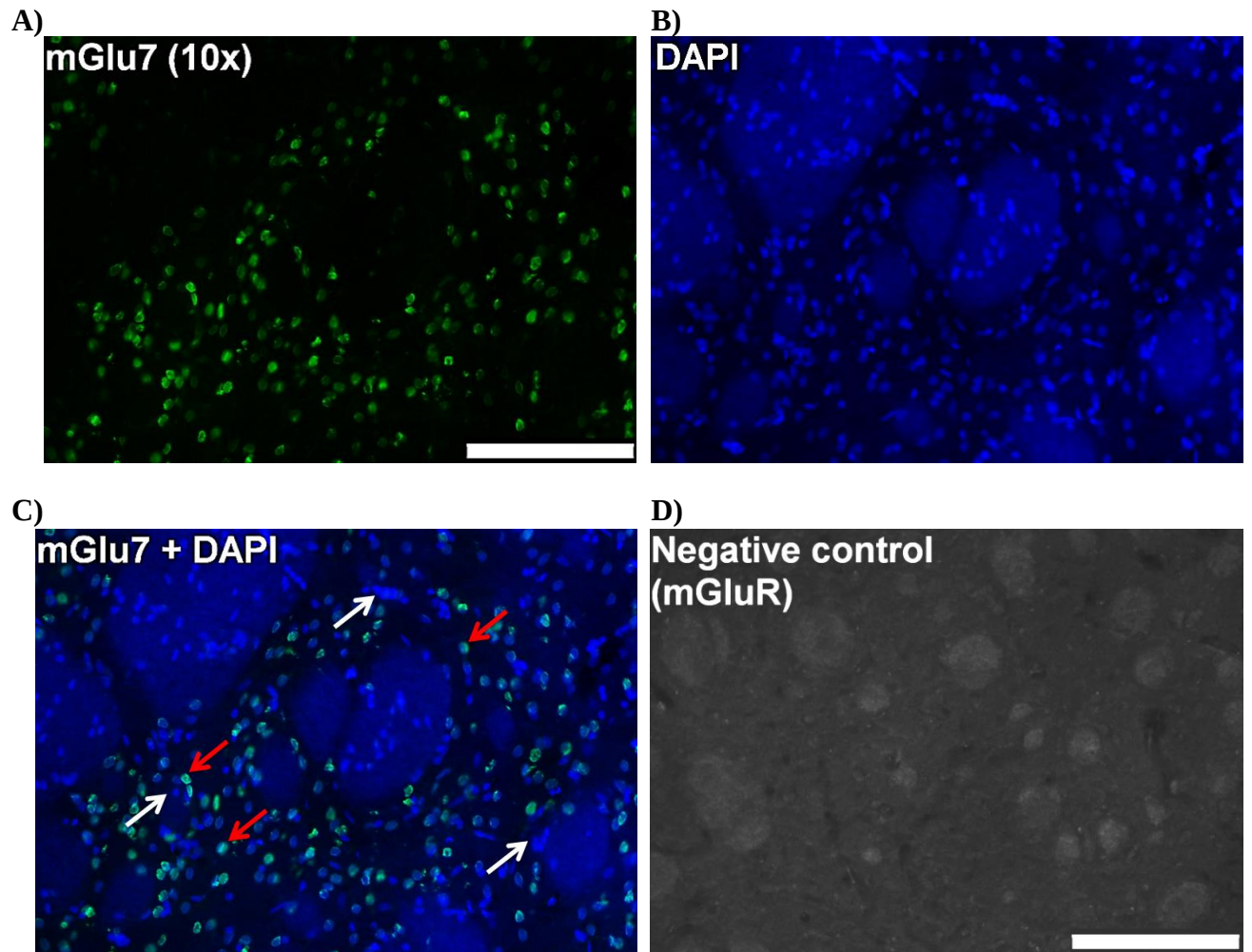


Figure 4.19 Identification of mGlu7 expression in the rat striatum. Fixed rat striatal slices (20 μm) were immunolabelled with a primary antibody targeting mGlu7 and tagged with an Alexa-488 conjugated secondary antibody (**A**, **C**). To visualize cell nuclei, tissue slices were also incubated with the nuclear stain DAPI (**B**, **C**). Negative control staining was obtained through omission of primary antibody incubation (**D**). Images were captured at 10x (**A**, **C**) or 20x (**B**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μm (10x). White arrows indicate cell nuclei with single DAPI fluorescent signal. Red arrows indicate cell nuclei with colocalization of DAPI signal and mGlu7 immunoreactivity.

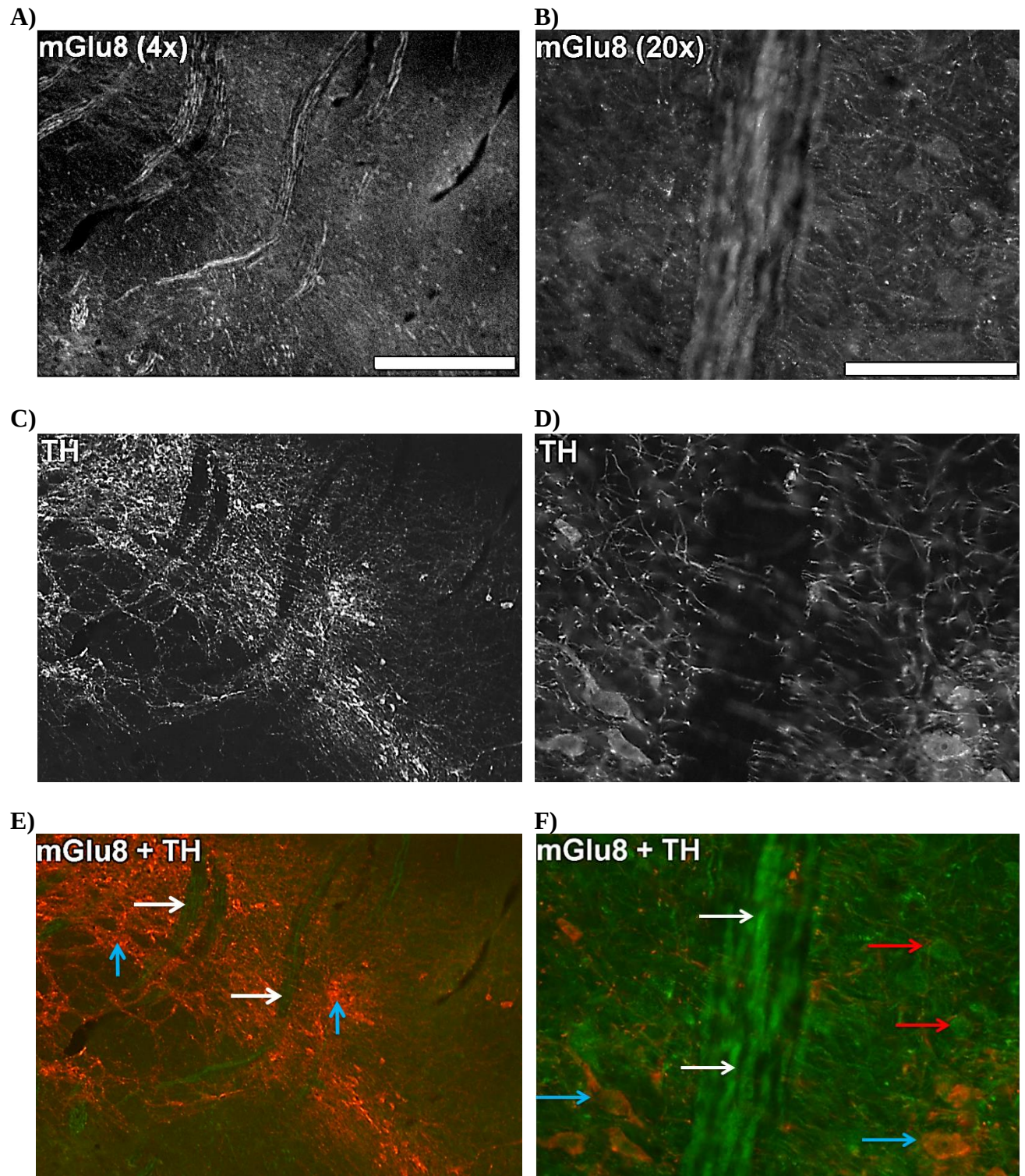


Figure 4.20 Identification of mGlu8 and TH expression in the rat substantia nigra. Fixed rat SNc slices (20 μ m) were immunolabelled for mGlu8 (**A, B**) (green staining in E, F) and tyrosine hydroxylase (**C, D**) (red staining in E, F). Images were captured at 10x (**A, C, E**) or 40x (**B, D, F**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 750 μ m (4x) or 200 μ m (20x). White and red arrows indicate fibres and cell bodies, respectively, with single mGlu8 immunoreactivity. Blue arrows indicate TH immunoreactivity.

slices (**fig. 4.21 A, B**). Unlike the SNc, mGlu8 reactivity in the striatum localized both to fibres and cell bodies. However, the mGlu8-positive fibres seemed to be restricted to striosomes, while mGlu8-positive cell bodies were found mainly surrounding striosomes. Intriguingly, the most clear and intense mGlu8 labelling was seen at the cortex of tissue slices spanning the SNc (**fig. 4.22A, C**) as well as the striatum (**fig. 4.22B, D**). Cortical mGlu8 immunoreactivity was restricted to cells with an oval-shaped cell body from which extended a single long process. The orientation of the cells appeared to be centrical, with cell bodies directed towards the centre of the tissue slice and the processes extending towards the periphery.

4.3.3 Microglial mGluR expression *in vivo*

Expression of mGluR by microglia *in vivo* was examined through double fluorescent labelling of rat brain tissue with antibodies targeting individual mGluR and the microglial marker CD11b. Both mGluR and microglia were clearly detected in labelled tissue slices. In addition, microglia displayed a highly ramified morphology with compact cell body. However, no co-localization between CD11b immunoreactivity and mGluR immunoreactivity was observed for any mGluR either in the SNc or in the striatum (**fig. 4.23-4.25**).

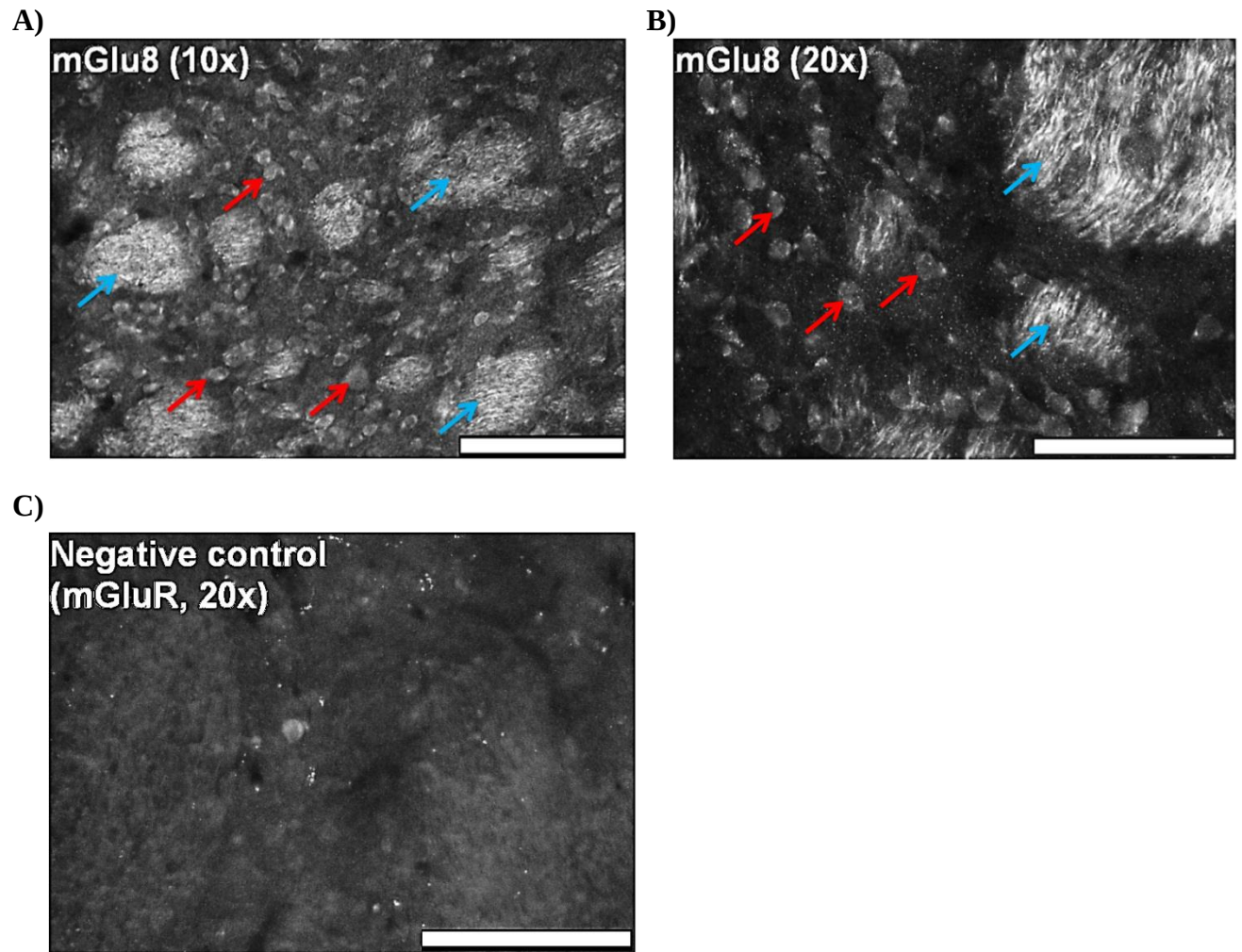


Figure 4.21 Identification of mGlu8 expression in the rat striatum. Fixed rat striatal slices (20 μ m) were immunolabelled with a primary antibody targeting mGlu8 and tagged with an Alexa-488 conjugated secondary antibody (**A, B**). Negative control staining was obtained through omission of primary antibody incubation (**C**). Images were captured at 10x (**A, C**) or 20x (**B**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μ m (10x) or 200 μ m (20x). Red and blue arrows indicate mGlu8 immunoreactivity in cell bodies and striatal fibres, respectively.

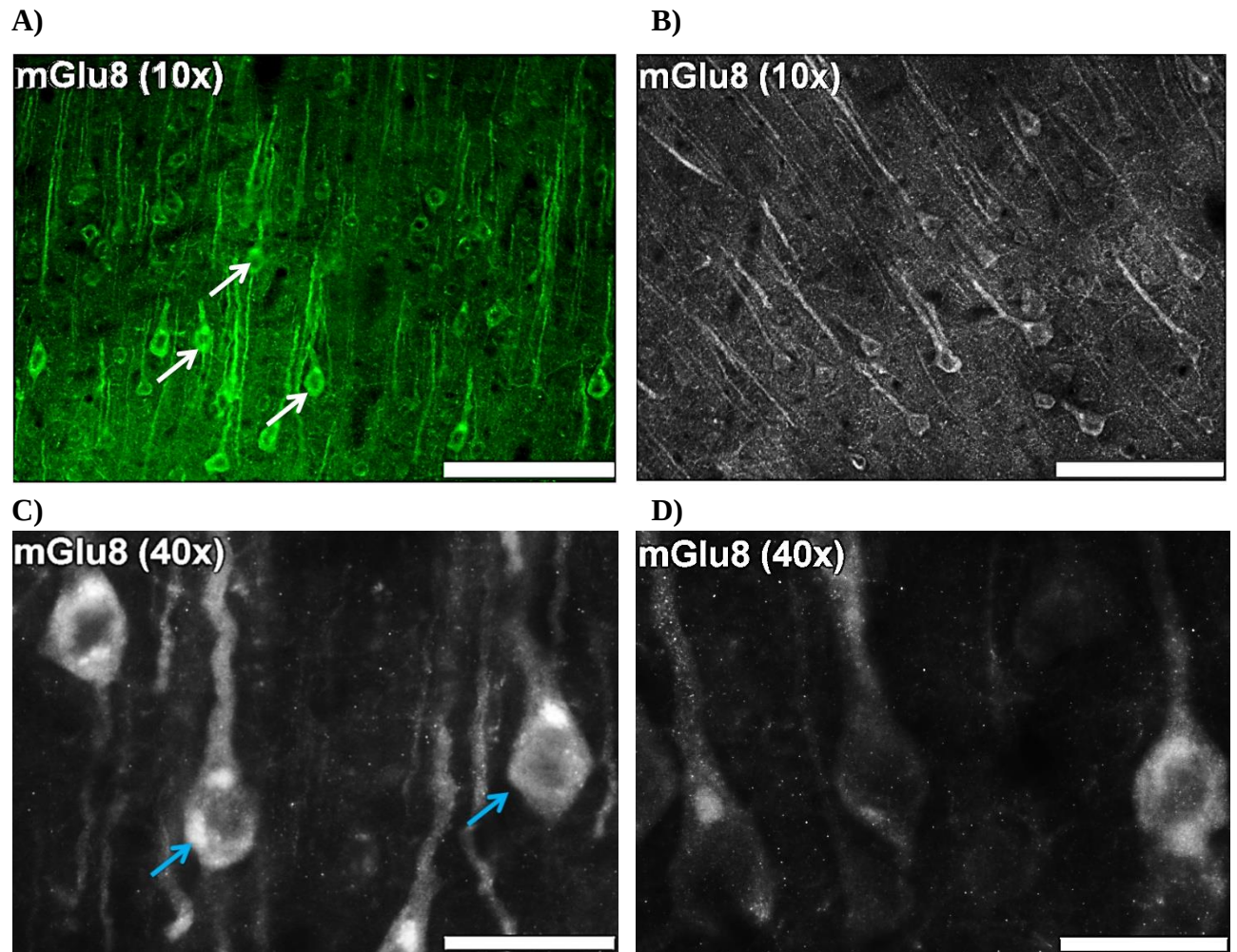


Figure 4.22 Identification of mGlu8 expression in the rat cortex. Fixed rat cortical slices (20 μm) were immunolabelled with a primary antibody targeting mGlu8 and tagged with an Alexa-488 conjugated secondary antibody. Cortical slices spanned either the SNc (**A, C**) or the striatum (**B, D**). Images were captured at 10x (**A, B**) or 40x (**C, D**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μm (10x) or 75 μm (40x). White and blue arrows indicate cortical cell bodies with mGlu8 immunoreactivity.

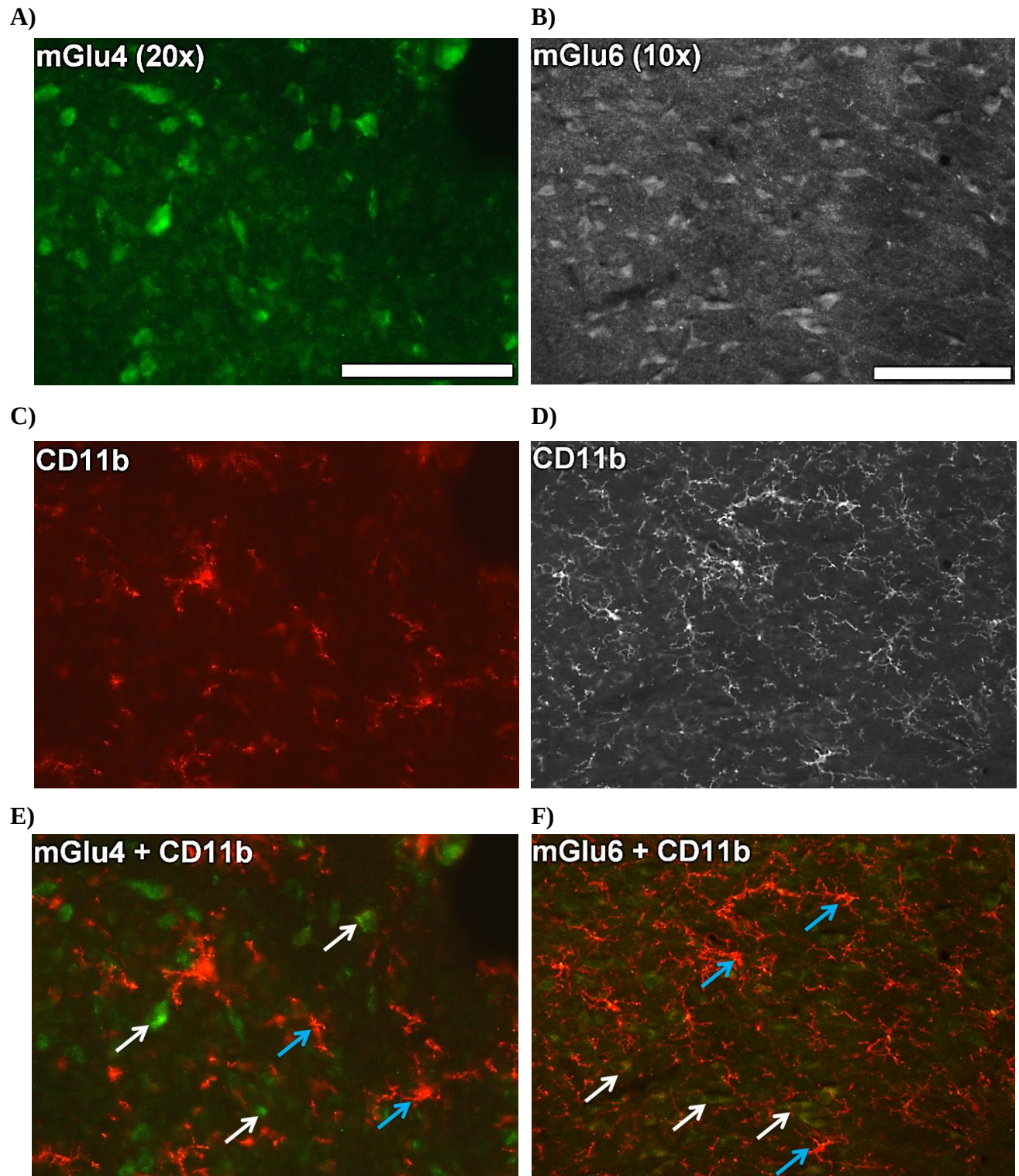


Figure 4.23 Identification of mGlu4 or mGlu6 microglial expression in the rat substantia nigra. Fixed rat SNc slices (20 μ m) were immunolabelled for mGlu4 (**A, E**) or mGlu6 (**B, F**) (green staining in **E, F**) and tyrosine hydroxylase (**C, D**) (red staining in **E, F**). Images were captured at 20x (**A, C, E**) or 10x (**B, D, F**) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μ m (10x) or 200 μ m (20x). White arrows indicate cell bodies with mGluR immunoreactivity. Blue arrows indicate microglial cells with CD11b immunoreactivity.

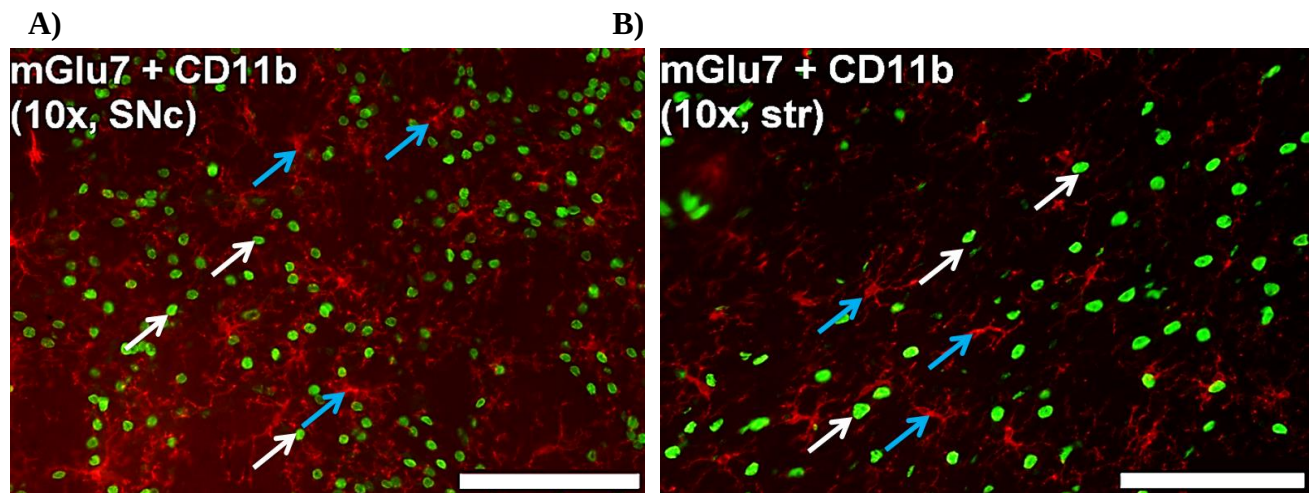


Figure 4.24 Identification of mGlu7 microglial expression in the rat substantia nigra and striatum. Fixed rat SNc (**A**) and striatal (**B**) slices (20 μ m) were immunolabelled for mGlu7 (green staining) and CD11b (red staining). Images were captured at 10x magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 300 μ m. White arrows indicate cells with mGlu7 immunoreactivity. Blue arrows indicate microglial cells with CD11b immunoreactivity.

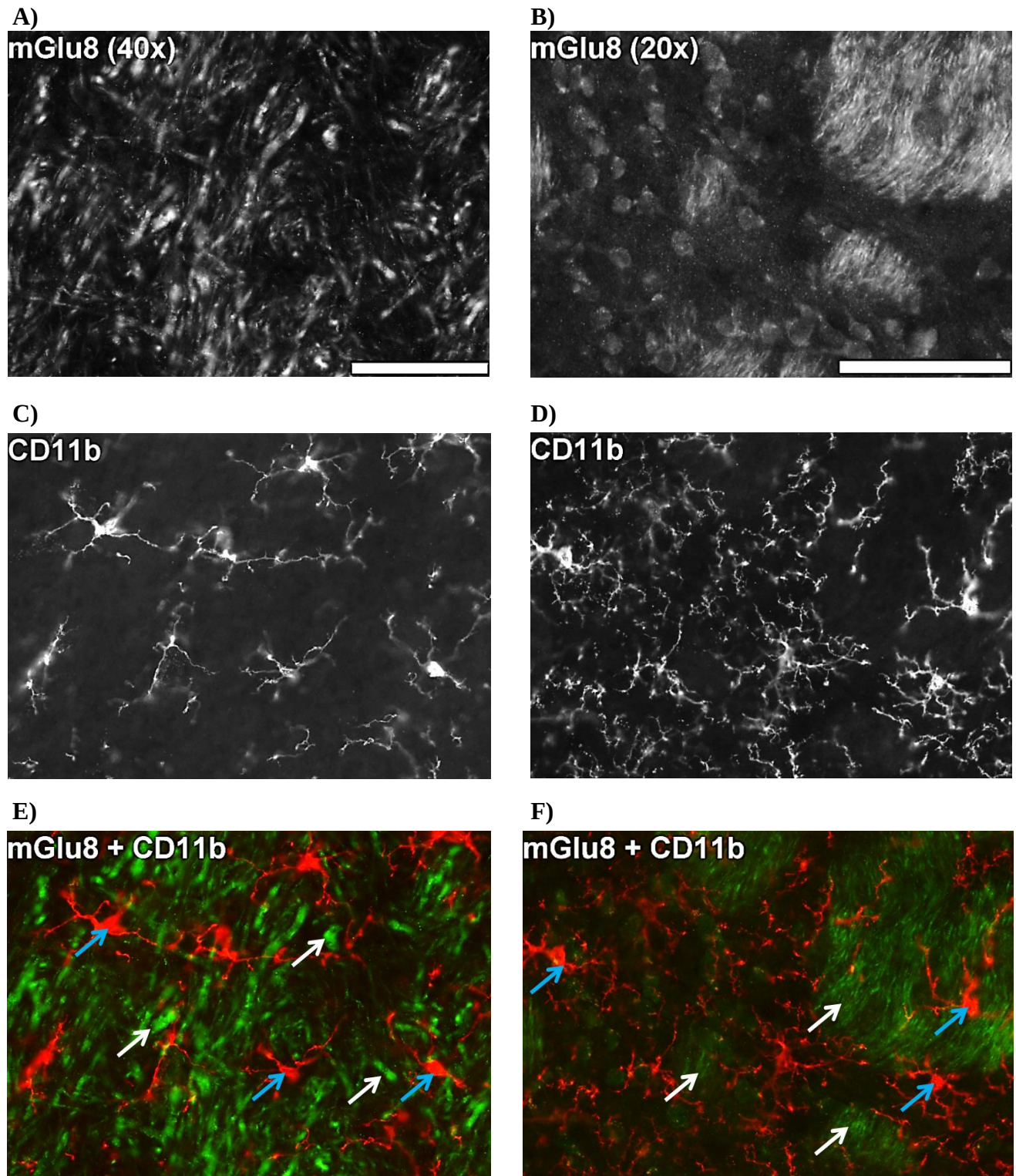


Figure 4.25 Identification of mGlu8 microglial expression in the rat substantia nigra and striatum. Fixed rat SNc (A, C, E) and striatal (B, D, F) slices (20 μ m) were immunolabelled for mGlu8 (green staining) and CD11b (red staining). Images were captured at 40x (A, C, E) or 20x (B, D, F) magnification using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera, and are representative of tissue staining from four different rat brains. Scale bars = 75 μ m (40x) or 200 μ m (20x). White arrows indicate fibers with mGlu8 immunoreactivity. Blue arrows indicate microglial cells with CD11b immunoreactivity.

4.4 Discussion

This chapter set out to examine the *in vitro* and *in vivo* expression of metabotropic glutamate receptors by microglia. The above results clearly demonstrate expression of mGluR across all three groups in N9 microglia as well as primary microglia. In addition, group III mGluR expression was demonstrated in the SNc and striatum of naïve rats. However, expression of group III mGluR was not detected in microglial cells.

4.4.1 Metabotropic glutamate receptor expression by N9 microglia

Cultured N9 microglia were shown to have positive immunoreactivity for both group I mGluR. Images captured at the same exposure indicated N9 microglia express higher levels of mGlu5 than mGlu1. Furthermore, high magnification images revealed mGlu1 and mGlu5 expression in N9 microglia is not homogenously distributed throughout the cell membrane, but is concentrated in certain loci, with much lower levels of expression in surrounding areas. This punctate pattern of distribution was more pronounced for mGlu5 receptors than mGlu1 receptors. Interestingly, a similar punctate expression pattern of mGlu5 has been described in cultured cerebellar granule neurons (Ango *et al.*, 2000) as well as enteric neurons (Liu and Kirchgeßner, 2000). However, at this point the function of such punctate expression pattern is unknown.

Similarly, N9 microglia expressed both mGlu2 and mGlu3 receptors, of which mGlu3 was more strongly expressed. Group II mGluR expression was more homogenously distributed through the cell membrane compared to group I mGluR expression.

Expression of group III mGluR by N9 microglia was the most surprising. While immunoreactivity was modest for mGlu4, there was significant labelling for mGlu6. Although this finding is in conflict with previous studies suggesting mGlu6 to be primarily expressed in the retina, previous *in vitro* studies utilizing cultured primary microglia have shown microglia are able to express mGlu6 mRNA and protein (Taylor *et al.*, 2003).

In addition, immunolabelling demonstrated cultured N9 microglia expressed significant levels of mGlu7 protein. Again, this finding appears to contradict a previous study suggesting cultured microglia lack mGlu7 expression, both at the mRNA and protein level (Taylor *et al.*, 2003). However, the discrepancy can be explained by the various isoforms recognized for mGlu7 generated through alternative splicing of the C-terminal (Schulz *et al.*, 2002). Indeed, in the study by Taylor *et al.*, mGlu7 mRNA and protein were detected with the use of a primer specific for mGlu7a and an antibody targeting the C- terminal of mGlu7a/b, respectively. Therefore, expression of other mGlu7 isoforms may have been overlooked. Given the fact that immunocytochemical labelling of N9 microglia in this thesis was carried out with an antibody targeting the protein's N-terminal, this false positive result would be avoided as the antibody would recognize all mGlu7 splice variants.

It is of particular interest that the expression pattern for mGlu7 in N9 microglia was mainly concentrated within the cell, with only faint immunolabelling of the cell body. The close co-localization seen with the nuclear stain DAPI strongly suggests mGlu7 immunoreactivity to localize primarily to the cell nucleus. Although mGluR are thought to be primarily plasma membrane proteins, previous studies have shown mGluR also localize the nuclear membrane, where they are functionally coupled to similar signalling mechanisms employed by plasma membrane mGluR (Kumar *et al.*, 2008; O'Malley *et al.*, 2003). Furthermore, similar nuclear localization of mGlu7 in neuronal cells has been evidenced by independent research groups (Dr. Anthony Vernon, King's College London, personal communication).

Despite previous reports of microglial mGlu8 expression, mGlu8 immunoreactivity in N9 microglia was not detected through ICC above negative control levels.

The results of group III mGluR expression by N9 microglia obtained through ICC were corroborated by immunolabelling of Western blot membranes of N9 microglia lysates. Hence, labelling of membranes with an anti-mGlu4 antibody revealed a specific band at the predicted molecular weight (100 kDa). The bands recognized by the mGlu4 antibody were not particularly

thick, reflecting the moderate immunoreactivity for mGlu4 seen with ICC. Similarly, labelling of membranes with an anti-mGlu7 antibody targeting a C-terminal epitope revealed a prominent band at the predicted molecular weight (100 kDa). Furthermore, a prominent band at the same molecular weight was detected in rat brain lysates. The lack of mGlu8 immunoreactivity detection by ICC was replicated by Western blot labelling, where incubation of membranes with an anti-mGlu8 failed to reveal bands at the predicted molecular weight (100 kDa).

4.4.2 Metabotropic glutamate receptor expression by primary microglia

Expression of mGluR in N9 microglia was corroborated in primary microglia extracted from neonatal rats. Similarly to N9 microglia, primary microglia were immunopositive for mGlu1 and mGlu5. Group I mGluR immunoreactivity in primary microglia was heterogeneously distributed through the cell membrane, though this effect was not as pronounced as in N9 microglia.

Primary microglia were also immunoreactive for group II and group III mGluR. Similarly to N9 microglia, immunoreactivity for mGlu3 was markedly more intense than mGlu2 immunoreactivity. Furthermore, mGlu4 immunoreactivity was the lowest of the detected group III receptors (mGlu4, 6, 7), again reflecting the labelling seen with N9 microglia. Although mGlu4 labelling in primary microglia was low, it was still above negative control, indicating low expression levels of mGlu4. Furthermore, mGlu7 expression was again convincingly detected in primary microglia employing a C-terminal targeted antibody. However, unlike N9 microglia, the mGlu7 expression pattern on primary microglia was not restricted to the nucleus, possibly reflecting an important difference between N9 and primary microglia. In addition, mGlu8 immunoreactivity was not detected in primary microglia, similarly to N9 microglia.

Importantly, double fluorescent labelling revealed all mGluR-positive were also immunoreactive for CD11b, demonstrating their microglial character and the efficacy of the isolation protocol.

4.4.3 *In vivo* expression of group III mGluR

Expression of group III mGluR was also examined through immunohistochemical labelling of SNc and striatum tissue. IHC labelling revealed moderate expression of mGlu4 in cell bodies within the SNc of naïve rats. Around half of the cells were also double labelled with anti-tyrosine hydroxylase antibodies, indicating their dopaminergic phenotype. The phenotype of the TH-negative cells cannot be ascertained presently, but most likely constitutes glutamatergic cells within the SNc. However, no mGlu4 immunoreactivity was detected in striatal tissue slices, as determined by comparable labelling to negative control striatal tissue.

Similarly to mGlu4, IHC labelling of SNc tissue from naïve rats revealed modest immunoreactivity against mGlu6 which was localized to cell bodies. Again, certain mGlu6-positive cell bodies were also immunoreactive for TH. However, unlike mGlu4, striatal slices were immunopositive. Interestingly, mGlu6 immunoreactivity was only detected in fibres surrounding striosomes. The striatum is divided into structures termed striosomes and the surrounding matrix, which are biochemically, neuroanatomically, and hence functionally, distinct (Crittenden and Graybiel, 2011; Graybiel, 1990). While neuronal networks within the striosomes are generally associated with limbic function, the surrounding matrix is more closely associated with sensorimotor function (Crittenden and Graybiel, 2011). Interestingly, previous immunolabelling studies have shown differential expression of ionotropic glutamate receptors within the striosomes and matrix (Martin *et al.*, 1993). Therefore, the segregation of mGluR expression between the striosomes and matrix may reflect the involvement of mGluR in striatal motor control.

Strikingly, mGlu7 immunoreactivity in both the SNc and the striatum highly resembled the ICC labelling in N9 microglia, *i.e.* highly restricted to the nucleus. Nuclear localization was demonstrated both by concentration of mGlu7 immunoreactivity within cell bodies immunoreactive for TH, as well as the close co-localization with the nuclear stain DAPI. Hence, the nuclear localization in the SNc and striatum indicate the nuclear localization in the N9 microglia is not

ectopic expression induced by the immortalization process of the N9 cell line. Interestingly, striatal mGlu7 immunoreactivity was restricted to the matrix surrounding the striosomes, similarly to mGlu6. The possibility of lack of immunoreactivity due to absence of cell bodies within the striosomes was ruled out by positive detection of cell nuclei within the striosomes.

Although mGlu8 immunoreactivity was detected in the SNc, this was seldom in cell bodies and most often in fibres crossing the SNc. Interestingly, mGlu8-positive fibres never co-localized with TH immunoreactivity. In fact, TH immunoreactivity disappeared in regions with mGlu8-positive fibres, perhaps indicating mGlu8-positive fibres were not functional within the SNc but were simply crossing it.

Despite being able to identify mGluR-immunoreactivity for all group III mGluR in cell bodies of both the SNc or the striatum, as well as identifying mGluR expression in cultured microglia, double labelling with CD11b failed to detect mGluR expression in microglia *in vivo*. However, the labelled microglia in the tissue slices had a highly ramified morphology with compact cell bodies, indicating a resting/surveying phenotype. In addition, even though primary and N9 microglia were shown in the last chapter to adopt a non-inflamed phenotype following plating, this may not fully represent a resting/surveying phenotype, as their ramifications were not as extended as *in vivo* (specially primary microglia), and their proliferative rate was higher than expected *in vivo* (specially N9 microglia). This lack of a true resting/surveying phenotype might confer in them an increased mGluR expression level. Though this might appear to preclude the use of N9 microglia to study microglial biology, this is not the case. Firstly, even though care was taken to ensure our microglial cells had a non-activated phenotype resembling *in vivo* microglia, a true replication of the *in vivo* phenotype is not attainable, as both the process of extracting/plating microglia and their lack of contact with non-microglial cells will inevitably confer in them a modified phenotype. Secondly, previous IHC studies on post-mortem tissue have indicated that, while resting/surveying microglia from control subjects do not express mGluR, microglia from multiple sclerosis patients, which have

a high level of microglial activation, express detectable levels of mGluR (Geurts *et al.*, 2003, 2005). Similarly, cortical and hippocampal mouse microglia fail to express mGlu5 under control conditions, but markedly express mGlu5 following traumatic brain injury (Byrnes *et al.*, 2012). Therefore, expression of microglial mGluR appears to be restricted to non-resting/surveying microglia. Hence, the lack of *in vivo* mGluR expression presented in this chapter might be due to the resting microglial phenotype. In order to verify this, IHC studies will need to be carried out in tissue from lesioned (*e.g.* 6-OHDA treated) rats. Furthermore, the detection of mGluR expression in our *in vitro* microglia appears to be due to lack of contact with non-microglia cells, as stimulation towards a pro-inflammatory phenotype by LPS treatment did not confer an increase in mGluR expression assessed through immunocytochemical staining (results not shown). Therefore, our control *in vitro* microglia might represent a microglial phenotype found in areas of early neurodegeneration, and which, after LPS treatment, convert to a pro-inflammatory phenotype seen in latter stages of neurodegeneration.

Interestingly, the lack of mGluR expression in resting/surveying microglia *in vivo* might add to the attractiveness of these receptors as therapeutic targets, as targeting only activated microglia will allow for modulation of the neurotoxic microglia phenotype (*i.e.* activated microglia) without affecting the supportive (*e.g.* neurotrophic) phenotype of resting/surveying microglia.

4.5 Conclusion

The results presented within this chapter clearly demonstrate, as hypothesized, N9 microglia, similarly to primary microglia, express protein for all three groups of metabotropic glutamate receptors, therefore are a suitable model to examine the functions of mGluR in microglial activation.

Furthermore, this chapter has shown mGlu7 has a peculiar expression pattern restricted to the nucleus both *in vitro* and *in vivo*. The nuclear localization of mGlu7 may confer it with particular biological and pharmacological characteristics. Indeed, this expression pattern may partly explain

the significantly lower affinity of mGlu7 for glutamate compared to other group III mGluR (Cartmell and Schoepp, 2000).

However, contrary to our hypothesis, this chapter has shown resting/surveying microglia *in vivo* are devoid of mGluR expression. If this is truly the case, mGluR expression opens up the possibility of targeting activated microglia specifically. This would allow for modulation of microglia producing pro-inflammatory and possibly neurotoxic compounds without affecting resting microglia which might be serving a neuroprotective role through production of neurotrophic factors.

Chapter V

Modulation of microglial reactivity by
individual group III mGluR

5.1 Introduction

Microglial activation is a tightly regulated process essential for host defence and tissue damage repair. Upon detection of pathogen infiltration, microglia are capable of adopting a pro-inflammatory phenotype characterized by increased expression of pro-inflammatory cytokines, such as IL-6, IL-1 β and TNF α , and pro-inflammatory enzymes, such as iNOS, as well as increased production of reactive nitrogen species (RNS) such as nitric oxide (NO) (reviewed by Kettenman *et al.*, 2011). Upregulation of inflammatory mediators is essential for proper pathogen clearance through induction of apoptosis and enhancement of the inflammatory response. However, if microglial activation is not properly regulated, it may lead to collateral neuronal damage resulting in neuronal death. Indeed, microglial activation has been associated with the progressive neuronal death seen in neurodegenerative diseases such as PD. Epidemiological studies have found an association between regular use of the non-steroidal anti-inflammatory drug Ibuprofen, which is capable of reducing microglial reactivity (Jin *et al.*, 2008), and lower risk of PD (Gao *et al.*, 2011; Chen *et al.*, 2005). In addition, blockade of microglial reactivity by minocycline is able to reduce neuronal loss in MPTP and 6-OHDA animal models of PD (Wu *et al.*, 2002; He *et al.*, 2001). Furthermore, direct microglial activation by intranigral administration of the bacterial cell wall component LPS leads to nigral dopaminergic cell death *in vivo* (Castaño *et al.*, 1998).

Interestingly, *in vitro* studies have shown that neuronal compounds released by degenerating dopaminergic neurons (*e.g.* MMP, neuromelanin, alpha-synuclein) are able to activate microglia towards a pro-inflammatory phenotype (Kim *et al.*, 2005, 2007; Wilm *et al.*, 2003; Lee *et al.*, 2010). Therefore, microglial activation, though probably not initiating neuronal cell death in the early stages of PD, may contribute towards a feed-forward cycle where an initial neuronal degeneration stimulates pro-inflammatory microglial activation, leading to further neuronal death, thereby establishing the chronic and progressive SNc degeneration seen in PD. Therefore, microglial activation may provide a viable therapeutic target against chronic neurodegenerative diseases.

Previous *in vitro* studies have shown that microglial metabotropic glutamate receptors (mGluR) are able to modulate microglial activation and neurotoxicity. Activation of group I mGluR is able to reduce the production of ROS and expression of pro-inflammatory cytokines, leading to a reduction in microglial neurotoxicity (Byrnes *et al.*, 2009; Farso *et al.*, 2009). Interestingly, activation of group II or group III receptors has been shown to have opposing effects on microglial neurotoxicity, despite putatively sharing signalling mechanisms. Group II activation results in enhanced neurotoxicity, partly through induction of TNF α /Fas ligand release (Taylor *et al.*, 2005). On the other hand, activation of group III leads to a decrease in microglial neurotoxicity (Pinteaux-Jones *et al.*, 2008; Taylor *et al.*, 2003).

In addition to differences between mGluR groups, *in vitro* studies employing receptor-specific ligands have revealed microglial neurotoxicity is differentially modulated by members of the same mGluR group. Accordingly, the inhibitory effects of the group I agonist DHPG on microglial activation can be reversed by co-treatment with the mGlu5 antagonist MTEP, but not the mGlu1 antagonist CPCCOEt (Loane *et al.*, 2009), indicating activation of mGlu5 but not mGlu1 is capable of reducing microglial reactivity. Similarly, treatment of microglia with the group II agonist DCGIV results in enhanced neurotoxicity, while treatment with the mGlu3-specific agonist NAAG protects neurons against microglial activation (Pinteaux-Jones *et al.*, 2008). Therefore, it has been proposed that activation of mGlu2 results in enhanced microglial reactivity and neurotoxicity, while activation of mGlu3 inhibits microglial reactivity and stimulates neuroprotection.

Given the opposing effects of activating different receptors within group I or group II on microglial activation and neurotoxicity, it is important to dissect the exact effects of activating individual group III mGluR on microglial activation and neurotoxicity if this group of receptors is to be developed as a therapeutic target against neurodegenerative diseases. Furthermore, as microglia are thought to exist in a state of chronic reactivity in PD brains, it is important to establish whether group III mGluR activation is able to modulate microglial reactivity once the microglial

inflammatory mechanism has been engaged. With that in mind, this chapter sets out to establish the effects of mGlu4/7/8 receptor-specific ligands on LPS-induced microglial activation.

- **Hypothesis:** The shift towards a pro-inflammatory N9 microglial phenotype stimulated by lipopolysaccharide treatment can be modulated by treatment with ligands targeting individual group III metabotropic glutamate receptors.

5.2 Experimental design

As microglia are thought to exist in a state of chronic activation in PD, mGluR ligands must be able to modulate microglial activation once the inflammatory process has been engaged if mGluR modulation of microglial activation is to be developed as a therapeutic strategy against PD. Additionally, mGluR ligands should also be able to prevent further activation of resting microglia. With that in mind, we set out to examine if ligands targeting individual group III mGluR were able to modulate microglial activation both before onset of microglial activation, as well as once microglial activation had been engaged. To that end, N9 microglia were treated with mGluR ligands 20 mins before LPS (500 ng/mL) treatment (henceforth termed ‘pre-treatment’) in order to investigate whether mGluR ligands could prevent microglial activation. In addition, N9 microglia were treated with LPS (500 ng/mL) for one hour before treatment with mGluR ligands (henceforth termed ‘post-treatment’) in order to examine whether mGluR ligands could modulate microglial activation once microglial activation was engaged (see section 2.1.9). Furthermore, microglia were activated with 5 ng/mL LPS in order to ensure any lack of effect of mGluR ligands was not due to excessive LPS signalling which might mask mGluR effects.

In order to verify that exposing N9 microglia to LPS for one hour was sufficient to engage the inflammatory mechanism, microglia were treated with LPS for one hour, after which time-point the cells were washed with sterile PBS to remove LPS and incubated in fresh complete microglial

medium. Activation was assessed through quantification of nitrite production (see section 2.1.15) and cellular morphology 18 hrs following LPS treatment.

The mGluR ligands employed in this thesis were the general group III mGluR orthosteric agonist L-AP4, the mGlu4-specific positive allosteric modulator VU0155041, the mGlu7-specific allosteric agonist AMN082 and the mGlu8-specific agonist DCPG (see section 2.1.8.2). The role of mGlu6 on microglial activation was not examined due to its putative exclusive expression in the substantia nigra *in vivo*. VU0155041 and AMN082 have not been used in *in vitro* microglial cultures before, therefore the doses employed in this thesis had to be determined empirically. Doses of L-AP4 employed in previous *in vitro* microglial cultures were taken as a reference point (Taylor *et al.*, 2003; Pinteaux-Jones *et al.*, 2008).

Modulation of microglial activation by mGluR ligands was assessed by comparing nitrite production, iNOS expression, and IL-6, IL-1 β and TNF α release between microglia treated or not with mGluR ligands 18 hrs following LPS treatment (see sections 2.1.9 and 2.1.15-17). An 18 hrs LPS stimulation period was chosen as the experimental end-point as a compromise between peak expression time-points of nitrite, iNOS, IL-6 and TNF α (Lin *et al.*, 2011; Byrnes *et al.*, 2009; Pinteaux-Jones *et al.*, 2008; Shigemoto-Mogami *et al.*, 2001; Zujovic *et al.*, 2000).

In order to confirm any observed effects of mGluR ligands on microglial activation were due to specific modulation of microglial activation and not simply increased cell death, microglial viability was assessed 18 hrs following LPS (500 ng/mL) and mGluR ligand treatment by quantification of intracellular NADH through MTS assays, assessment of cellular membrane integrity through Trypan blue exclusion assays and LDH release assays, and quantification of cellular protein content through Bradford assays (see sections 2.1.11-14).

Statistical significance was set at $p < 0.05$, calculated with one-way ANOVA with Tukey's *post-hoc* test using SPSS software, unless otherwise stated (see section 2.1.23).

5.3 Results

5.3.1 Microglial reactivity is differentially modulated by individual group III mGluR

5.3.1.1 *Effect of one-hour LPS treatment on N9 microglial activation*

Treatment with 500 ng/mL LPS for one hour markedly activated microglia when assessed 18hrs following LPS treatment, as assessed by a significant and robust increase in nitrite production (**fig. 5.1**) and a shift towards an activated amoeboid morphology (images not shown). The increase in nitrite production was subsequent to one hour LPS treatment, as nitrite concentration did not differ between untreated and LPS-treated microglia one hour after LPS treatment (**fig. 5.1**).

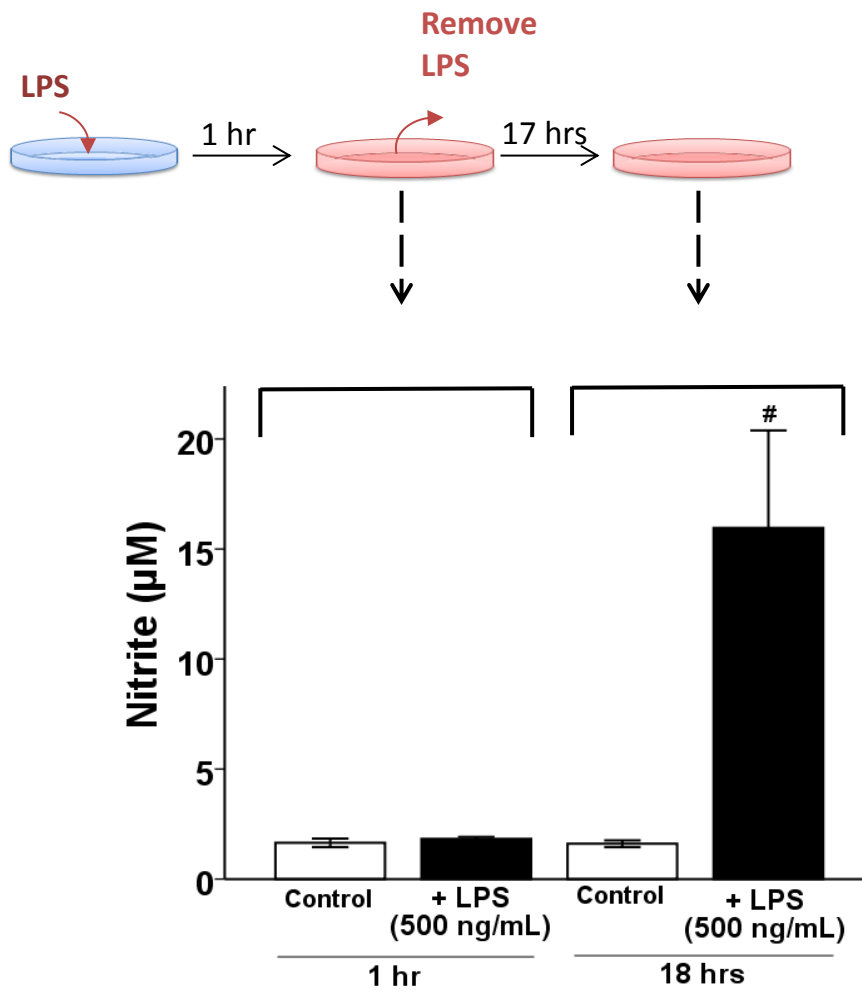


Figure 5.1 N9 microglial activation by one hour LPS stimulation. N9 microglia were exposed to 500 ng/mL LPS for one hour, after which time-point cells were washed and replaced with fresh medium to remove LPS, and the microglia incubated for a further 17 hrs in LPS-free medium. Nitrite concentration in the cell culture medium were quantified through the Griess assay at 1 hr (prior to removal of LPS) and 18 hrs following LPS treatment. Results are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate. # denotes $p < 0.05$ vs. respective control as determined by Student's T-test for independent samples. $F = 0.001$ (1 hr), 14.53 (18 hrs)

5.3.1.2 *Effect of L-AP4 treatment on nitrite production, iNOS expression and IL-6, IL-1 β*

and TNF α release by LPS-activated N9 microglia

Treatment of N9 microglia with L-AP4 1 hr following LPS (500 ng/mL) treatment led to a modest and dose-dependent decrease in nitrite production 18 hrs following LPS treatment. Interestingly, the decrease in nitrite production adopted an inverted bell-shaped curve, with strongest effect seen at 10 μ M L-AP4 (73.4% of LPS response), whilst higher L-AP4 concentrations (100 μ M) did not affect nitrite production (**fig. 5.2A**). Inhibition of nitrite production by L-AP4 treatment 1 hr following LPS treatment was accompanied by a decrease in iNOS expression at the same time-point (**fig. 5.2B**). Similarly, preliminary data indicated L-AP4 treatment 20 mins before LPS treatment induced a modest decrease in nitrite production. However, this effect was lost with 100 μ M L-AP4 (**fig. 5.2C**). L-AP4 treatment of LPS (500 ng/mL)-activated N9 microglia failed to modulate IL-6 release (**fig. 5.3A**), IL-1 β release (**fig. 5.3B**) and TNF α release (**fig. 5.3C**). Similarly, L-AP4 treatment failed to modulate TNF α release stimulated by 5 ng/mL LPS at 18 hrs following LPS activation (**fig. 5.3D**).

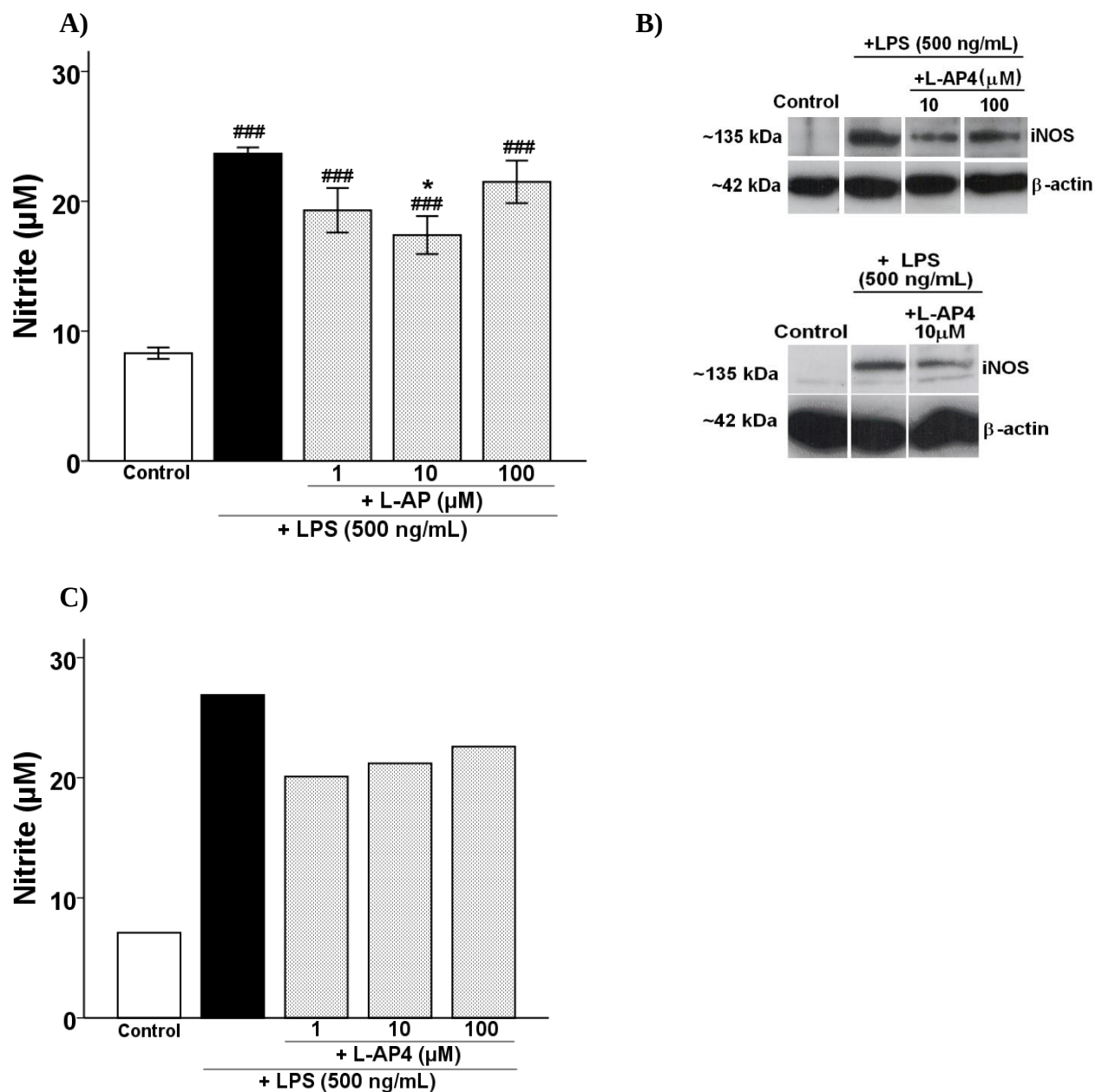


Figure 5.2 Effect of L-AP4 treatment on nitrite production and iNOS expression by LPS-activated N9 microglia. N9 microglia were exposed to LPS (500 ng/mL) for one hour prior to treatment with the general group III mGluR orthosteric agonist L-AP4. Nitrite concentration in the cell culture medium was quantified through the Griess assay at 18 hrs following LPS activation (**A**). Expression of iNOS by N9 microglia at 18 hrs following LPS activation was assessed through immunolabelling of Western blots of microglial cell lysates from control or LPS (500 ng/mL)-activated N9 microglia treated with L-AP4 one hour following LPS activation. To confirm equal protein loading, Western blots were also labelled with anti- β -actin (**B**). Nitrite concentration in the cell culture medium of N9 microglia treated with L-AP4 20 mins prior to LPS (500 ng/mL) activation were also quantified through the Griess assay at 18 hrs following LPS activation (**C**). Results are displayed mean $\pm$ SEM of three (**A**) or two (**C**) independent experiments measured in triplicate. Images in (**B**) are from two independent experiments. ### denotes $p < 0.005$ vs. control. * denotes $p < 0.05$ vs. LPS. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 21.43$

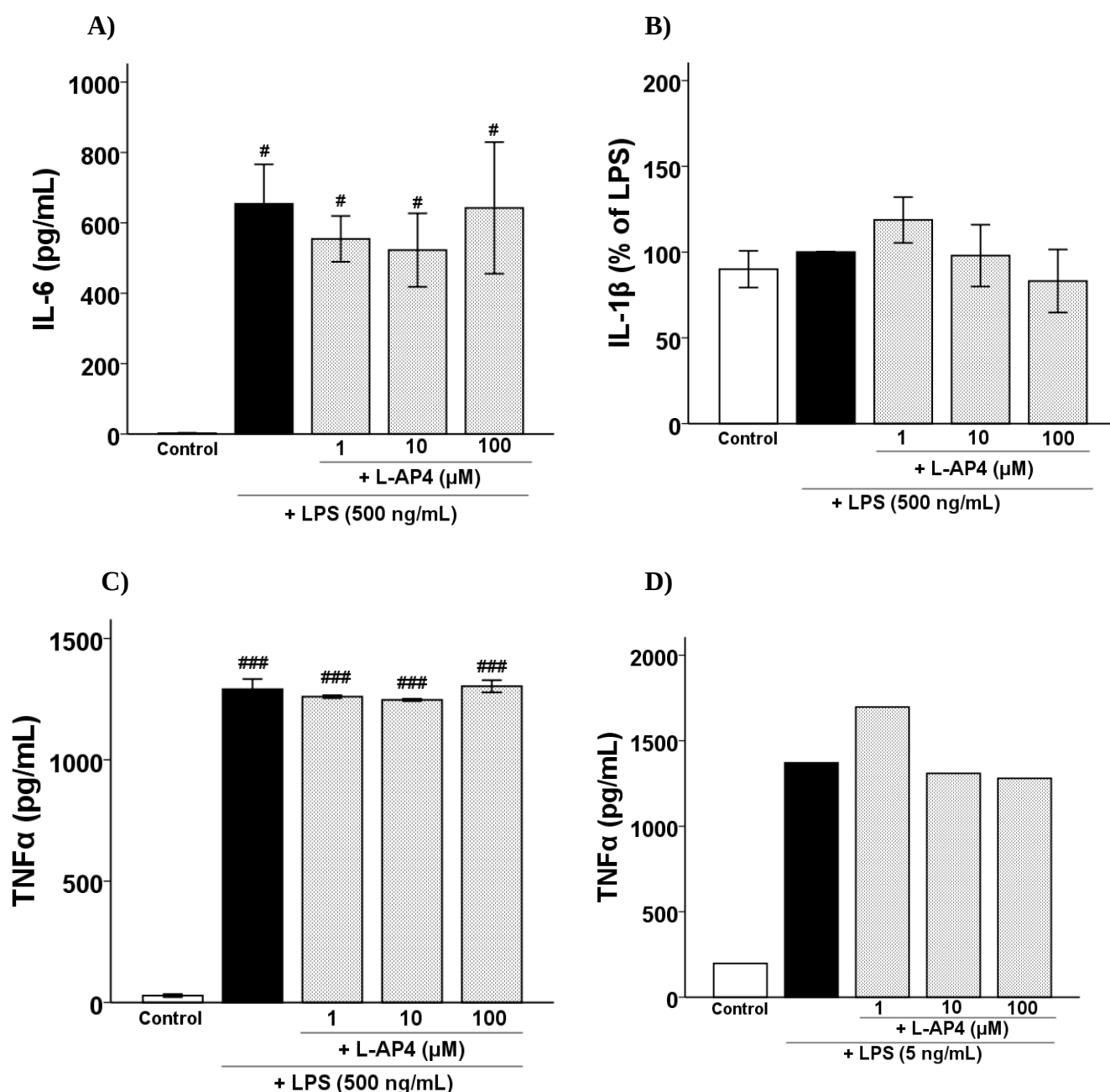


Figure 5.3 Effect of L-AP4 treatment on IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with the general group III mGluR orthosteric agonist L-AP4 1 hr following LPS activation and IL-6 (**A**), IL-1 β (**B**) and TNF α (**C**) release into the cell culture medium was quantified at 18 hrs following LPS activation through an ELISA. Similar quantification of TNF α was carried out when N9 microglia were activated with 5 ng/mL LPS (**D**). Results are displayed as mean $\pm$ SEM of three (A-C) or two (D) independent experiments measured in duplicate. #, ### denote $p < 0.05$, 0.005 vs. control, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 5.83$ (A), 0.94 (B), 632.3 (C).

5.3.1.3 *Effect of AMN082 treatment on nitrite production, iNOS expression and IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia*

Treatment of microglia with AMN082 1 hr following LPS (500 ng/mL) activation induced a marked decrease in nitrite production 18 hrs following LPS treatment, though statistical significance was only achieved at the highest concentration (10 μ M, 54% of LPS response) (**fig. 5.4A**). Inhibition of nitrite production by AMN082 treatment was accompanied by a marked reduction in iNOS expression at the same time-point (**fig. 5.4B**). Though treatment with AMN082 20 mins before LPS activation resulted in inhibition of nitrite production, this failed to reach statistical significance, even at the highest concentration tested (**fig. 5.4C**). Treatment of N9 microglia with 5 ng/mL LPS led to an increase in nitrite production 18 hrs following LPS activation which was markedly inhibited by AMN082 treatment 1 hr following LPS activation. However, statistical significance was only reached with the highest concentration tested (10 μ M, 63.7% of LPS response) (**fig. 5.4D**).

Treatment with the mGlu7-specific allosteric antagonist MMPIP 25 mins before AMN082 treatment reversed the AMN082-induced reduction in LPS-stimulated nitrite production (**fig. 5.5C**).

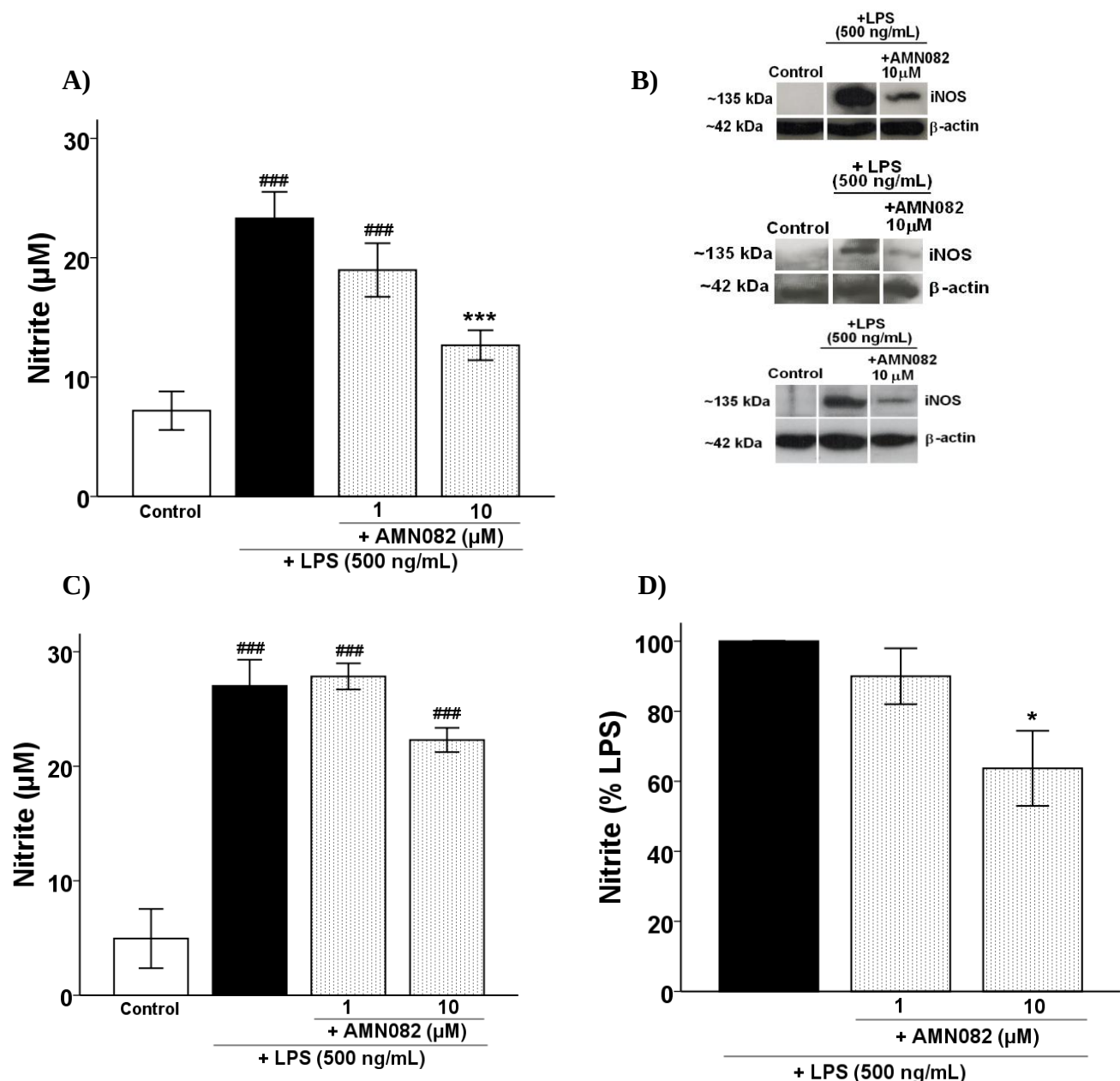


Figure 5.4 Effect of AMN082 treatment on nitrite production and iNOS expression in LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with the mGlu7-specific allosteric agonist AMN082 one hour following LPS activation and nitrite concentration in the cell culture medium quantified through the Griess assay at 18 hrs following LPS activation (**A**). Expression of iNOS by N9 microglia at 18 hrs following LPS activation was assessed through immunolabelling of Western blots of microglial cell lysates from control or LPS (500 ng/mL)-activated N9 microglia treated with AMN082 one hour following LPS activation. To confirm equal protein loading, Western blots were labelled with anti-β-actin (**B**). Nitrite concentration in the cell culture medium of N9 microglia treated with AMN082 20 mins prior to LPS (500 ng/mL) activation was also quantified through the Griess assay at 18 hrs following LPS activation (**C**). Nitrite levels were similarly quantified following AMN082 treatment 1 hr after activating microglia with 5 ng/mL LPS (**D**). Results are displayed as mean $\pm$ SEM of 4 (A) or 3 (C,D) independent experiments measured in triplicate. Images in (**B**) are from three independent experiments. ### denotes $p < 0.005$ vs. control. *, **, *** denote $p < 0.05$, 0.01, 0.005 vs. LPS, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 14.14$ (A), 31.78 (C), 5.91 (D).

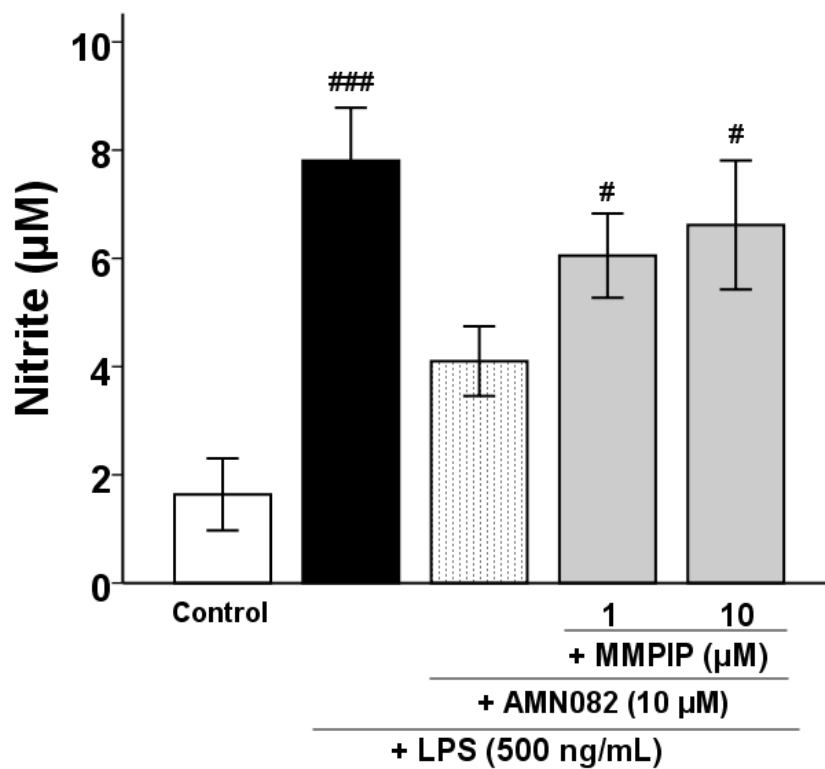


Figure 5.5 Effect of MMPIP treatment on AMN082-stimulated inhibition of nitrite production by LPS-activated N9 microglia. Nitrite concentration in the cell culture medium of LPS (500 ng/mL)-activated N9 microglia treated with the allosteric antagonist MMPIP were quantified through the Griess assay at 18 hrs after LPS activation. Results are displayed as mean $\pm$ SEM of 3 independent experiments measured in triplicate. #, ### denote $p < 0.05$, 0.005 vs. control, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 7.61$

AMN082 treatment of N9 microglia 1 hr following LPS (500 ng/mL) activation markedly inhibited IL-6 release 18 hrs following LPS activation (**fig. 5.6A**). Furthermore, AMN082 pre-treatment induced a more robust inhibition of IL-6 release 18 hrs following LPS treatment (**fig. 5.6B**). The effect of both treatments however only reached statistical significance at the highest AMN082 concentration tested (10 μ M). Conversely, AMN082 treatment 1 hr following LPS (500 ng/mL) activation failed to modulate IL-1 β release 18 hrs following LPS treatment (**fig. 5.6C**). Although AMN082 treatment 1 hr following LPS activation failed to modulate TNF α release stimulated by 500 ng/mL LPS 18 hrs following LPS treatment (**fig. 5.6D**), it produced a strong trend towards inhibition of TNF α release stimulated by 5 ng/mL at the same time-point (**fig. 5.6E**). Although a statistically significant difference failed to be reached between LPS treatment and 10 μ M AMN082 treatment, the latter treatment reduced TNF α release to such an extent as to remove statistical significance between AMN082 treatment and control treatment. Interestingly, 5 ng/mL LPS stimulated a comparable increase in TNF α release to 500 ng/mL.

5.3.1.4 *Effect of VU0155041 treatment on nitrite production, iNOS expression and IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia*

VU0155041 treatment of N9 microglia one hour following LPS (500 ng/mL) activation induced a marked decrease in nitrite production 18 hrs following LPS treatment. Interestingly, the effect of VU0155041 post-treatment on nitrite production adopted an inverse bell-shaped curve, with 10 μ M VU0155041 eliciting maximal nitrite production inhibition, and the effect being lost at higher concentrations (**fig. 5.7A**).

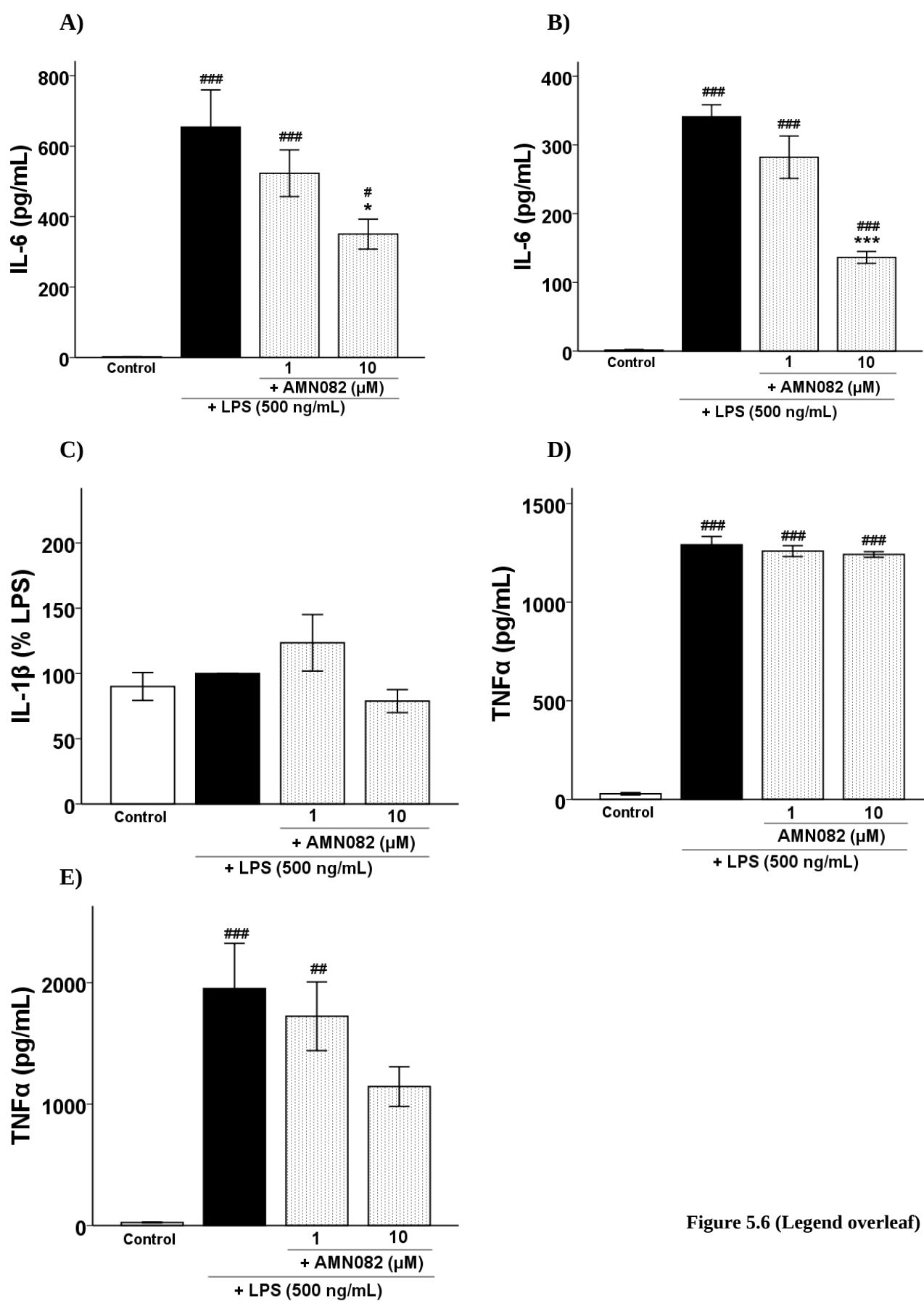


Figure 5.6 (Legend overleaf)

Nitrite production inhibition by VU0155041 was accompanied by a reduction in iNOS protein expression at the same time point (**fig. 5.7B**). VU0155041 treatment of N9 microglia 20 mins before LPS (500 ng/mL) activation induced a more robust inhibition of LPS-stimulated nitrite production 18 hrs following LPS activation. Interestingly however, N9 microglia did not become desensitized to VU0155041 pre-treatment with respect to nitrite production inhibition, as strongest inhibitory effect was achieved with 100 μ M VU0155041 (**fig. 5.7C**). In addition, a preliminary experiment indicated treatment of N9 microglia with 5 ng/mL LPS led to an increase in nitrite production 18 hrs following LPS activation which was markedly inhibited by VU0155041 treatment 1hr following LPS activation (**fig. 5.7D**).

Figure 5.6 Effect of AMN082 treatment on IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia (plated at 600×10^3 cells per well in 6 well plates) were treated with the mGlu7-specific allosteric agonist AMN082 1 hr following LPS (500 ng/mL) activation and release of IL-6 (**A**), IL-1 β (**C**) and TNF α (**D**) into the cell culture medium quantified through an ELISA at 18 hrs following LPS activation. IL-6 release into the cell culture medium by LPS (500 ng/mL)-activated N9 microglia (plated at 450×10^3 cells per well in 6 well plates) was also quantified by an ELISA at 18 hrs following LPS activation when treated with AMN082 20 mins prior to LPS activation (**B**). TNF α release by N9 microglia (plated at 600×10^3 cells per well in 6 well plates) treated with AMN082 1 hr following 5 ng/mL LPS activation was quantified by an ELISA at 18 hrs after LPS activation (**E**). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05, 0.01, 0.005$ vs. control, respectively. *, *** denote $p < 0.05, 0.005$ vs. LPS, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 18.36$ (A), 69.68 (B), 2.19 (C), 556.3 (D), 12.07 (E).

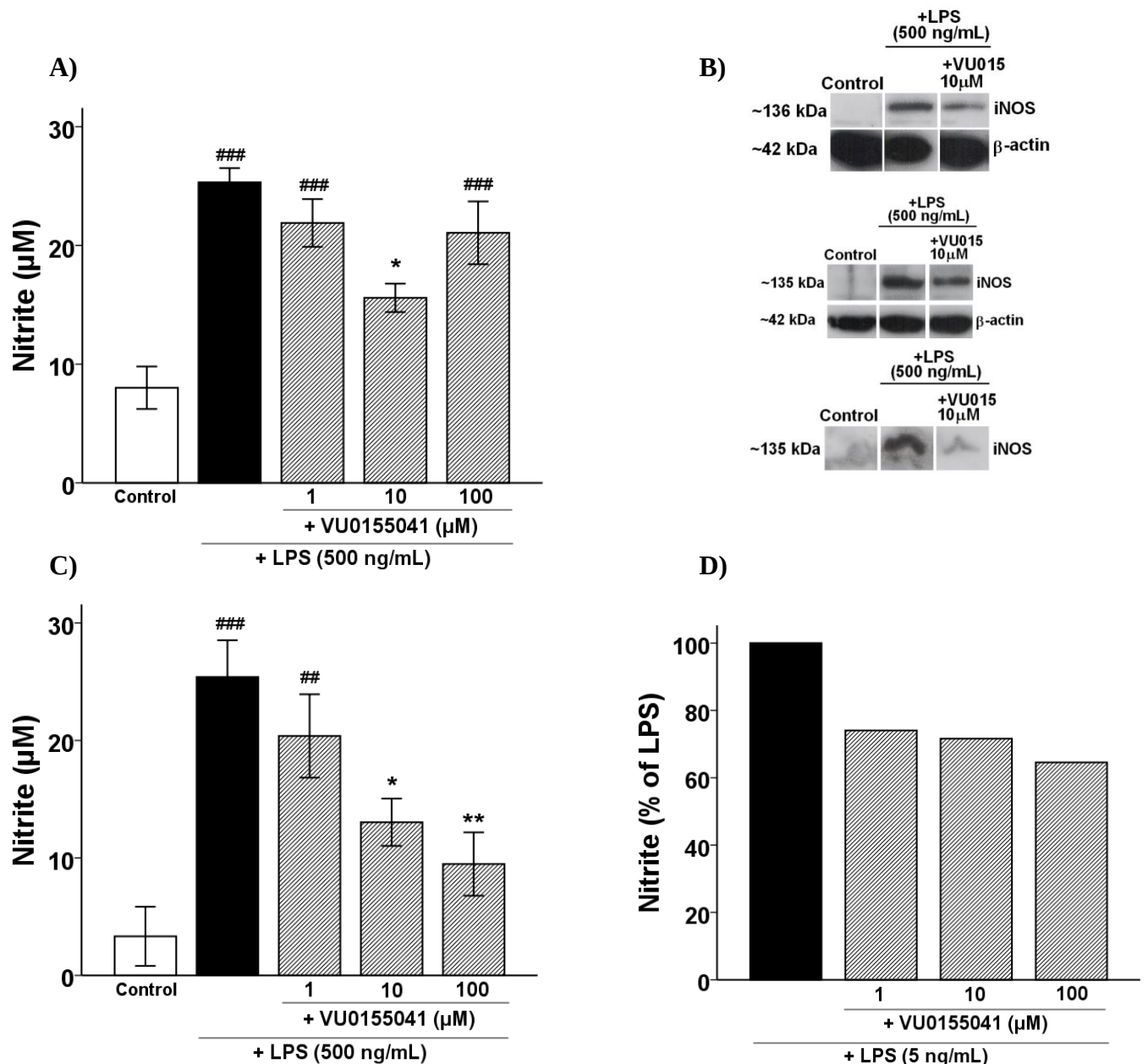


Figure 5.7 Effect of VU0155041 treatment on nitrite production and iNOS expression in LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with the mGlu4-specific positive allosteric modulator VU0155041 one hour following LPS activation, and nitrite concentration in the cell culture medium 18 hrs following LPS activation was quantified through the Griess assay (**A**). Expression of iNOS by N9 microglia at 18 hrs following LPS activation was assessed through immunolabelling of Western blots of microglial cell lysates from control or LPS (500 ng/mL)-activated N9 microglia with or without VU0155041 treatment one hour following LPS treatment. To confirm equal protein loading, Western blots were labelled with anti-β-actin (**B**). Nitrite concentration in the cell culture medium of N9 microglia treated with VU0155041 20 mins prior to LPS (500 ng/mL) activation were also quantified 18 hrs following LPS activation through the Griess assay (**C**). Similarly, nitrite production by N9 microglia was quantified 18 hrs following activation with 5 ng/mL LPS with or without VU0155041 treatment 1 hr following LPS activation (**D**). The effect of the general group III mGluR orthosteric antagonist M-SOP on VU0155041-stimulated decrease in nitrite production was investigated by treating LPS (500 ng/mL)-activated N9 microglia with M-SOP 20 mins prior to VU0155041 treatment (1 hr following LPS activation) and quantifying nitrite concentration in the culture medium through the Griess assay at 18 hrs following LPS exposure (**E**). Results are displayed as mean $\pm$ SEM of three (**A**, **C**, **E**) or one (**D**) independent experiments measured in triplicate. Images in (**B**) are from three independent experiments. ###, ## denote $p < 0.01$, 0.005 vs. control, respectively. *, **, *** denote $p < 0.05$, 0.01 , 0.005 vs. LPS, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 13.35$ (**A**), 10.25 (**C**).

VU0155041 treatment of N9 microglia 1 hr following LPS (500 ng/mL) activation induced an increase in IL-6 release 18 hrs following LPS treatment. Although there was a clear trend of increasing IL-6 release with increasing VU0155041 treatment, this did not reach statistical significance (**fig. 5.8A**). However, VU0155041 treatment 20 mins before LPS (500 ng/mL) activation led to a more pronounced and statistically significant enhancement of IL-6 release by N9 microglia 18 hrs following LPS treatment (**fig. 5.8B**). Similarly, VU0155041 treatment 1 hr following LPS (500 ng/mL) activation led to a marked and dose-dependent increase in IL-1 β release 18 hrs following LPS activation (**fig. 5.8C**). VU0155041 treatment of N9 microglia 1 hr following LPS activation failed to modulate TNF α release stimulated by both 500 ng/mL LPS (**fig. 5.8D**) and 5 ng/mL LPS (**fig. 5.8E**).

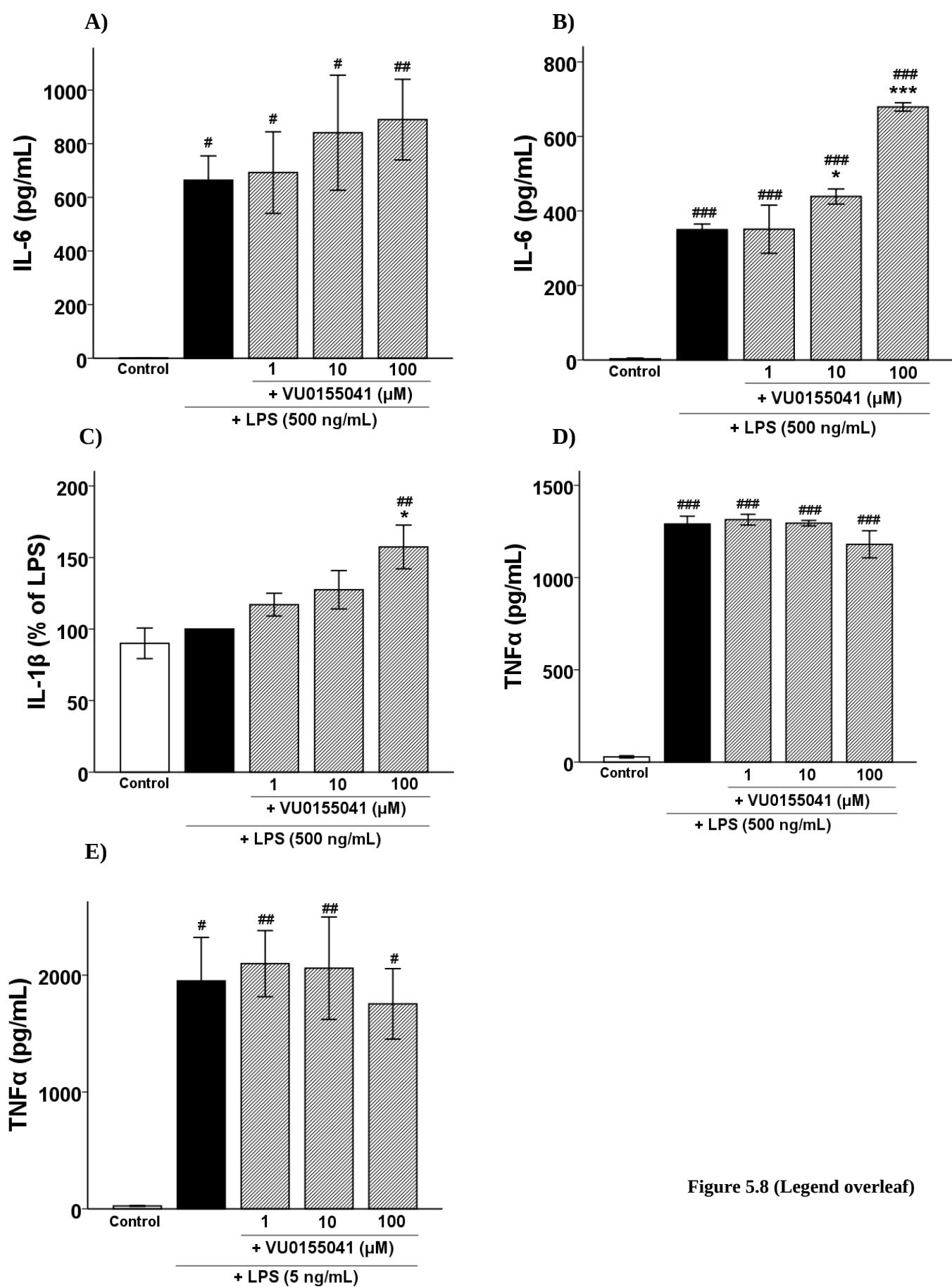


Figure 5.8 (Legend overleaf)

5.3.1.5 *Effect of AMN082 and VU0155041 co-treatment on nitrite production and iNOS*

expression by LPS-activated N9 microglia

Simultaneous treatment of N9 microglia with AMN082 and VU0155041 one hour following LPS (500 ng/mL) activation induced additive inhibition of nitrite production 18 hrs following LPS treatment which was lower than inhibition by treatment with either ligand alone (**fig. 5.9A**). An additive inhibitory effect of simultaneous AMN082 and VU0155041 treatment was also seen on iNOS protein expression 18 hrs following LPS treatment (**fig. 5.9B**). However, simultaneous AMN082 and VU0155041 treatment of N9 microglia 20 mins before LPS (500 ng/mL) activation failed to induce an additive inhibitory effect on nitrite production 18 hrs following LPS treatment, with comparable inhibition between combined treatment and VU0155041 treatment alone (**fig. 5.9C**).

5.3.1.6 *Effect of DCPG treatment on nitrite production by LPS-activated N9 microglia*

Treatment of N9 microglia with the mGlu8-specific orthosteric agonist DCPG one hour following LPS (500 ng/mL) treatment failed to significantly modulate nitrite production 18 hrs following LPS treatment (**fig. 5.10**).

Figure 5.8 Effect of VU0155041 treatment on IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia (plated at 600×10^3 cells per well in 6 well plates) were treated with the mGlu4-specific positive allosteric modulator VU0155041 1 hr following LPS (500 ng/mL) activation and release of IL-6 (**A**), IL-1 β (**C**) and TNF α (**D**) into the cell culture medium quantified through an ELISA at 18 hrs following LPS exposure. IL-6 release into the cell culture medium by LPS (500 ng/mL)-activated N9 microglia (plated at 450×10^3 cells per well in 6 well plates) was also quantified by an ELISA at 18 hrs following LPS activation when treated with VU0155041 20 mins prior to LPS activation (**B**). TNF α release by N9 microglia (plated at 600×10^3 cells per well in 6 well plates) treated with VU0155041 one hour following LPS (5 ng/mL) activation was quantified by an ELISA at 18 hrs after LPS activation (**E**). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05$, 0.01, 0.005 vs. control, respectively. *, ***, **** denote $p < 0.05$, 0.005 vs. LPS, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. F = 5.83 (A), 136.9 (B), 5.8 (C), 188.7 (D), 7.67 (E).

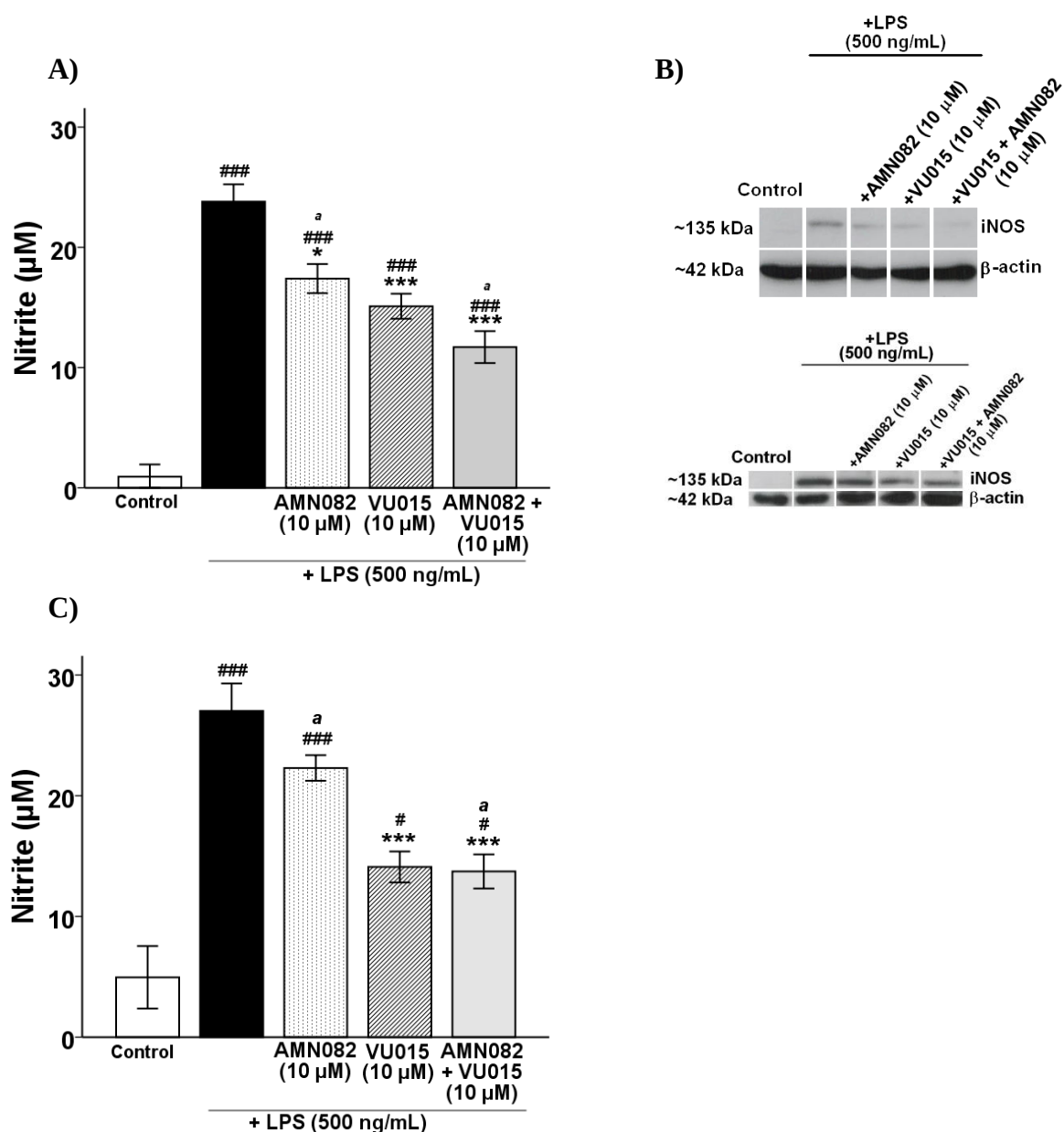


Figure 5.9 Effect of simultaneous AMN082 and VU0155041 treatment on nitrite production and iNOS expression by LPS-activated N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with the mGlu7-specific allosteric agonist AMN082, the mGlu4-specific positive allosteric modulator VU0155041 or both 1 hr following LPS activation. Nitrite concentration in the culture medium was quantified through the Griess assay at 18 hrs following LPS activation (**A**). iNOS expression at the same time-point was assessed by immunolabelling Western blots from cell lysates of control or LPS-activated N9 microglia treated with AMN082, VU0155041 or both 1 hr following LPS activation. To confirm equal protein loading, the Western blots were labelled with anti-β-actin (**B**). Nitrite concentration in the cell culture medium of N9 microglia treated with AMN082, VU0155041 or both 20 mins prior to LPS (500 ng/mL) activation was also quantified through the Griess assay at 18 hrs following LPS activation (**C**). Results are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate (**A**, **C**, **D**). Images in (**B**) are from two independent experiments. #, ### denote $p < 0.05$, 0.005 vs. control, respectively. *, *** denote $p < 0.05$, 0.005 vs. LPS, respectively. Letters denote $p < 0.05$ between corresponding conditions. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 20.1$ (**A**), 21.82 (**C**).

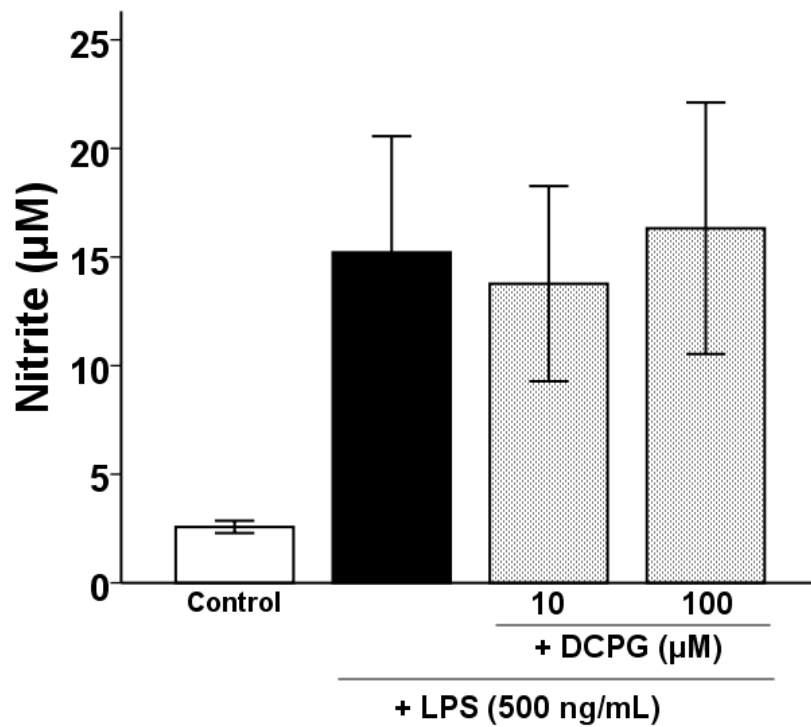


Figure 5.10 Effect of DCPG treatment on nitrite production by LPS-activated N9 microglia. N9 microglia were activated with LPS (500 ng/mL) for one hour prior to treatment with the mGlu8-specific orthosteric agonist DCPG. Nitrite concentration in the cell culture medium was quantified through the Griess assay at 18 hrs following LPS activation. Results are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 1.96$.

5.3.1.7 *Effect of mGluR ligand treatment on microglial cell viability*

N9 microglial cell viability 18 hrs following LPS activation was not affected by post-treatment with L-AP4, AMN082 or VU0155041 at the highest concentration employed to modulate microglial reactivity, as assessed by lack of effect on intracellular NADH (**fig. 5.11A**), trypan blue exclusion (**fig. 5.11B**), LDH release (**fig. 5.11C**) and cellular protein concentration (**fig. 5.11D**) compared to LPS treatment alone. However, at higher concentrations (100 μ M), AMN082 post-treatment led to a trend towards decreased N9 microglial cell viability, as assessed by decreased NADH levels (**fig. 5.11A**), significantly increased TB incorporation (**fig. 5.11B**) and reduced cellular protein (**fig. 5.11D**).

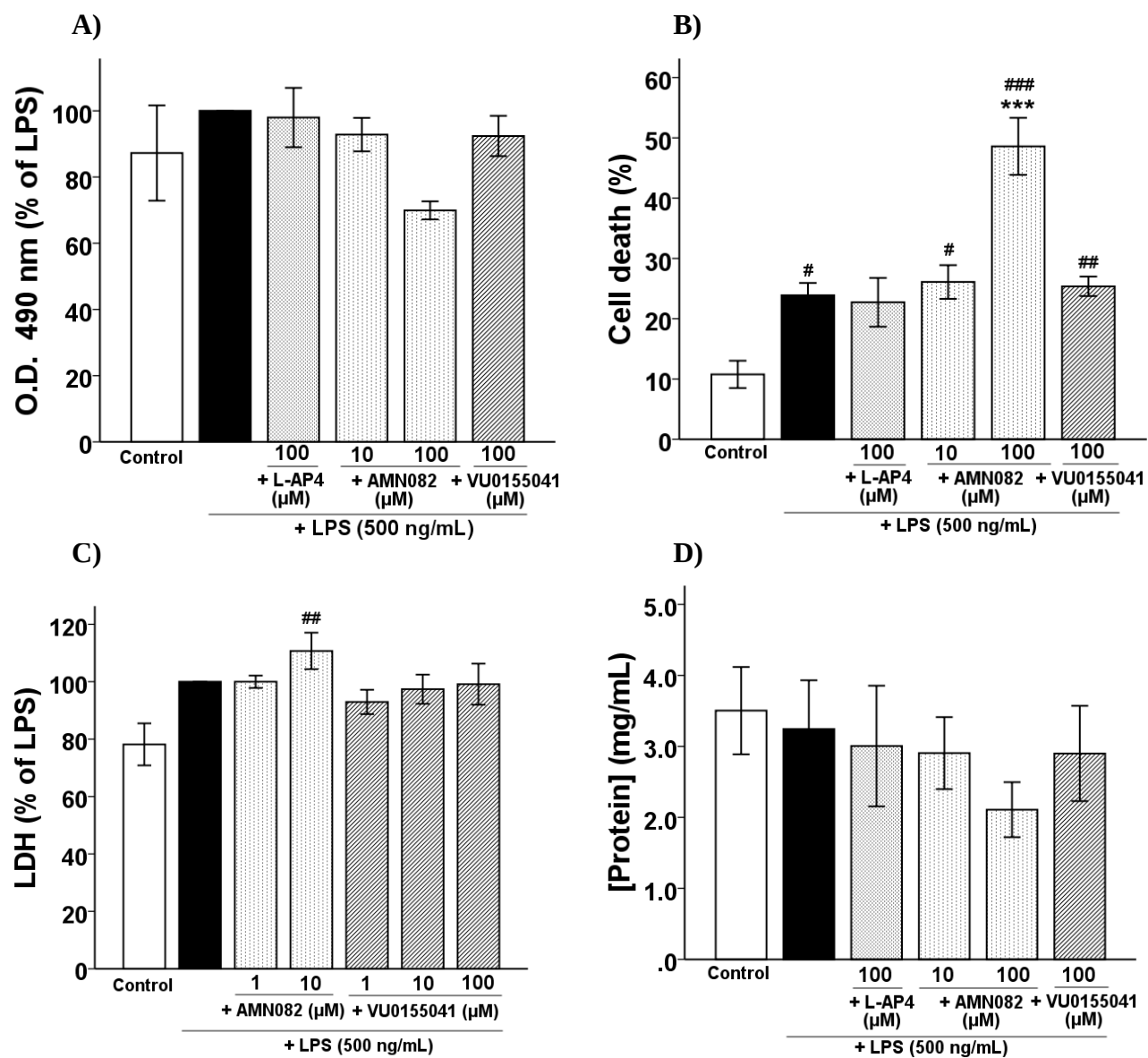


Figure 5.11 (Legend on following page)

Similarly, N9 microglial cell viability 18 hrs following LPS activation was not affected by simultaneous treatment with 10 μ M AMN082 and 10 μ M VU0155041, as assessed by lack of effect on NADH levels (**fig. 5.12A**) and cellular protein concentration (**fig. 5.12B**) compared to LPS treatment alone. N9 microglial cell viability 18 hrs following LPS activation was similarly not affected by treatment with M-SOP, as assessed by lack of effect on NADH levels compared to LPS treatment alone (**fig. 5.12C**).

Figure 5.11 Effect of L-AP4, AMN082 or VU0155041 treatment on N9 microglial cell viability. LPS (500 ng/mL)-activated N9 microglia were treated with the general group III mGluR orthosteric agonist L-AP4, the mGlu7-specific allosteric agonist AMN082 or the mGlu4-specific positive allosteric modulator VU0155041 one hour following LPS activation and cell viability assessed at 18 hrs after LPS activation. Cell viability was assessed by quantification of intracellular NADH through the MTS assay (**A**), assessment of membrane integrity through a trypan blue exclusion assay (**B**) and quantification of LDH release (**C**), and quantification of cellular protein content through the Bradford assay (**D**). Microglia were either plated in 96 well plates at 10×10^3 cells per well (**A**) or in 6 well plates at 600×10^3 cells per well (**B-D**). For trypan blue exclusion assays, floating and adhered cells were collected, incubated with trypan blue and cell death determined as the percentage of cells incorporating trypan blue. For cellular protein quantification, cells were lysed at the appropriate time-point and protein concentration in the lysates quantified through the Bradford assay. Results are displayed as $\pm$ SEM of three independent experiments measured in duplicate (**A,B, D**) or triplicate (**C**). #, ##, ### denote $p < 0.05$, 0.005 vs. control, respectively. *** denotes $p < 0.005$ vs. LPS, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 1.97$ (A), 18.53 (B), 3.5 (C), 0.55 (D).

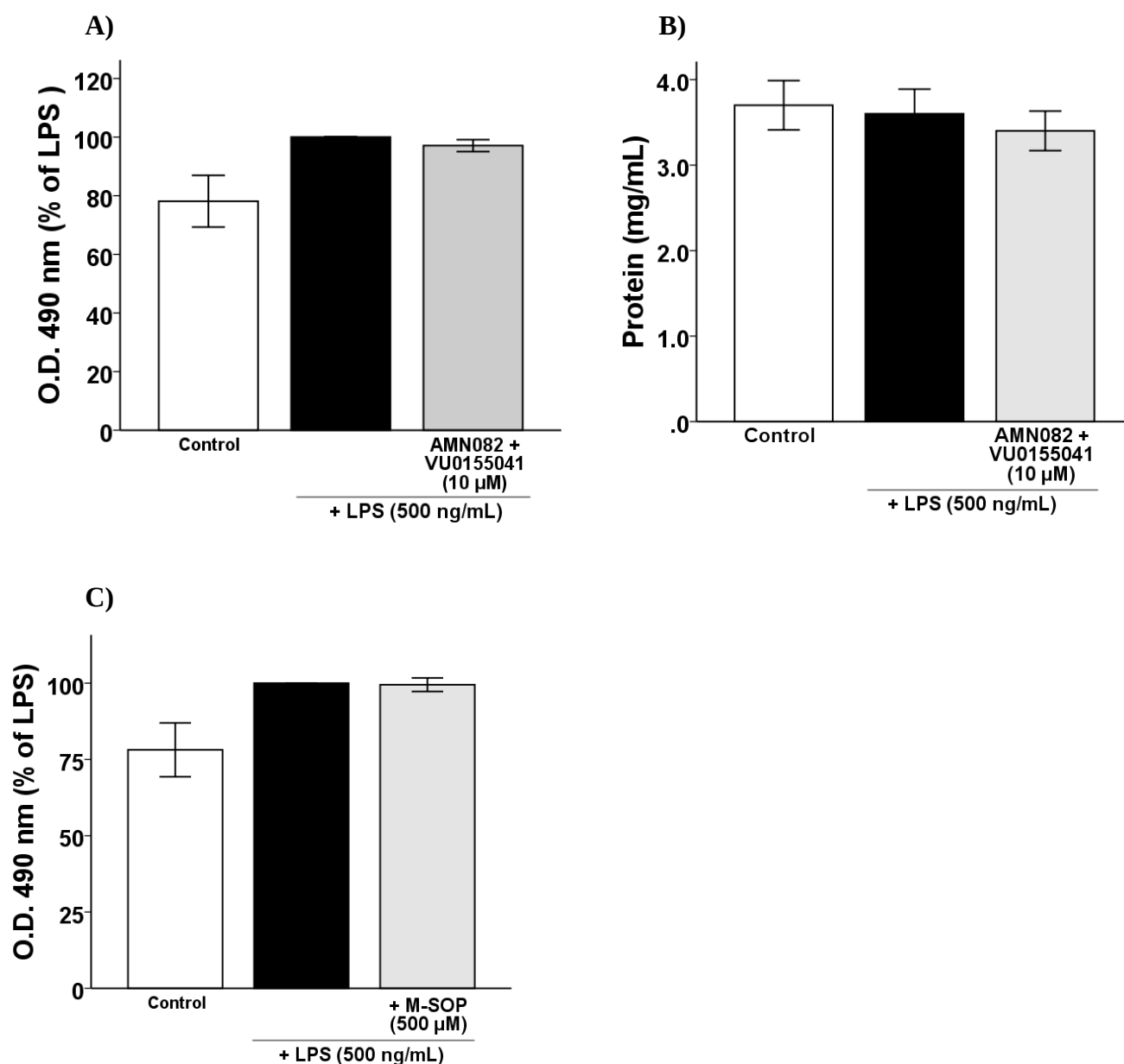


Figure 5.12 Effect of simultaneous AMN082 and VU0155041 treatment or M-SOP treatment on LPS-activated N9 microglial cell viability. LPS (500 ng/mL)-activated N9 microglia were simultaneously treated with the mGlu7-specific allosteric agonist AMN082 and the mGlu4-specific positive allosteric modulator VU0155041 one hour following LPS activation and intracellular NADH quantified through an MTS assay **(A)** or lysed to quantify cellular protein content through the Bradford assay **(B)** at 18 hrs following LPS activation. Similarly, LPS (500 ng/mL)-activated N9 microglia were treated with the general group III mGluR orthosteric antagonist M-SOP forty mins following LPS activation and NADH levels measured through an MTS assay at 18 hrs following LPS activation **(C)**. For MTS assays, N9 microglia were plated in 96 well plates at 10×10^3 cells per well. For protein quantification, N9 microglia were plated in 6 well plates at 600×10^3 cells per well. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. **##**, **###** denote $p < 0.01$, 0.005 vs. control. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 5.17$ (A), 0.37 (B), 5.66 (C).

5.4 Discussion

The present results clearly demonstrate that activation of individual group III mGluR is able to robustly modulate microglial reactivity even after microglial reactivity has been engaged.

5.4.1 N9 microglia activation is set in motion within 1 hr of LPS treatment

A one hour LPS treatment proved sufficient to engage the microglial inflammatory mechanism, as evidenced by an increase in nitrite production and a shift to an activated amoeboid morphology 18 hrs following LPS treatment, even if LPS was removed after 1hr treatment.

5.4.2 Nitric oxide synthesis is reduced by mGluR treatment

Treatment of N9 microglia with the group III general agonist L-AP4 one hour after LPS treatment resulted in a modest yet significant decrease in nitrite production, indicating nitric oxide (NO) synthesis had been reduced. The reduction in nitrite production was accompanied by a decrease in the expression of the NO-synthesizing enzyme iNOS, further suggesting a *bona fide* reduction in NO synthesis, as opposed to decreased nitrite accumulation or alternative NO metabolism (Ignarro, 1990; Marletta *et al*, 1988). Interestingly, L-AP4 failed to reduce nitrite production and iNOS expression at high concentrations (100 μ M), suggesting high L-AP4 concentrations may stimulate receptor internalization or stimulate inhibition of its signalling transduction mechanisms by a negative feedback loop (Iacovelli *et al.*, 2004). Furthermore, preliminary experiments indicated treating N9 microglia with L-AP4 20mins before LPS treatment resulted in a similar modest decrease in nitrite production which was reduced at higher L-AP4 concentrations.

Similarly to L-AP4 treatment, treatment of N9 microglia with the mGlu7-specific allosteric agonist AMN082 one hour following LPS treatment resulted in a robust and dose-dependent

decrease in nitrite production and iNOS protein expression. Unexpectedly, AMN082 treatment of N9 microglia 20mins before LPS treatment resulted in a less marked reduction in nitrite production which lost statistical significance.

The inhibitory effects of AMN082 on nitrite production were shown to be receptor-dependent, as the mGlu7-specific allosteric antagonist MMPIP (Suzuki *et al.*, 2007) reversed the AMN082-induced reduction in nitrite production. During MMPIP antagonism studies, N9 microglia produced lower levels of nitrite both under control and LPS-treatment conditions. These experiments were carried out with cells nearing their passage limit (25 passages), therefore the age of the culture could have affected their rate of nitrite production. Crucially however, the microglia still responded robustly to LPS-treatment with an increase in nitrite production, which could still be markedly inhibited by AMN082 treatment.

Similarly to AMN082, treatment of N9 microglia with the mGlu4-specific positive allosteric modulator VU0155041 stimulated a reduction in nitrite production. VU0155041 treatment 1 hr following LPS treatment induced a marked inhibition of nitrite production which decreased at higher VU0155041 concentrations (100 μ M). Although this effect could be explained by receptor internalization (Iacovelli *et al.*, 2004), this seems unlikely, as increasing VU0155041 concentrations did not lose efficacy with respect to modulation of IL-6 release (see below). VU0155041 treatment 20mins before LPS treatment induced a more pronounced reduction in nitrite production than treatment 1 hr following LPS treatment. Surprisingly, inhibition of nitrite production did not decrease with high VU0155041 concentrations under these conditions.

As with L-AP4 and AMN082, nitrite production inhibition by VU0155041 treatment one hour following LPS treatment was accompanied by a decrease in iNOS protein expression.

As expected, simultaneous treatment of N9 microglia with AMN082 and VU0155041 one hour following LPS treatment inhibited nitrite production and iNOS protein expression more robustly than treatment with either ligand independently, indicating additive NO-synthesis inhibition by

simultaneous activation of mGlu4 and mGlu7. However, simultaneous AMN082 and VU1055041 treatment 20 mins before LPS treatment inhibited nitrite production to an equal extent than treatment with VU0155041 alone, reflecting the decreased potency of AMN082 and the increased potency of VU0155041 to inhibit nitrite production under pre-treatment conditions.

Unlike L-AP4, AMN082 or VU0155041, treatment of N9 microglia with the mGlu8-specific orthosteric agonist DCPG one hour following LPS treatment failed to modulate nitrite production, perhaps reflecting the inability to detect mGlu8 expression in N9 microglia in this thesis (see previous chapter).

5.4.3 Metabotropic glutamate receptor ligands differentially modulate pro-inflammatory cytokine release

While treatment of N9 microglia with L-AP4 one hour following LPS treatment failed to modulate IL-6 release, activation of individual group III mGluR differentially modulated IL-6 release. Accordingly, treating N9 microglia with AMN082 one hour following LPS treatment stimulated a marked decrease in IL-6 release. Furthermore, AMN082 treatment 20 mins before LPS treatment resulted in a more pronounced decrease in IL-6 release. In contrast, treatment with VU0155041 one hour following LPS treatment stimulated a marked increase in IL-6 release, though this did not reach statistical significance. However, treatment with VU0155041 20mins before LPS treatment led to a robust and statistically significant enhancement of IL-6 release. The opposing effects of mGlu4 and mGlu7 activation on LPS-stimulated IL-6 release could account for the lack of effect of L-AP4 on IL-6 release, as L-AP4 would activate both receptors and hence cancel out any effect on IL-6 release. Although the enhancing effect of VU0155041 on IL-6 release was weaker than the inhibitory effect of AMN082 at the same concentration, L-AP4 is known to activate mGlu4 with a potency ~100x greater than mGlu7 (reviewed by Cartmell and Schoepp, 2000).

While LPS treatment successfully up-regulated NO/iNOS, IL-6 and TNF α production in N9 microglia, it caused only a modest increase in IL-1 β release which did not reach statistical significance. Although it is well established IL-1 β expression in microglia may be enhanced by LPS treatment (*e.g.* Bachstetter *et al.*, 2011; Nakamura *et al.*, 1999), previous studies with immortalized microglia have shown that LPS is ineffective at stimulating IL-1 β release under certain circumstances (Nagai *et al.*, 2001).

TNF α release stimulated by 500 ng/mL LPS was not affected by treatment with L-AP4, AMN082 or VU0155041 one hour after LPS treatment. Interestingly however, if TNF α release was stimulated by a lower LPS dose (5 ng/mL), AMN082 treatment induced a marked decrease in TNF α release. On the other hand, similar treatment with VU0155041 or L-AP4 failed to modulate TNF α release stimulated by 5 ng/mL LPS. Interestingly, TNF α release stimulated by 5 ng/mL LPS was comparable to TNF α release stimulated by 500 ng/mL. As was established in chapter III, LPS-stimulated TNF α release was comparable through the LPS concentration range examined (50-1000 ng/mL) (**see fig 3.5**). Therefore, TNF α release in N9 microglia may become saturated with LPS concentrations even as low as 5 ng/mL. Interestingly, comparable stimulation of TNF α release by a wide range of LPS concentrations (100 ng/mL-5 μ g/mL) was also seen in primary microglia (**see fig. 3.1B**). Therefore, 500 ng/mL LPS may have over-stimulated TNF α release to such an extent as to mask the effects of mGluR ligands, which would come to light under a decreased LPS stimulation. From the present results, it cannot be determined if the decrease in TNF α release by AMN082 was due solely to a decrease in release, or if this was a result from decreased protein synthesis or decreased gene transcription.

Hence, the results presented in this chapter demonstrate that, despite putatively sharing signalling mechanisms, activation of mGlu4 and mGlu7 will affect microglial phenotype differently. Interestingly, a recent study investigating the effects of L-AP4, AMN082 or VU0155041 treatment

on anaesthesia-induced neurotoxicity on cultured primary hippocampal neurons demonstrated treatment with L-AP4 (up to 50 μ M) or AMN082 (up to 10 μ M) prevented neuronal cell death induced by the anaesthetic sevoflurane, while treatment with VU0155041 (up to 100 μ M) did not affect neuronal cell death, again demonstrating the differential cellular effects of group III mGluR despite their putative shared signalling mechanism (Wang *et al.*, 2012).

5.4.4 Reduction in N9 microglial reactivity by mGluR ligands was not due to reduced cellular viability

In order to verify that the reduction in NO synthesis and cytokine release was due to specific reduction in microglial activation and not due to decreased cellular viability, N9 microglia were subjected to two independent viability assays following LPS and ligand treatment. Cell viability was not affected by any mGluR ligand at concentrations employed for modulation of microglial reactivity, as assessed by comparable NADH levels and trypan blue exclusion. Therefore, mGluR ligand treatment reduces microglial activation without reducing cell viability. Interestingly, LPS treatment induced a significant increase in the percentage of cells incorporating trypan blue. Although this could be interpreted as an increase in cell death, this does not seem to be the case, as LPS did not reduce cellular protein content. Instead, the increase in percentage of cells incorporating trypan blue can be attributed to an increase in microglial proliferation, which would lead to an increase in accumulation of 'spent' cells in the culture medium. This interpretation is supported by the fact that cellular metabolism was increased upon LPS treatment. Indeed, it is known that microglial activation may lead to an increase in microglial proliferation (Ooi *et al.*, 2010; Gerhmann *et al.*, 1994).

5.5 Conclusion

Taken together, the results within this chapter support our hypothesis regarding modulation of N9 microglial reactivity by treatment with group III mGluR ligands. Furthermore, they demonstrate mGluR ligands have the capacity to modulate microglial activation even after microglial activation has been engaged. Previous studies utilizing mGluR ligands to modulate microglial activation have employed simultaneous treatment of microglia with activating compounds (*e.g.* LPS, myelin) and mGluR ligands (*e.g.* Taylor *et al.*, 2003, 2005; Pinteaux-Jones *et al.*, 2008). Therefore, the results within this chapter offer novel insight into the potential of mGluR as modulators of microglial activation in neurodegenerative diseases where chronic microglial activation is thought to take place.

In addition, the present results add to our original hypothesis in that they clearly demonstrate that microglial reactivity is differentially modulated by individual group III mGluR. Hence, activation of mGlu7 was able to reduce all parameters of microglial activation examined, while activation of mGlu4 inhibited NO/iNOS synthesis, but had no effect on TNF α release and actually significantly increased IL-6 and IL-1 β release.

Therefore, it is important to carefully select which mGluR to target if modulation of these receptors is to be developed as a viable therapeutic strategy. However, future work will have to also examine the effects of individual group III mGluR activation on expression of other important reactive oxygen species (*e.g.* superoxide), as well as their effect on the neurotrophic potential of microglia by examining their effect on expression of important factors for neuronal survival (*e.g.* GDNF).

Chapter VI

Modulation of microglia-mediated
neurotoxicity by mGluR ligands

6. Modulation of microglial neurotoxicity by group III mGluR

6.1 Introduction

A growing body of evidence suggests inflammation and microglial activation plays an important role in the chronic and progressive neuronal loss seen in neurodegenerative diseases such as Parkinson's disease (PD). For instance, blockade of microglial reactivity with minocycline reduces neuronal degeneration both in the MPTP and the 6-OHDA animal models of PD (Wu *et al.*, 2002; He *et al.*, 2001). Similarly, intranigral LPS injection induces loss of dopaminergic neurons in the rat substantia nigra (SN) (Castaño *et al.*, 1998). In addition, regular use of the non-steroidal anti-inflammatory drug Ibuprofen is associated with a lower risk of developing PD (Gao *et al.*, 2011; Chen *et al.*, 2005). Furthermore, minocycline treatment in humans has shown modest therapeutic potential in phase II clinical trials (NINDS NET-PD study, 2006, 2008).

Activated microglia are capable of increasing nitric oxide production and release of pro-inflammatory factors such as TNF α , IL-6 and IL-1 β in order to kill and clear invading pathogens. However, excessive production of these factors, as is the case during chronic microglial activation, may lead to collateral neuronal damage and neurodegeneration. For instance, *in vitro* studies have shown TNF α is able to induce death of cultured hippocampal (Yang *et al.*, 2002) and cerebellar granule neurons (Taylor *et al.*, 2005). Furthermore, *in vitro* LPS treatment of mesencephalic glia/neuron cultures leads to specific dopaminergic neuronal loss in a TNF α -dependent manner (McCoy *et al.*, 2006). Similarly, *in vivo* studies have shown overexpression of TNF α in the SN of rats leads to progressive loss of TH⁺ neurons (De Lella Ezcurra *et al.*, 2010). Importantly, TNF α expression has been shown to be up-regulated in PD patients (Mogi *et al.*, 1994), and specifically in glial cells of the SN of PD patients (Boka *et al.*, 1994).

Similarly, IL-6 levels have been found to be elevated in the striatum (Mogi *et al.*, 1994) and cerebrospinal fluid (CSF) (Muller *et al.*, 1998) of PD. Furthermore, elevated serum IL-6 levels have been found in PD patients, which correlate with decreased physical performance (Scalzo *et al.*,

2010). Importantly, IL-6 signalling has been shown to promote microgliosis both *in vitro* (Shafer *et al.*, 2002; Streit *et al.*, 2000) and *in vivo* (Pang *et al.*, 2006; Galiano *et al.*, 2001). Furthermore, direct IL-6 treatment of cultured cerebellar granule neurons enhances neuronal cell death (Conroy *et al.*, 2004; Qiu *et al.*, 1998). Similarly, incubation of motor neurons with medium derived from IL-6 treated rat microglia reduces neuronal survival (Kradky *et al.*, 2008).

In addition to pro-inflammatory cytokines, nitric oxide has also been associated with the progressive neurodegeneration seen in PD. For instance, there is higher expression of inducible nitric oxide synthase (iNOS) (Knott *et al.*, 2000; Hunot *et al.*, 1996) and higher levels of nitrated proteins (Good *et al.*, 1998; Duda *et al.*, 2000) in the SN of PD patients and MPTP-treated animals (Ferrante *et al.*, 1999; Pennathur *et al.*, 1999). Furthermore, disruption of iNOS activity is able to provide neuroprotection in various *in vivo* models of neurodegeneration (Ladecola *et al.*, 1997; Liberatore *et al.*, 1999; Broom *et al.*, 2011).

The neurotoxic effects of NO are primarily mediated through nitration and impairment of proteins important for cell survival. Indeed, several proteins associated with PD (*e.g.* α -synuclein, parkin, DJ-1, mitochondrial complex-I) have been shown to be able to be nitrated by NO or its derivatives *in vitro* and *in vivo* (Good *et al.*, 1998; Przedborski *et al.*, 2001), generating highly stable species resistant to degradation by proteolysis (Souza *et al.*, 2000), loss of neuroprotective function (Chung *et al.*, 2004) or decreased mitochondrial electron transport chain function (Clementi *et al.*, 1998; Dahm *et al.*, 2006; Chinta *et al.*, 2007).

Apart from directly damaging cellular components, NO is capable of stimulating neuronal cell death through promotion of phagocytosis by microglia. Accordingly, release of the NO-derivative peroxynitrite from activated microglia is able to promote exposure of phosphatidylserine (PS) in membranes of healthy neurons, leading to microglial phagocytosis of PS-exposing neurons and hence neuronal loss (Neher *et al.*, 2011). Interestingly, blockade of microglial phagocytosis

prevented removal of PS-exposing neurons and subsequent survival of neurons, indicating microglia were releasing sub-lethal levels of peroxynitrite which did not constitutively damage neurons.

In addition to promoting neuronal death, nitration of neuronal proteins associated with PD has been shown to be able to induce microglial activation and neurotoxicity (Reynolds *et al.*, 2008a, b). Therefore, NO production by activated microglia may establish a feed-forward mechanism resulting in increased levels of nitrated proteins, increased microglial activation, and hence increased NO production.

Given the fact that pro-inflammatory factors produced by activated microglia are able to induce neuronal degeneration, this chapter sets out to determine if the effects of mGluR ligands on microglial NO/iNOS production and IL-6, IL-1 β and TNF α release established in the previous chapter translates to modulation of microglial neurotoxicity *in vitro*.

- **Hypothesis:** The modulation of N9 microglial reactivity and release of pro-inflammatory factors by ligands targeting individual group III metabotropic glutamate receptors translates into modulation of microglial toxicity towards dopaminergic neurons.

6.2 Experimental design

The neuronal cell lines SH-SY5Y and N27 (see section 2.1.5) were employed in this thesis to examine microglial neurotoxicity. The SH-SY5Y cell line is an immortalized undifferentiated human neuroblastoma cell line capable of differentiating fully into a dopaminergic neuronal phenotype. The N27 cell line is a viral vector immortalized dopaminergic cell line derived from fetal rat midbrain cells. SH-SY5Y cells were purchased from the Health Protection agency. N27 cells were a kind gift from Dr. Nabil Hajji, Imperial College London.

In order to investigate whether the effects of mGluR ligands on N9 microglial reactivity translated to modulation of neuronal viability, neuronal cells were incubated for 48 hrs with

complete microglial medium (see section 2.1.1) derived from N9 microglial cultures (*i.e.* microglial conditioned medium, MCM) activated with LPS (LPS-MCM) with or without mGluR ligand treatment (mGluR/LPS-MCM) (see section 2.1.8), and neuronal viability compared to neurons incubated with MCM derived from control (non-activated) N9 microglia (C-MCM). The same LPS activation and mGluR treatment protocols employed in previous chapters (see section 5.2) were followed for neurotoxicity studies. The effects of mGluR treatment both 1 hr after or 20 minutes before LPS activation on microglial neurotoxicity were examined. In order to avoid transfer of microglial cells into the neuronal cultures, the MCM was cleared of floating microglial cells by centrifugation (600g x 10 minutes) prior to transferring it to neuronal cultures.

As group III mGluR are believed to decrease cAMP production, the effects of increased cAMP production on microglial neurotoxicity were examined by treating LPS (100 ng/mL)-activated microglia with the adenylyl cyclase activator forskolin (for a detailed description of forskolin protocols, see chapter VII and section 2.1.18).

Neuronal viability was assessed after 48 hours incubation with MCM through quantification of intracellular NADH levels and cellular protein content through MTS and Bradford assays, respectively (see sections 2.1.12 and 2.1.14, respectively). In addition, neuronal cell death was confirmed by assessment of cellular membrane integrity through quantification of released neuronal LDH (see section 2.1.13). Briefly, neuronal medium was transferred to a 96 well plate (10 μ L in triplicate) and incubated with LDH reaction mixture to evaluate LDH activity (LDH activity was taken as directly proportional to LDH concentration). To control for low LDH in the culture medium due to low cell number owing to cell death, released LDH was normalized to total LDH in the culture well (*i.e.* intracellular plus released LDH). Total LDH was quantified by complete lysis of neurons through addition of lysing buffer and quantifying LDH in the culture medium as above.

In order to examine the direct effects of mGluR ligands and LPS carried over in the MCM on neuronal viability, mGluR ligands and LPS were incubated with complete microglial medium in the

absence of N9 microglia for 18 hrs before transfer to neurons, and neuronal viability assessed after 48 hrs as above.

As treatment of N9 microglia with VU0155041 led to a marked increase in IL-6 release, the role of direct neuronal treatment with IL-6 on neuronal viability was also examined. Employing recombinant mouse IL-6 protein, neurons were incubated with increasing IL-6 concentrations in complete microglial medium (48 hrs) and cellular viability assessed as above. In addition, to examine whether IL-6 enhanced the effects of other microglial factors release by LPS-activated N9 microglia, neurons were incubated (48 hrs) with LPS-MCM in the presence of increasing IL-6 concentrations, and cellular viability assessed through quantification of cellular protein content.

6.3 Results

6.3.1 Microglial neurotoxicity is differentially modulated by individual group III mGluR

6.3.1.1 Effect of LPS treatment on N9 microglial neurotoxicity towards N27 and SH-SY5Y neurons

Incubation of N27 neurons (48 hrs) with microglial conditioned medium (MCM) derived from LPS (500 ng/mL)-activated N9 microglia (LPS-MCM) led to a marked decrease in neuronal viability as assessed by decreased NADH levels compared to neurons incubated with MCM from control N9 microglia (C-MCM) (70.8% of C-MCM response) (**fig. 6.1**). Incubation of SH-SY5Y neurons with LPS-MCM for an equal time period failed to induce neuronal cell death, as assessed by equal NADH levels between neurons incubated with LPS-MCM and C-MCM (**fig. 6.1**).

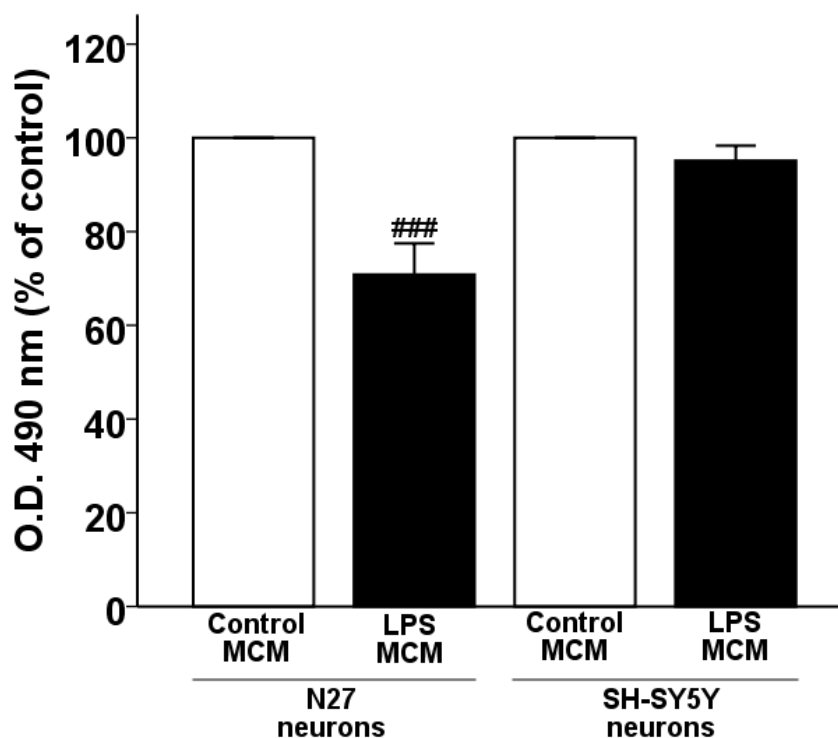


Figure 6.1 Effect of LPS treatment on N9 microglial neurotoxicity towards the SH-SY5Y and N27 dopaminergic cell lines. The effect of LPS treatment on microglial neurotoxicity towards the undifferentiated neuronal cell line SH-SY5Y or the dopaminergic neuronal cell line N27 was investigated by incubating (48 hrs) neurons with microglial conditioned medium (MCM) derived from control or LPS (500 ng/ml)-activated N9 microglia and quantifying NADH levels through an MTS assay. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. ### denotes $p < 0.005$ vs. respective C-MCM. Statistical significance was determined by one way ANOVA with Tukey's post-hoc test. $F = 14.31$.

6.3.1.2 Effect of L-AP4 treatment on LPS-stimulated N9 microglial neurotoxicity

Preliminary experiments indicated treatment of N9 microglia with the group III-wide agonist L-AP4 one hour following LPS (500 ng/mL) activation did not affect N9 microglial neurotoxicity towards N27 neurons, as assessed by lack of difference in NADH levels (**fig. 6.2A**) or neuronal protein content (**fig. 6.2B**) between neurons incubated (48 hrs) with LPS-MCM and neurons incubated with MCM derived from LPS and L-AP4 treated N9 microglia (L-AP4/LPS-MCM).

6.3.1.3 Effect of AMN082 treatment on LPS-stimulated N9 microglial neurotoxicity

Treatment of N9 microglia with the mGlu7-specific allosteric agonist AMN082 one hour following LPS (500 ng/mL) activation induced a slight reduction in microglial neurotoxicity towards N27 neurons, as assessed by a modest increase in NADH levels in neurons incubated (48 hrs) with MCM derived from LPS and AMN082 treated N9 microglia (AMN082/LPS-MCM) compared to neurons incubated with LPS-MCM (**fig. 6.3A**). Although AMN082 treatment failed to reach a statistically significant difference with respect to LPS-MCM, it did eliminate the statistically significant difference with respect to C-MCM. Direct treatment of N27 neurons with 10 μ M AMN082 (48 hrs) did not affect neuronal NADH levels compared to C-MCM (**fig. 6.3A**). Similarly, though there was a slight increase in cellular protein content in N27 neurons incubated (48 hrs) with AMN082/LPS-MCM compared to N27 neurons incubated with LPS-MCM, this failed to reach statistical significance compared to LPS-MCM (**fig. 6.3B**). However, statistical significance with respect to C-MCM was eliminated. Direct treatment of N27 neurons (48 hrs) with 10 μ M AMN082 did not affect cellular protein content compared to N27 neurons incubated (48 hrs) with C-MCM (**fig. 6.3B**).

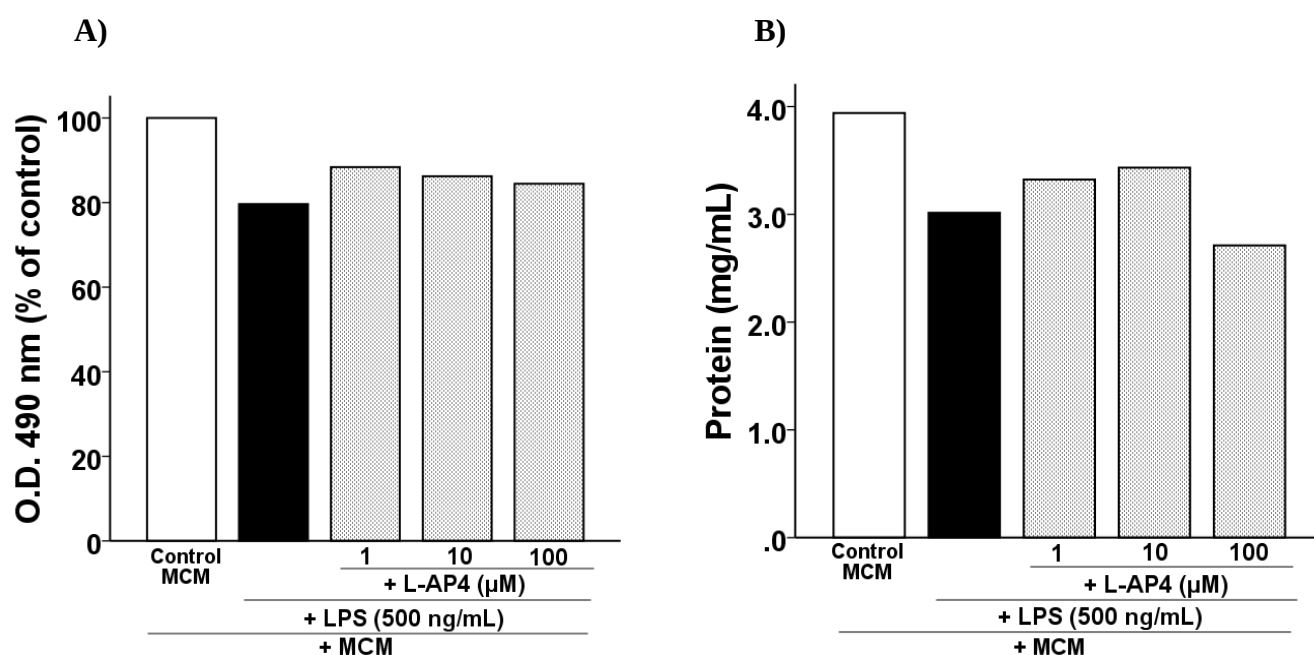


Figure 6.2 Effect of L-AP4 treatment on LPS-stimulated N9 microglial neurotoxicity. N27 dopaminergic neurons were incubated (48 hrs) with microglial conditioned medium (MCM) derived from LPS (500 ng/mL)-activated N9 microglia treated or not with the general group III mGluR orthosteric agonist L-AP4, and neuronal viability assessed through quantification of NADH through the MTS assay **(A)** and cellular protein content of cell lysates through the Bradford assay **(B)**. Results are displayed as mean of two independent experiments measured in duplicate.

Similarly, AMN082 treatment of LPS (500 ng/mL)-activated microglia failed to reduce neurotoxicity towards N27 neurons as assessed by comparable LDH release between neurons incubated with AMN082/LPS-MCM and neurons incubated with LPS-MCM (**fig. 6.3C**). Incubation of N27 neurons with LPS (500 ng/mL) and AMN082 (10 μ M) did not increase LDH release compared to neurons incubated with C-MCM (**fig. 6.3C**). However, while AMN082 treatment of N9 microglia failed to modulate microglial neurotoxicity elicited by 500 ng/mL LPS activation, treatment with AMN082 one hour after LPS activation led to a marked and statistically significant reduction in microglial neurotoxicity on N27 neurons when microglial activation was stimulated by 5 ng/mL LPS, as assessed by reduced LDH release from neurons incubated (48 hrs) with AMN082/LPS-MCM compared to neurons incubated with LPS-MCM (a reduction of 12.1 percentage points) (**fig. 6.3D**).

Treatment of N9 microglia with AMN082 twenty minutes before LPS (500 ng/mL) activation did not affect LPS-stimulated N9 microglial neurotoxicity on N27 neurons. NADH levels were comparable between N27 neurons incubated (48 hrs) with MCM from AMN082-pretreated LPS-activated N9 microglia (pre-AMN082/LPS-MCM) and N27 neurons incubated with LPS-MCM (**fig. 6.3E**). Similarly, cellular protein content was comparable between N27 neurons incubated with pre-AMN082/LPS-MCM and LPS-MCM (**fig. 6.3F**).

6.3.1.4 Effect of VU0155041 treatment on LPS-stimulated N9 microglial neurotoxicity

Treatment of N9 microglia with the mGlu4-specific positive allosteric modulator VU0155041 one hour following LPS (500 ng/mL) activation led to a significant increase in microglial neurotoxicity. Incubation of N27 neurons (48 hrs) with MCM from N9 microglia treated with 100 μ M VU0155041 one hour following LPS (500 ng/mL) activation (100 μ M VU0155041/LPS-MCM) led to a marked decrease in NADH levels compared to N27 neurons incubated with LPS-MCM (~

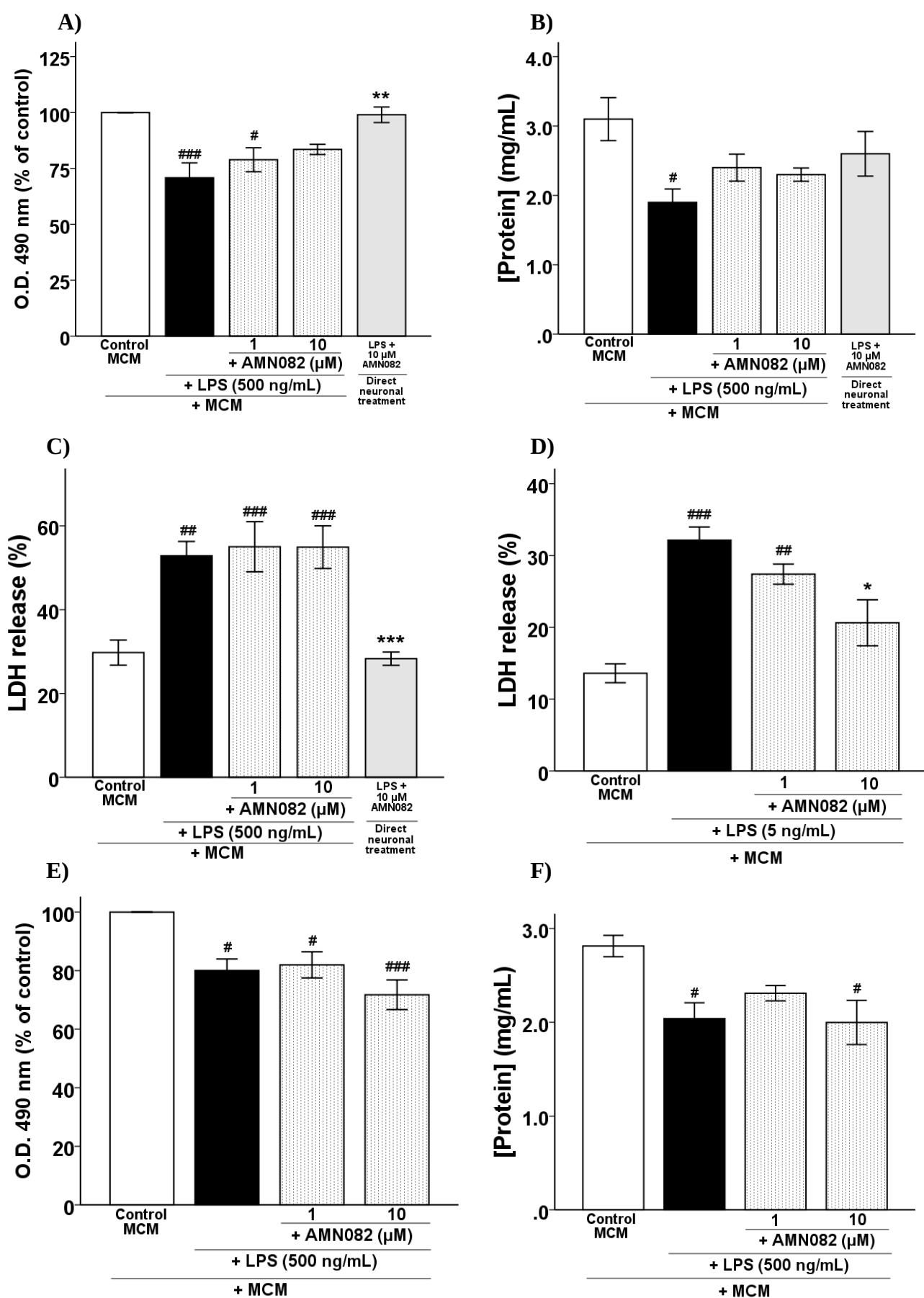


Figure 6.3 (Legend on following page)

60% of LPS-MCM response). However, lower VU1055041 concentrations (1 and 10 μ M) did not affect NADH levels (**fig. 6.4A**). Incubation of N27 neurons (48 hrs) with 100 μ M VU0155041 and LPS (500 ng/mL) did not affect NADH levels compared to N27 neurons incubated with C-MCM (**fig. 6.4A**). Similarly, incubation of N27 neurons (48 hrs) with 100 μ M VU0155041/LPS-MCM led to a marked decrease in cellular protein content compared to N27 neurons incubated with LPS-MCM (46.8% of LPS response). However, though incubating neurons with LPS-MCM did result in lower protein content, this failed to reach statistical significance. Again, lower VU0155041 concentrations (1 and 10 μ M) did not affect cellular protein content (**fig. 6.4B**). Incubation of N27 neurons (48 hrs) with 100 μ M VU0155041 and LPS did not affect cellular protein content compared to incubation with C-MCM (**fig. 6.4B**). In addition, neuronal cell death was confirmed by an increase in released LDH from N27 neurons incubated with 100 μ M VU0155041/LPS-MCM compared to neurons incubated with LPS-MCM (~ 14 percentage points increase). At lower concentrations (1 and 10 μ M), VU0155041 treatment failed to increase LDH release (**fig. 6.4C**). Incubation of neurons (48 hrs) with 100 μ M VU0155041 and LPS did not increase LDH release compared to neurons incubated with C-MCM (**fig. 6.4C**).

Treatment of N9 microglia with VU0155041 twenty minutes before LPS (500 ng/mL) activation led to a similar increase in microglial neurotoxicity on N27 neurons compared to VU0155041

Figure 6.3 Effect of AMN082 treatment on LPS-stimulated N9 microglial neurotoxicity. N27 dopaminergic neurons were incubated (48 hrs) with microglial conditioned medium (MCM) derived from LPS-activated N9 microglia (activated with 500 ng/mL LPS (A-C) or 5 ng/mL LPS (D)) treated or not with the mGlu7-specific allosteric agonist AMN082 one hour following LPS activation, and neuronal viability assessed through quantification of NADH through the MTS assay (**A**), cellular protein content through the Bradford assay (**B**) and assessment of membrane integrity through LDH release assays (**C, D**). In order to examine direct neuronal effects of AMN082 and LPS carried over in the culture medium, N27 neurons were also incubated (48 hrs) with medium supplemented with AMN082 and LPS. Neuronal viability was also assessed through the MTS assay (**E**) and Bradford assay (**F**) when incubated (48 hrs) with MCM derived from LPS (500 ng/mL)-activated N9 microglia treated or not with AMN082 twenty minutes prior to LPS activation. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05$, 0.01, 0.005 vs. C-MCM, respectively. *, **, *** denote $p < 0.05$, 0.01, 0.005 vs. LPS-MCM, respectively. Statistical significance was determined by a one-way ANOVA with Tukey's *post-hoc* test. $F = 9.05$ (A), 3.4 (B), 11.32 (C), 15.08 (D), 9.27 (E), 5.44 (F).

treatment one hour following LPS activation. Incubation of N27 neurons with MCM from N9 microglia treated with 100 μ M VU0155041 twenty minutes before LPS (500 ng/mL) activation (100 μ M pre-VU0155041/LPS-MCM) led to a marked decrease in NADH levels compared to N27 neurons incubated with LPS-MCM. However, incubation with MCM from N9 microglia treated with lower VU0155041 concentrations (1 and 10 μ M) did not affect microglial neurotoxicity (**fig. 6.4D**). Similarly, incubation (48 hrs) of N27 neurons with pre-VU0155041/LPS-MCM led to a pronounced trend of decreasing cellular protein content compared to N27 neurons incubated with LPS-MCM, though this failed to reach statistical significance (**fig. 6.4E**).

6.3.1.5 *Effect of forskolin treatment on LPS-stimulated N9 microglial neurotoxicity*

Simultaneous treatment of N9 microglia with forskolin and LPS (100 ng/mL) resulted in a significant reduction in microglial neurotoxicity on N27 neurons, as evidenced by reduced LDH release from neurons incubated (48 hrs) with MCM derived from N9 microglia treated with LPS (100 ng/mL) and forskolin (100 μ M) (forskolin/LPS-MCM) compared to neurons incubated with LPS-MCM (7.1 percentage point decrease) (**fig. 6.5**).

6.3.1.6 *Effect of IL-6 treatment on N27 neuronal viability*

In order to examine the role of IL-6 on microglial neurotoxicity, N27 neurons were incubated (48 hrs) with increasing concentrations of recombinant IL-6 protein and cellular viability assessed through quantification of intracellular NADH and cellular protein content. IL-6 treatment failed to affect neuronal NADH levels (**fig. 6.6A**) and cellular protein content (**fig. 6.6B**) through all concentrations examined (5 pg/mL- 40 ng/mL).

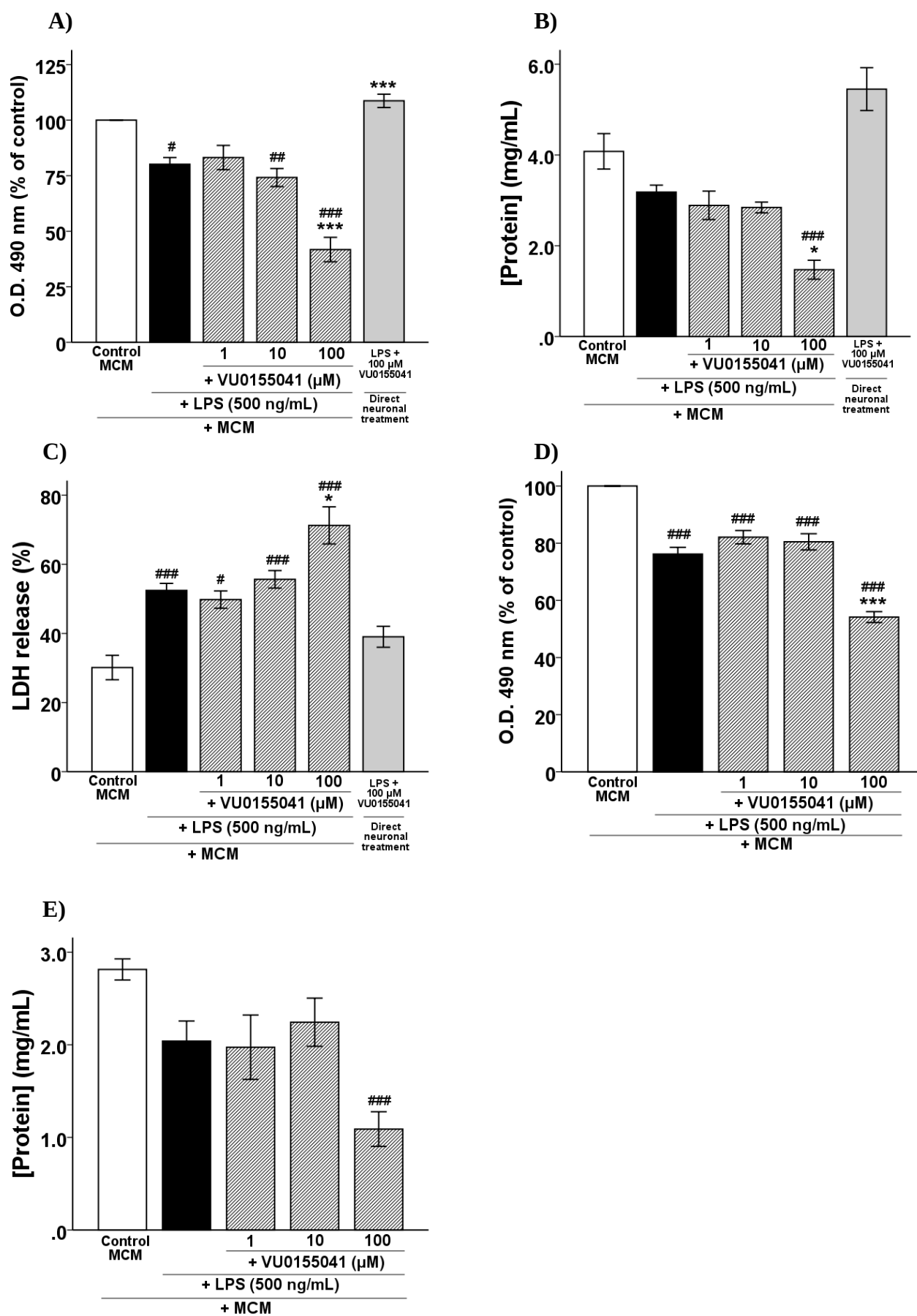


Figure 6.4 (Legend on following page)

Figure 6.4 Effect of VU0155041 treatment on LPS-stimulated N9 microglial neurotoxicity. N27 dopaminergic neurons were incubated (48 hrs) with microglial conditioned medium (MCM) derived from LPS (500 ng/mL)-activated N9 microglia treated or not with the mGlu4-specific positive allosteric modulator VU0155041 one hour following LPS activation, and neuronal viability assessed through quantification of NADH through the MTS assay **(A)**, cellular protein content of cell lysates through the Bradford assay **(B)** and assessment of membrane integrity through LDH release assays **(C)**. In order to investigate direct neuronal effects of LPS and VU0155041 carried over in the culture medium, N27 neurons were also incubated (48 hrs) with medium supplemented with VU0155041 and LPS in the absence of microglia **(A-C)**. In addition, neuronal viability was assessed through the MTS assay **(D)** and Bradford assay **(E)** when incubated (48 hrs) with MCM derived from LPS (500 ng/mL)-activated N9 microglia treated or not with VU0155041 twenty minutes prior to LPS activation. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05$, 0.01, 0.005 vs. C-MCM, respectively. *, *** denote $p < 0.05$, 0.005 vs. LPS-MCM, respectively. Statistical significance was determined by a one-way ANOVA with Tukey's *post-hoc* test. F = 34.5 (A), 19.4 (B), 17.89 (C), 59.8 (D), 6.82 (E).

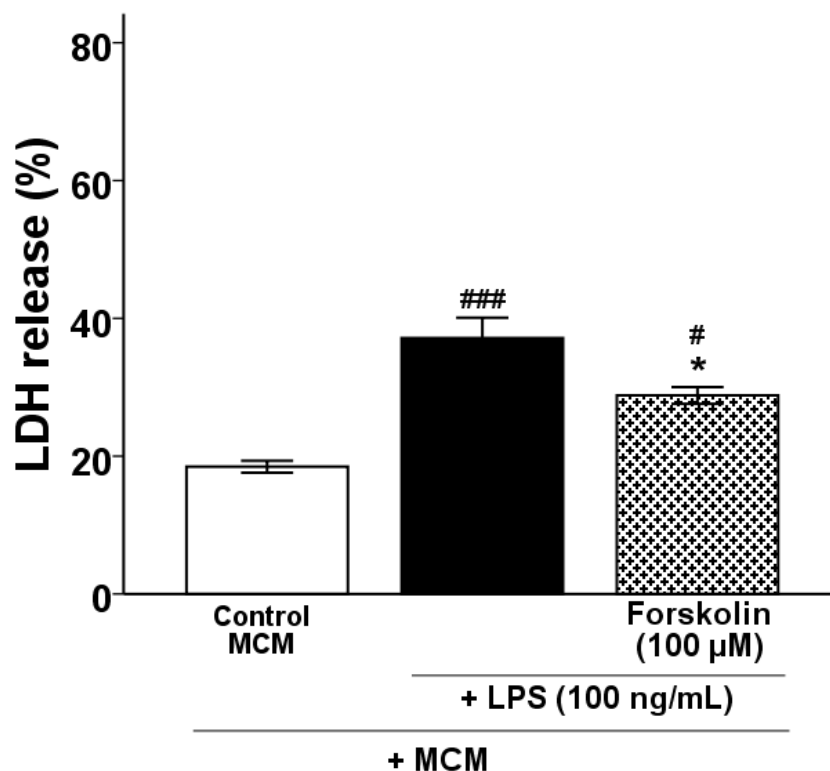


Figure 6.5 Effect of forskolin treatment on LPS-stimulated N9 microglial neurotoxicity. To examine the effect of increasing cAMP on LPS-stimulated microglial neurotoxicity, N27 dopaminergic neurons were incubated (48 hrs) with microglial conditioned medium (MCM) derived from LPS (100 ng/mL)-activated N9 microglia with or without simultaneous treatment with the adenylyl cyclase activator forskolin. Neuronal viability was determined through quantification of released LDH into the culture medium to assess membrane integrity. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ### denote $p < 0.05$, 0.005 vs. C-MCM, respectively. * denotes < 0.05 vs. LPS-MCM. Statistical significance was determined by a one-way ANOVA with Tukey's *post-hoc* test. $F = 24.1$

In order to investigate whether increased IL-6 concentration in the MCM stimulated by VU0155041 treatment enhanced the neurotoxic effects of microglial factors released by LPS stimulation, N27 neurons were incubated with LPS-MCM supplemented with increasing concentrations of recombinant IL-6 protein (10 pg/mL- 40 ng/mL) for 48 hrs, after which time cellular protein content was quantified. IL-6 failed to enhance the neurotoxic effects of LPS-MCM over all concentrations examined, as assessed by equal cellular protein content (**fig. 6.6C**).

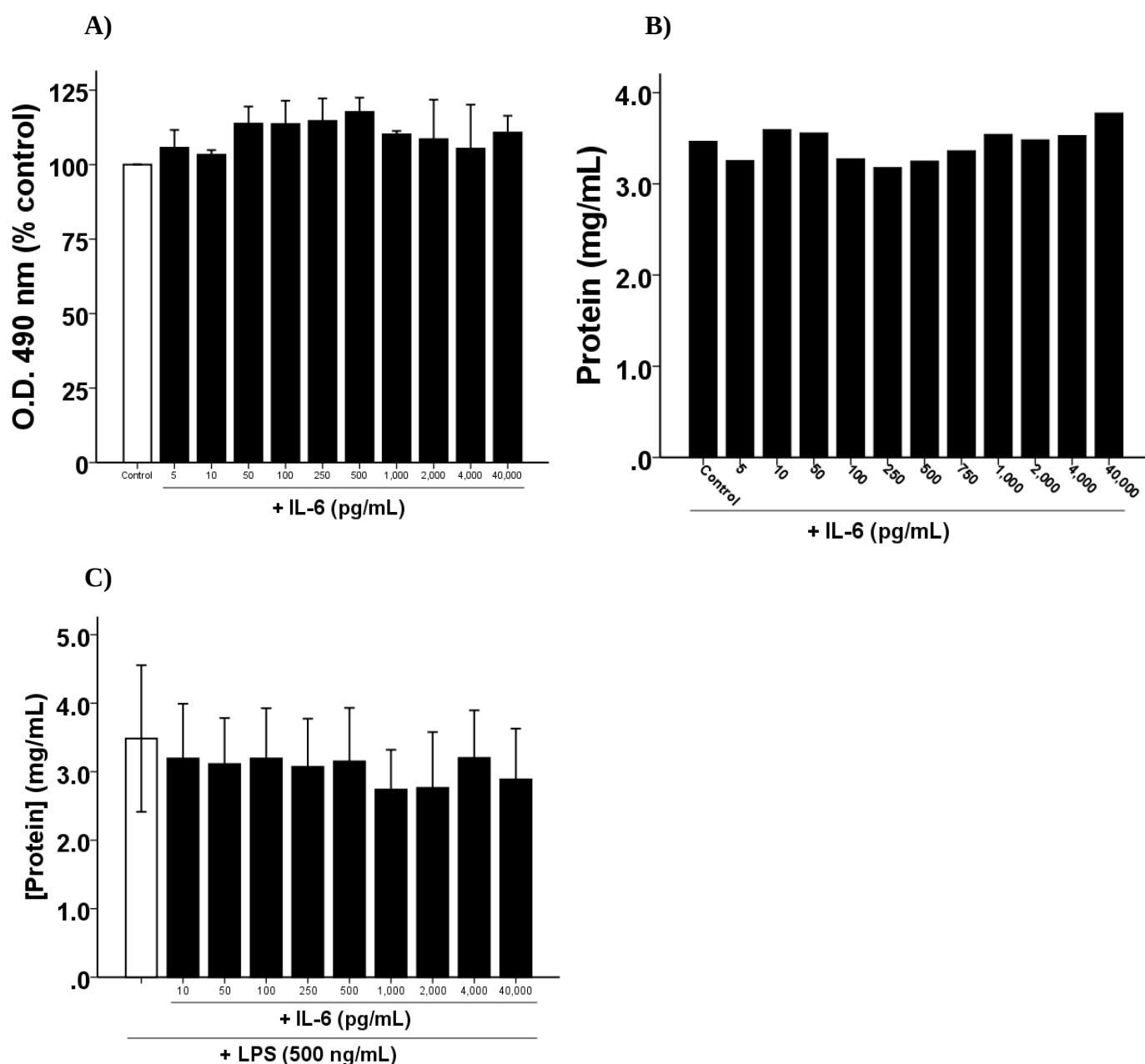


Figure 6.6 Effect of IL-6 treatment on N27 neuronal cell viability. In order to examine the role of microglial IL-6 in N9 microglial neurotoxicity, N27 dopaminergic neurons were incubated (48 hrs) with increasing concentrations of recombinant IL-6 protein and neuronal cell viability assessed by quantification of mitochondrial activity through the MTS assay **(A)** and cellular protein content of cell lysates through the Bradford assay **(B)**. In addition, to examine whether microglial IL-6 enhances the neurotoxic effects of other microglial-derived factors, N27 neurons were incubated (48 hrs) with microglial conditioned medium (MCM) from LPS (500 ng/ml)-activated N9 microglia supplemented with recombinant IL-6 protein, and cellular protein content of cell lysates quantified through the Bradford assay **(C)**. Results are displayed as mean $\pm$ SEM of three independent experiments (A, C) or as mean of two independent experiments (B), each performed in duplicate. Statistical significance was determined by a one-way ANOVA with Tukey's *post-hoc* test. $F = 0.5$ (A), 0.09 (C).

6.4 Discussion

As reported in previous studies, incubation of neuronal cultures with MCM derived from LPS-activated N9 microglia (LPS-MCM) led to a significant increase in neuronal cell death compared to neurons incubated with MCM derived from control N9 microglia (C-MCM), indicating the increase in pro-inflammatory cytokine release by LPS treatment of N9 microglia resulted in a neurotoxic microglial phenotype. Interestingly, LPS-MCM only proved toxic to N27 neurons, without affecting cellular viability of the undifferentiated SH-SY5Y neuronal cell line, as assessed by intracellular NADH levels. This result could point to important differences between the physiology of dopaminergic and non-dopaminergic neurons, where expression of certain proteins in dopaminergic cells renders them more susceptible to certain toxic factors. Indeed, a previous *in vitro* study has shown that undifferentiated SH-SY5Y neurons are less susceptible to degeneration induced by the histone de-acetylase inhibitor trichostatine-A compared to N27 neurons (Wang *et al.*, 2009). In the present study, the increased susceptibility of N27 neurons to LPS-MCM may arise due to increased pro-inflammatory cytokine receptor expression, thereby rendering N27 neurons more responsive to microglia-derived proteins. Therefore, all subsequent experiments investigating the effect of microglial mGluR activation on microglial neurotoxicity were carried out in the N27 neuronal cell line.

Previous studies have suggested general activation of group III mGluR on microglia leads to reduced microglial neurotoxicity towards cerebellar granule cells (Pinteaux-Jones *et al.*, 2008; Taylor *et al.*, 2003). However, the present results indicate that general activation of group III mGluR on N9 microglia by the group III general orthosteric agonist L-AP4 did not module LPS-stimulated microglial neurotoxicity towards dopaminergic N27 neurons. The lack of neurotoxicity modulation could be attributed to failure of L-AP4 to modulate LPS-stimulated pro-inflammatory cytokine release (IL-6, IL-1 β and TNF α , see chapter V). Although L-AP4 treatment did reduce NO/iNOS production, the short-half life of reactive nitrite species would most likely preclude them

from playing a salient role under the medium-transfer protocol employed during this study. However, the present results could also be attributed to differential susceptibility of dopaminergic neurons and cerebellar granule neurons to microglial factors.

Given that AMN082 and VU0155041 differentially modulated N9 microglial reactivity, their effects on N9 microglial neurotoxicity towards N27 neurons were investigated.

Although AMN082 treatment of N9 microglia failed to robustly modulate microglial neurotoxicity stimulated by 500 ng/mL LPS, it robustly decreased microglial neurotoxicity stimulated by 5 ng/mL LPS, as assessed by significantly decreased LDH release levels. Importantly, this LPS dose still induced a significant neurotoxic phenotype on N9 microglia. It is interesting that modulation of microglial neurotoxicity by AMN082 closely mirrors the effects of AMN082 on LPS-stimulated TNF α release. Indeed, the neurotoxic properties of TNF α towards dopaminergic neurons is well documented (*e.g.* McCoy *et al.*, 2006; De Lella Ezcurra *et al.*, 2010). Hence, from the AMN082 studies it could be surmised that mGlu7 activation leads to decreased microglial neurotoxicity due to decreased TNF α release.

Contrary to AMN082, treatment of N9 microglia with VU0155041 led to a robust enhancement of LPS-stimulated microglial neurotoxicity. Interestingly though, enhancement of microglial neurotoxicity by VU0155041 treatment was seen even with 500 ng/mL LPS stimulation, whereas VU0155041 treatment failed to increase TNF α release stimulated by the same LPS dose. In this case, modulation of microglial neurotoxicity by VU0155041 more closely resembled VU0155041-mediated modulation of IL-6 and IL-1 β release. However, though VU0155041 pre- and post-treatment led to a comparable enhancement of LPS-stimulated neurotoxicity, VU0155041 pre-treatment led to a significantly more robust increase in IL-6 release. Furthermore, direct treatment of N27 neurons with recombinant IL-6 up to a concentration of 40 ng/mL (exceeding the VU0155041-stimulated IL-6 concentration in the LPS-MCM) failed to elicit neuronal cell death. Similarly, incubation of N27 neurons with LPS-MCM supplemented with recombinant IL-6 failed to

reduce neuronal cell protein compared to neurons incubated with LPS-MCM only, suggesting increased IL-6 does not directly stimulate neuronal cell death nor does it enhance the neurotoxic effects of distinct LPS-stimulated microglial factors (*e.g.* TNF α). Hence, the neurotoxic effects of VU0155041 may rest in the increased release of IL-1 β more than IL-6. Regardless of which specific factor(s) induce VU0155041-stimulated microglial neurotoxicity, these results may explain the lack of complete neuroprotection seen in a recent study employing *in vivo* models of PD (Betts *et al.*, 2012), where supranigral administration of VU0155041 led to a 40% reduction in loss of TH⁺ neurons. Hence, VU0155041 might have provided neuroprotection when acting on mGlu4 receptors expressed in neurons (by reducing excitotoxicity), but might have counteracted this protective effect when acting on microglial receptors by increasing release of pro-inflammatory factors. Although there was a decrease in the number of IBA-1⁺ microglia in the Betts *et al.* study, this apparent reduction in microglial reactivity might be due to a decrease in neuronal factor-stimulated microglial activation. Hence, if VU0155041 were only acting on neuronal receptors without activating microglial receptors, a markedly greater level of neuroprotection might have been seen.

Interestingly, even though forskolin treatment also led to a robust increase in IL-6 and IL-1 β release by N9 microglia (see chapter VII), incubation of N27 neurons with MCM from forskolin/LPS treated microglia actually resulted in a slight decrease in neuronal cell death compared to LPS-MCM. Crucially however, forskolin treatment also led to a marked decrease in LPS-stimulated TNF α release from N9 microglia (see chapter VII).

Therefore, the differential effects of AMN082, VU0155041 and forskolin on LPS-stimulated microglial neurotoxicity can be attributed to their differential modulation of cytokine release, as well as the varying potency of cytokines to induce neuronal effects *e.g.* TNF α has a potency several magnitudes greater than IL-6 to induce CNS effects such as hyperalgesia (Ferreira *et al.*, 1997). Hence, while AMN082 may reduce IL-6 release stimulated by 500 ng/mL LPS, it fails to modulate IL-1 β and TNF α release stimulated by the same LPS dose. Therefore, any reduction in

neurotoxicity which might be brought about by decreased IL-6 release would be masked by the high levels of IL-1 β and TNF α in the MCM, resulting in lack of modulation of neurotoxicity under this experimental set up. However, AMN082 treatment of microglia activated by 5 ng/mL LPS stimulates a robust decrease in nitrite production and TNF α release (see chapter V), leading to a marked decrease in microglial neurotoxicity. On the other hand, while VU0155041 may not affect TNF α release, its enhancement of IL-1 β (coupled or not with increased IL-6 release) may result in enhanced neurotoxicity either through direct neuronal effects or through potentiation of neuronal loss elicited by different factors (Chen *et al.*, 2010; Godoy *et al.*, 2008). Indeed, previous *in vitro* and *in vivo* studies have demonstrated synergistic cellular effects of IL-1 β and TNF α (Bluthe *et al.*, 1994; Moore *et al.*, 2001).

Similarly, the modest reduction in microglial neurotoxicity induced by forskolin treatment may be due its opposing effects on IL-6/1 β and TNF α decrease from microglia. Hence, while a decrease in TNF α release would result in decreased neurotoxicity towards N27 neurons, this would be counteracted by the increase in IL-1 β and IL-6 release.

However, at this point, the involvement of other microglial factors in the evidenced modulation of LPS-stimulated microglial neurotoxicity by AMN082 and VU0155041 cannot be discarded. In particular, modulation of microglial-derived neurotrophic support may play a pivotal role during neuronal cell death associated with microglial activation. In line with this argument, it is known that general activation of microglial group III mGluR with the group-wide agonist L-AP4 stimulates expression of the trophic factor GDNF (Liang *et al.*, 2010), known to strongly promote dopaminergic cell survival. Therefore, stimulation of individual receptors may differentially regulate GDNF expression, leading to increased or decreased trophic support on neuronal cells.

6.5 Conclusion

In accordance with previous studies, this chapter shows that microglial activation by LPS leads to a neurotoxic phenotype, thereby further implicating microglial reactivity as an important contributor to chronic neuronal cell death, as well as highlighting its potential as a therapeutic target for neurodegenerative diseases. In addition however, these results demonstrate that microglial reactivity is particularly damaging to dopaminergic neurons, perhaps reflecting the specific dopaminergic loss seen in PD. Most importantly though, the results presented within this chapter clearly validate our hypothesis demonstrating that the differential modulation of microglial reactivity by activation of mGlu4 or mGlu7 translates to increased or decreased microglial neurotoxicity on dopaminergic neurons, respectively. Therefore, these results indicate care must be taken when selecting individual group III mGluR as therapeutic targets against PD, since activation of *e.g.* mGlu4 on neurons may provide neuroprotection through reduction of glutamate release from the STN, but may increase neurodegeneration through exacerbation of microglial neurotoxicity. Indeed, these results may explain the lack of complete neuroprotection by VU0155041 treatment in a recent *in vivo* study employing a rodent model of PD (Betts *et al.*, 2012).

Chapter VII

Signalling mechanisms engaged
by microglial group III mGluR

7. Signalling mechanisms engaged by microglial group III mGluR

7.1 Introduction

Inflammatory reactions in macrophages are known to be orchestrated by the transcription factor nuclear factor kappa-B (NFκB) (*e.g.* Lawrence *et al.*, 2005) by modulating the expression of a wide range of pro-inflammatory proteins, including IL-1β, IL-6, TNFα and iNOS. NFκB is composed of five independent proteins, namely p50, p52, p65, RelB and c-Rel, which share a conserved Rel-homology domain important for DNA binding and may homo- or hetero-dimerize to form the functional NFκB complex (Baldwin, 1996). NFκB exists in an inactive state sequestered in the cytosol by the protein inhibitor of kappa B (IκB). Upon appropriate signalling, IκB is phosphorylated by IκB kinases (IKK) and targeted for degradation by the proteasome, thereby allowing NFκB to translocate to the nucleus and bind to promoter sequences of pro-inflammatory genes.

The bacterial cell wall component lipopolysaccharide (LPS) stimulates microglial reactivity through binding to the microglial pattern recognition receptor Toll-like receptor (TLR)-4. LPS binding to TLR-4 stimulates an inflammatory signalling cascade involving the TLR-4 associated adaptor protein MyD-88 activating IKK, leading to degradation of IκB and nuclear translocation of NFκB, ultimately stimulating the expression of pro-inflammatory proteins (*e.g.* IL-6/1β, TNFα and iNOS). In addition however, TLR-4 ligation is capable of activating an NFκB-independent signalling pathway through recruitment of MAP kinases and activation of the transcription factor Activator Protein (AP)-1 (reviewed by Takeda and Akira, 2004).

Due to the central role of NFκB in regulation of inflammation, NFκB activity is tightly regulated at several levels, including nuclear translocation of the IκB-dissociated proteins (King *et al.*, 2011), DNA binding ability (Hou *et al.*, 2003) and dimer composition (Wall *et al.*, 2009). Cyclic AMP, a ubiquitous second messenger molecule, modulates inflammatory reactions at least partly through regulation of NFκB activity. Production of cAMP by the enzyme adenylyl cyclase (AC) leads to

cAMP binding to the enzyme protein kinase (PK)-A, thereby promoting dissociation of the regulatory and catalytic PK-A units and enzyme activation. Active PK-A phosphorylates threonine and serine residues on a wide range of proteins, including NFκB. However, despite extensive research, the role of cAMP in inflammation remains controversial, with strong evidence for both a pro-inflammatory and anti-inflammatory role (Spooren *et al.*, 2010; Tasken *et al.*, 2006; Chen *et al.*, 1999; Mustafa and Olson, 1998). Hence, cAMP production may have anti-inflammatory effects, for instance driving down iNOS expression in macrophages (Mustafa and Olson, 1998) or inhibiting T-cell activation (Tasken *et al.*, 2006). However, cAMP production may also exert pro-inflammatory effects, such as enhanced expression of the pro-inflammatory proteins IL-6 and COX-2 (Spooren *et al.*, 2010) and increased nitrite production (Chen *et al.*, 1999). The contradictory effects of cAMP on inflammation may result from its varied effects on NFκB activity. For instance, cAMP may increase NFκB-dependent gene expression by PK-A-mediated phosphorylation of nuclear p65 subunit, resulting in enhanced transactivation and expression of inflammatory genes (Gao *et al.*, 2010). Conversely, *in vitro* studies have shown that enhancers of cAMP production (*e.g.* forskolin, β-adrenergic receptor agonists) can prevent activation of IKK and hence degradation of IκB (Chong *et al.*, 2002; Farmer *et al.*, 2000; Wu *et al.*, 2007), thereby preventing NFκB signalling. In addition, phosphorylation of serine-276 on the NFκB subunit p65 by PK-A results in a decreased rate of nuclear translocation independent of IκB modulation, leading to a reduced expression of pro-inflammatory genes (King *et al.*, 2011; Neumann *et al.*, 1995). Furthermore, PK-A activity in macrophages can lead to increased nuclear translocation of the inhibitory NFκB dimer p50/p50, thereby reducing NFκB-dependent gene expression (Wall *et al.*, 2009).

Activation of group III mGluR, thought to couple to a G_i protein, leads to modulation of cAMP production, thereby providing a mechanism for modulation of microglial inflammation. However, given the differential effects of individual group III mGluR activation on microglial reactivity and neurotoxicity, as established in the previous chapters, it is important to examine the effects of NFκB

modulation and cAMP production on N9 microglial reactivity, as well as the effects of individual group III mGluR activation on cAMP production, in order to shed light on the possible differences in signalling mechanisms underlying the different effects of individual group III mGluR on N9 microglial phenotype.

- **Hypothesis:** Modulation of lipopolysaccharide-stimulated N9 microglial activation by ligands targeting individual group III metabotropic glutamate receptors occurs through a decrease in cAMP production, leading to modulation of NF κ B signalling.

7.2 Experimental design

In order to examine the role of NF κ B activity on LPS-stimulated N9 microglial reactivity, NF κ B activity was inhibited with the use of the cell permeable compound ammonium pyrrolidine dithiocarbamate (APDC), known to inhibit NF κ B activity by preventing I κ B dissociation, without affecting AP-1 activation (Liu *et al.*, 1999; Schreck *et al.*, 1992) (see section 2.1.8.2.3). N9 microglia were treated with APDC directly into the cell culture medium one hour after (post-treatment) or 20 minutes before (pre-treatment) LPS treatment. Microglial reactivity was assessed through quantification of nitrite production, iNOS expression and IL-6, IL-1 β and TNF α release into the culture medium 18 hrs following LPS treatment (see sections 2.1.15-19).

In order to examine the effect of group III mGluR activation on cAMP production, N9 microglia were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IMX) to prevent cAMP degradation, followed by simultaneous incubation (30 minutes) with the adenylyl cyclase (AC) activator forskolin and mGluR ligands (see section 2.1.18). In mGluR antagonism studies, the mGlu7-specific allosteric antagonist MMP-IP was added simultaneously with IMX to ensure receptor occupancy before agonist treatment. Cyclic AMP production was quantified through an ELISA kit. In order to examine whether glutamate in the culture medium interfered with the cAMP assay,

experiments were repeated incubating the cells in Locke's buffer. The same experimental protocol was followed, except cells were washed with and incubated in Locke's buffer for 30 minutes before the addition of IMX. All subsequent treatments were also carried out in Locke's buffer.

The role of the adenylyl cyclase-cAMP-PKA signalling pathway in modulation of N9 microglial reactivity by group III mGluR was examined by pharmacological modulation of individual components within the pathway. The effect of activating adenylyl cyclase on LPS-induced microglial reactivity was examined by simultaneous incubation of N9 microglia with LPS and forskolin. Since 500 ng/mL LPS induces a maximal increase in nitrite production (see chapter III), the LPS concentration was reduced to 100 ng/mL to ensure an enhancing effect of cAMP on microglial reactivity could be detected. Microglial reactivity was assessed through quantification of nitrite production, iNOS expression and IL-6, IL-1 β and TNF α release 18 hrs following LPS treatment. In addition, the role of the adenylyl cyclase-cAMP-PKA signalling pathway in group III mGluR signalling was examined through treatment with the PK-A inhibitor H-89 or the adenylyl cyclase inhibitor MDL-12330. H-89 has been widely used in PK-A inhibition studies due to its effective cell membrane permeability and specificity for PK-A (reviewed by Murray, 2008). H-89 inhibits PK-A through competitive binding to the ATP-binding site of the catalytic sub-unit of PK-A, thereby preventing ATP binding and hence PK-A-mediated phosphorylation of target proteins. However, though widely used to inhibit PK-A, H-89 is known to lose its specificity for PK-A at high (μ M) concentrations (*e.g.* Davies *et al.*, 2000; Chijiwa *et al.*, 1990). Hence, in this thesis, H-89 was employed at nanomolar concentrations to minimize non-specific inhibitory actions. MDL-12330 has been widely employed as an adenylyl cyclase inhibitor (Guellaen *et al.*, 1977; Hunt and Evans, 1980). However, at high concentrations ($> 10 \mu$ M) MDL-12330 is known to inhibit Ca²⁺ entry (van Rossum *et al.*, 2000), as well as glycine transport into glial cells (Gadea *et al.*, 1999). Therefore, similarly to H-89, MDL-12330 was used at nanomolar concentrations to minimize non-specific effects.

N9 microglia were treated with H-89 or MDL-12230 forty minutes after LPS (500 ng/mL) activation, followed by treatment with AMN082 or VU0155041 one hour after LPS activation. Microglial reactivity was assessed at 18 hrs following LPS activation through quantification of nitrite production by the Griess assay and TNF α release into the culture medium by an ELISA.

7.3 Results

7.3.1 Inhibition of NF κ B replicates group III mGluR-modulation of microglial activation

7.3.1.1 *Effect of APDC treatment on nitrite production and iNOS expression by LPS-activated N9 microglia*

Treatment of N9 microglia with APDC one hour following LPS (500 ng/mL) treatment led to a marked and dose dependent reduction in nitrite production 18 hrs following LPS treatment compared to LPS treatment alone. Interestingly, the effect of APDC on nitrite production adopted an inverted bell-shaped curve, with maximal inhibition achieved with 5 μ M APDC (67.2% of LPS response) and waning with higher concentrations (**fig. 7.1A**). Western blot analysis of N9 lysates revealed the reduction in nitrite production by APDC treatment was accompanied by an inhibition of iNOS protein expression at the same time point (**fig. 7.1B**). Similarly, treatment of N9 microglia with APDC 20 minutes before LPS treatment led to a reduction in nitrite production, though this did not reach statistical significance. However, unlike APDC post-treatment, the effect of APDC pre-treatment on nitrite production did not adopt an inverted bell-shaped curve, with the highest concentration examined (10 μ M) stimulating the strongest reduction in nitrite production (**fig. 7.1C**). In addition, preliminary experiments demonstrated treatment of N9 microglia with 5 ng/mL LPS led to a robust increase in nitrite production 18 hrs following LPS activation which was inhibited by APDC treatment 1 hr following LPS activation (**fig. 7.1D**).

7.3.1.2 Effect of APDC treatment on IL-6 and TNF α release by LPS-activated N9 microglia

Treatment of N9 microglia with APDC one hour after LPS (500 ng/mL) treatment led to a robust decrease in IL-6 release 18 hrs following LPS treatment. Interestingly, IL-6 release inhibition stimulated by APDC treatment adopted an inverted bell-shaped curve, with 5 μ M eliciting the strongest inhibition (58.8% of LPS response) (**fig. 7.2A**) reminiscent of APDC-stimulated nitrite production inhibition. APDC treatment of N9 microglia one hour following LPS activation failed to modulate TNF α release stimulated by 500 ng/mL LPS 18 hrs following LPS treatment (**fig. 7.2B**). However, a similar APDC treatment produced a strong trend towards inhibition of TNF α release 18 hrs following stimulation with 5 ng/mL LPS. Though this failed to reach statistical significance with respect to LPS, it abolished the statistically significant difference with respect to control condition (**fig. 7.2C**). Interestingly, TNF α release inhibition by APDC was comparable through all APDC concentrations examined (1-10 μ M), though statistical significance was not reached for any concentration.

7.3.1.3 Effect of APDC treatment on N9 microglial cell viability

Treatment of N9 microglia with APDC at concentrations employed to modulate microglial reactivity did not affect microglial cellular viability 18 hrs following LPS treatment, as assessed by unaltered NADH levels (**fig. 7.3A**), trypan blue exclusion (**fig. 7.3B**), and cellular protein content (**fig. 7.3C**). However, high APDC concentrations (50 μ M) proved toxic to N9 microglia, as assessed by markedly decreased NADH levels (**Fig. 7.3A**).

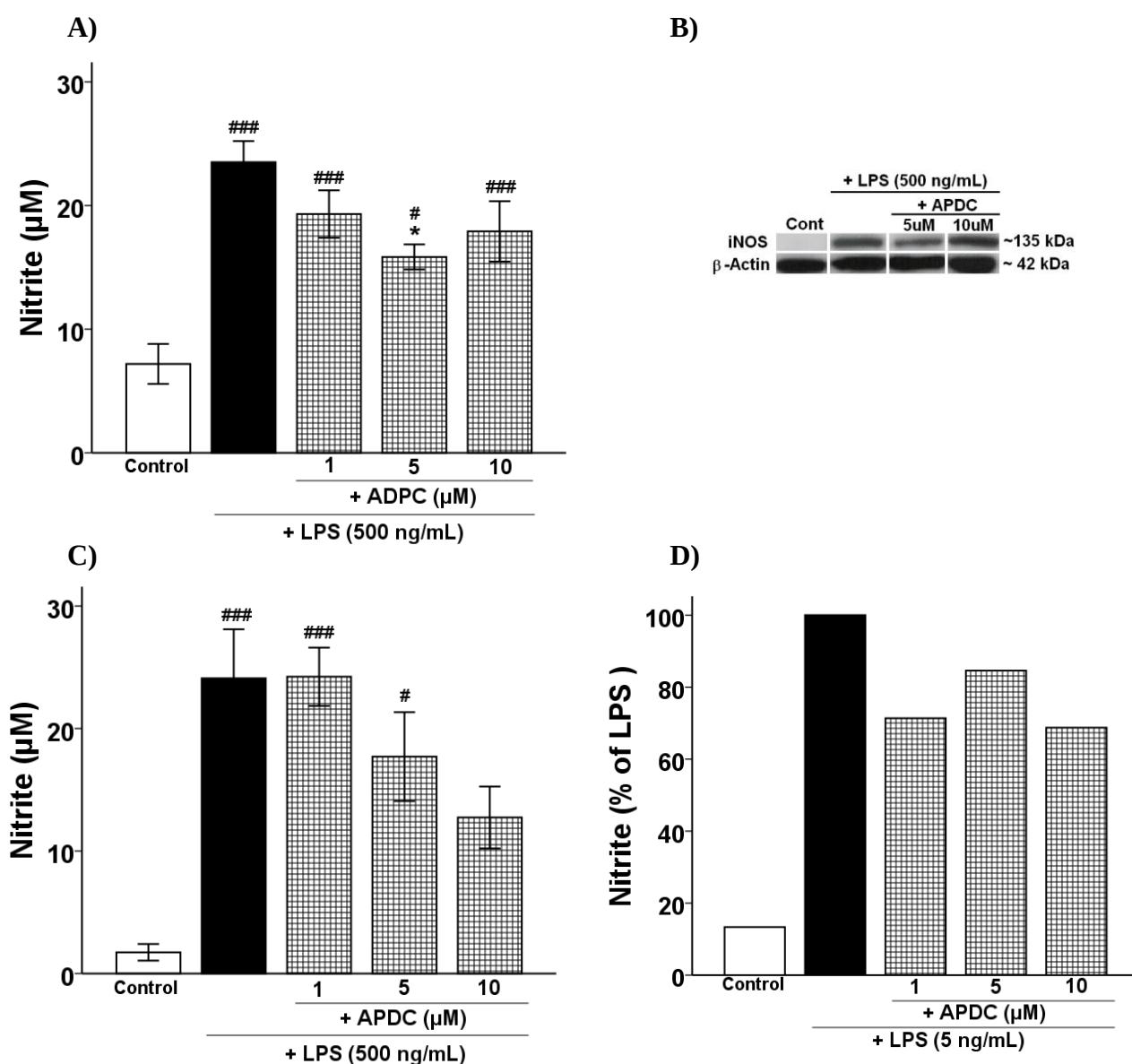


Figure 7.1 Effect of APDC treatment on nitrite production and iNOS expression by LPS-activated N9 microglia.

The NFκB inhibitor APDC was employed to investigate the role of NFκB in LPS-stimulated increase of nitrite production and iNOS expression by N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with APDC one hour following (A) or 20 minutes prior to (C) LPS activation, and nitrite levels in the culture medium quantified through the Griess assay at 18 hrs after LPS activation. Similarly, nitrite production was quantified when N9 microglia were activated by 5 ng/mL LPS and treated with APDC 1hr following LPS activation (D). The effect of APDC on iNOS protein expression by N9 microglia at 18 hrs following LPS activation was assessed through immunolabelling of Western blots of microglial cell lysates from LPS (500 ng/mL)-activated N9 microglia treated with APDC one hour following LPS activation. To confirm equal protein loading, Western blots were labelled with anti-β-actin (B). Results are displayed as mean ± SEM of three (A, C) or one (D) independent experiment(s) measured in triplicate. #, ### denote $p < 0.05$, 0.005 vs. control, respectively. * denotes $p < 0.05$ vs. LPS condition. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 11.3$ (A), 10.5 (C).

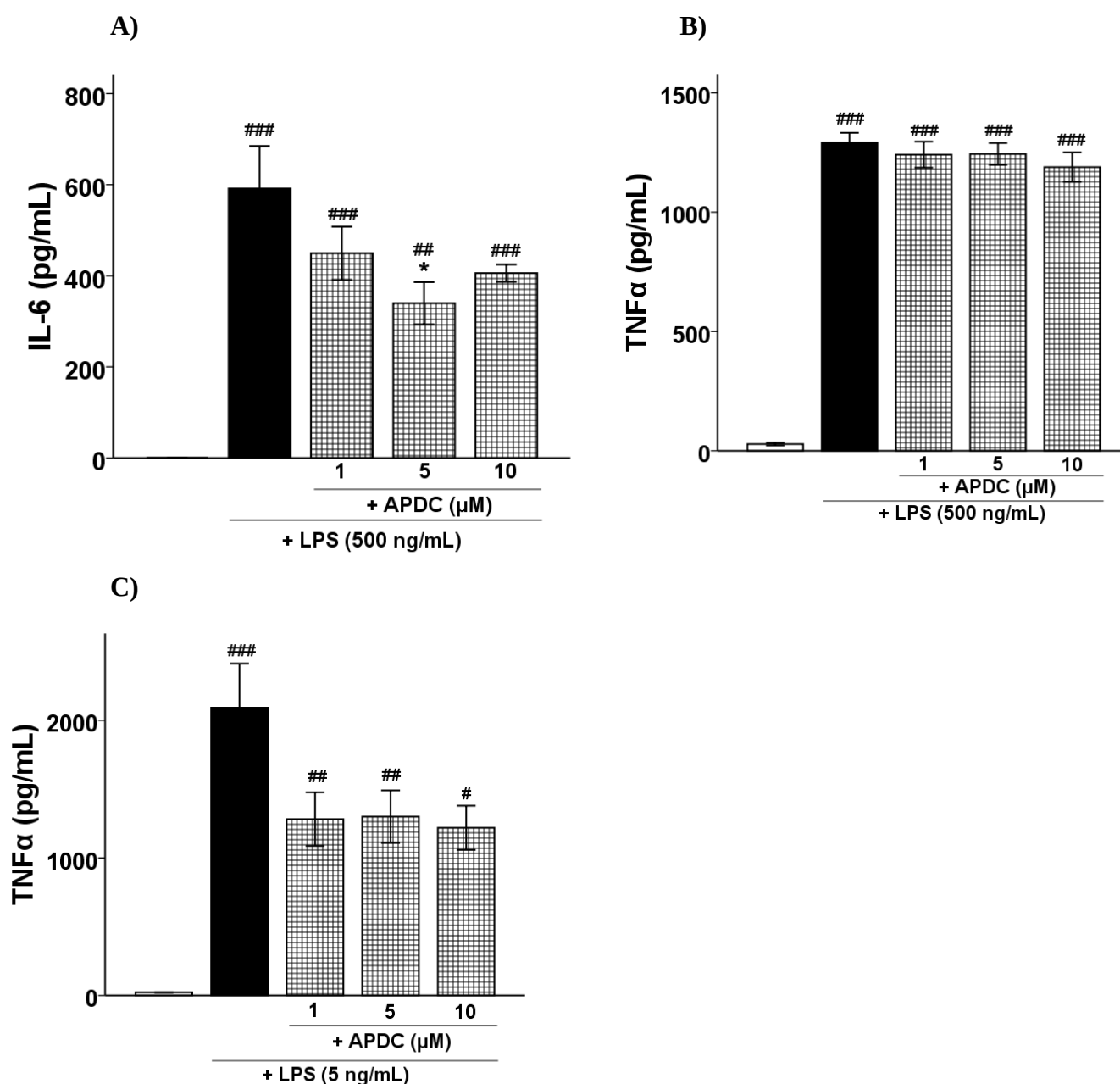


Figure 7.2 Effect of APDC treatment on IL-6 and TNFα release by LPS-activated N9 microglia. The inhibitor of NFκB nuclear translocation APDC was employed to investigate the role of NFκB in LPS-stimulated increase in IL-6 and TNFα release by N9 microglia. LPS (500 ng/mL)-activated N9 microglia were treated with APDC one hour following LPS activation, and IL-6 (**A**) and TNFα (**B**) release into the culture medium quantified through an ELISA at 18 hrs after LPS activation. The effect of APDC on TNFα release by N9 microglia stimulated with 5 ng/mL LPS was similarly investigated (**C**). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05$, 0.01, 0.005 vs. control, respectively. * denotes $p < 0.05$ vs. LPS. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 16.6$ (A), 138.0 (B), 13.6 (C).

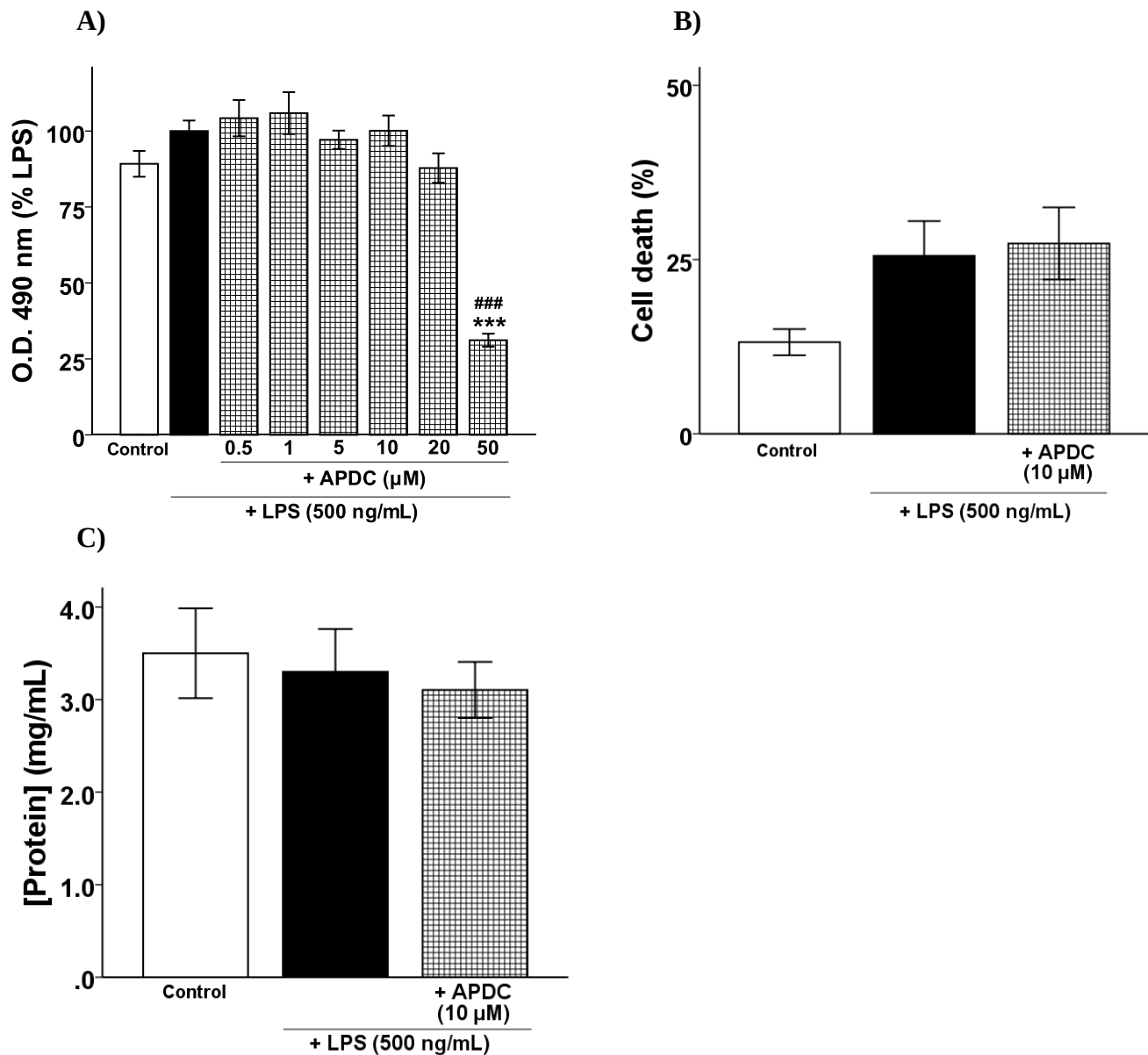


Figure 7.3 Effect of APDC treatment on N9 microglial cell viability. LPS (500 ng/mL)-activated N9 microglia were treated with the inhibitor of NFκB nuclear translocation APDC one hour following LPS activation and cell viability assessed at 18 hrs after LPS activation. Cell viability was assessed by quantification of intracellular NADH levels through the MTS assay **(A)**, assessment of membrane integrity through a trypan blue exclusion assay **(B)** and quantification of cellular protein content through the Bradford assay **(C)**. Microglia were either plated in 96 well plates at 10×10^3 cells per well **(A)** or in 6 well plates at 6×10^5 cells per well **(B, C)**. For trypan blue exclusion assays, floating and adhered cells were collected, incubated with trypan blue and cell death determined as the percentage of cells incorporating trypan blue. For cellular protein quantification, cells were lysed at the appropriate time-point and protein concentration in the lysates quantified through the Bradford assay. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. **###** denotes $p < 0.005$ vs. control. ******* denotes $p < 0.005$ vs. LPS condition. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 3.23$ (B), 0.22 (C).

7.3.2 L-AP4, AMN082 and VU0155041 increase forskolin-stimulated cAMP accumulation in N9 microglia

7.3.2.1 Effect of group III mGluR ligands on forskolin-stimulated cAMP accumulation in N9 microglia incubated in cell culture medium

In order to investigate the functional coupling of group III mGluR to adenylyl cyclase in N9 microglia, the effect of mGluR ligands on forskolin-stimulated cAMP accumulation was investigated. Treatment of N9 microglia with L-AP4 enhanced the cAMP accumulation stimulated by 10 μ M forskolin. However, a statistically significant difference with respect to control condition was only reached with the highest L-AP4 concentration (**fig. 7.4A**). Treatment of N9 microglia with the mGlu7-specific allosteric agonist AMN082 (10 μ M) led to an increase in forskolin (10 μ M)-stimulated cAMP accumulation. Similarly to L-AP4, a statistically significant difference with respect to control condition was only reached with AMN082 treatment. Furthermore, treatment with the mGlu7-allosteric antagonist MMPIP 20 minutes prior to AMN082 treatment abolished the AMN082-stimulated cAMP accumulation (**fig. 7.4B**).

Treatment of N9 microglia with the mGlu4-specific positive allosteric modulator VU0155041 led to a marked enhancement of forskolin (10 μ M)-stimulated cAMP accumulation. However, only the highest VU0155041 concentration examined (100 μ M) reached statistical significance with respect to forskolin treatment (**fig. 7.4C**). Treatment of N9 microglia with the mGlu8-specific orthosteric agonist DCPG failed to enhance cAMP accumulation even at the highest concentration (**fig. 7.4D**), both with respect to control condition and to forskolin treatment.

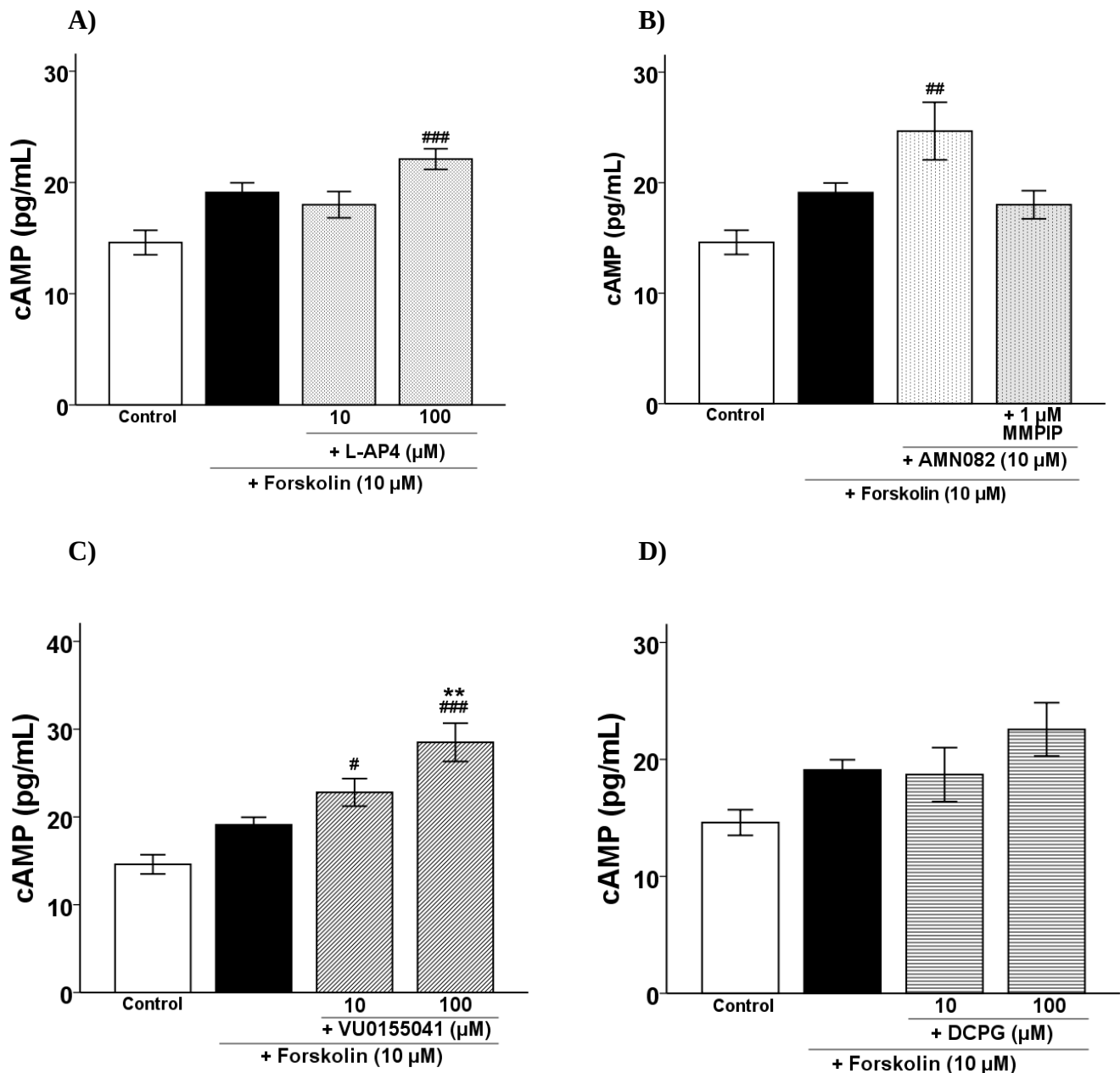


Figure 7.4 Effect of L-AP4, AMN082, VU0155041 or DCPG treatment on forskolin-stimulated cAMP accumulation in N9 microglia incubated in culture medium. To investigate the functional coupling of group III mGluR to adenylyl cyclase in N9 microglia, the effect of treatment of N9 microglia with the general group III mGluR orthosteric agonist L-AP4, the mGlu7-specific allosteric agonist AMN082, the mGlu4-specific positive allosteric modulator VU0155041 or the mGlu8-specific orthosteric agonist DCPG on intracellular cAMP accumulation stimulated by the adenylyl cyclase activator forskolin was investigated. N9 microglia were stimulated with forskolin (10 μM, 30 min) in the presence of a cAMP degradation-inhibitor (3-isobutyl-1-methylxanthine) with simultaneous treatment with L-AP4 (**A**), AMN082 (**B**), VU0155041 (**C**) or DCPG (**D**). When employed, the mGlu7-specific allosteric antagonist MMPiP was added to the culture medium 20 minutes prior to AMN082 treatment. Microglia were subsequently lysed and intracellular cAMP quantified through an ELISA. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ##, ### denote $p < 0.05$, 0.01 , 0.005 vs. control, respectively. ** denotes $p < 0.01$ vs. forskolin treatment, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 9.1$ (A), 6.76 (B), 15.14 (C), 9.92 (D).

7.3.2.2 Effect of group III mGluR ligands on forskolin-stimulated cAMP accumulation under Locke's buffer incubation

In order to investigate whether cell culture medium components interfered with forskolin/mGluR-stimulated cAMP production, the cAMP assay was repeated incubating microglia with Locke's buffer instead of cell culture medium. Treatment of N9 microglia with L-AP4 under these conditions led to a similar increase in forskolin-stimulated cAMP accumulation. However, though there was a clear trend towards increasing cAMP levels, L-AP4 treatment failed to reach statistical significance (**fig. 7.5A**). Similarly, treatment of N9 microglia with AMN082 (**fig 7.5B**) or VU0155041(**fig. 7.5C**) led to a strong trend towards increasing cAMP accumulation following forskolin treatment, though this did not reach statistical significance. Treatment of N9 microglia with DCPG failed to modulate cAMP accumulation even at the highest concentration examined (100 μ M) (**fig. 7.5D**).

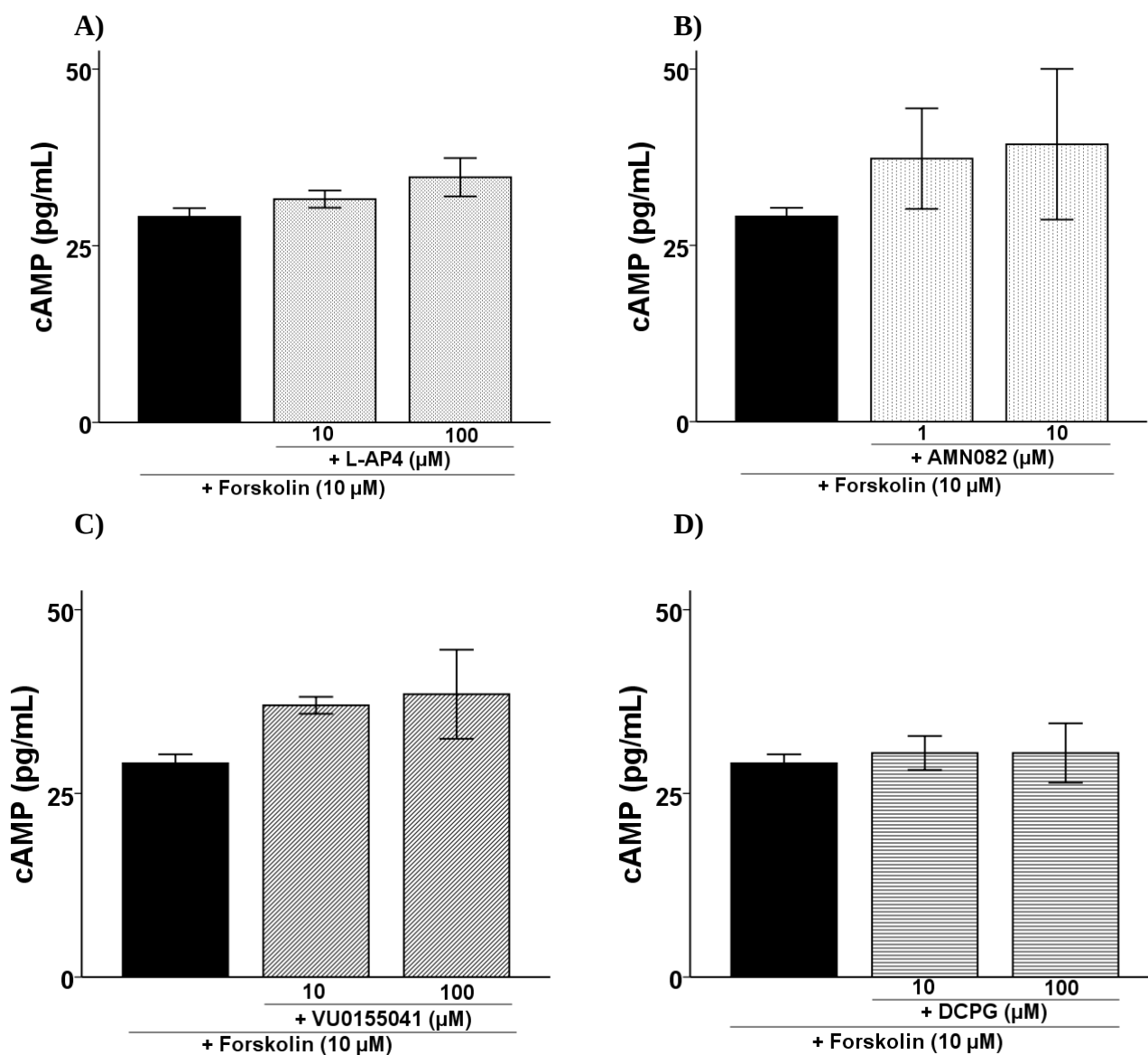


Figure 7.5 Effect of L-AP4, AMN082, VU0155041 or DCPG treatment on forskolin-stimulated cAMP accumulation in N9 microglia incubated with Locke's buffer. To investigate whether cell culture medium components interfered with forskolin/mGluR-stimulated cAMP production, cAMP assays were repeated incubating microglia in Locke's buffer. Functional coupling of group III mGluR to adenylyl cyclase in N9 microglia incubated in Locke's buffer was subsequently investigated by examining the effect of treatment of N9 microglia with the general group III mGluR orthosteric agonist L-AP4, the mGlu7-specific allosteric agonist AMN082, the mGlu4-specific positive allosteric modulator VU0155041 or the mGlu8-specific orthosteric agonist DCPG on intracellular cAMP accumulation stimulated by forskolin. N9 microglia were stimulated with forskolin (10 μM, 30 min) in the presence of a cAMP degradation-inhibitor with simultaneous treatment with L-AP4 (A), AMN082 (B), VU0155041 (C) or DCPG (D). Microglia were subsequently lysed and intracellular cAMP quantified through an ELISA. Results are displayed as mean $\pm$ SEM of two independent experiments measured in duplicate. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 1.99$ (A), 0.47 (B), 1.74 (C), 0.08 (D).

7.3.3 Adenylyl cyclase activation replicates group III mGluR-modulation of microglial activation

7.3.3.1 *Effect of forskolin treatment on nitrite production and iNOS expression in LPS-activated N9 microglia*

N9 microglia simultaneously treated with forskolin and LPS (100 ng/mL) had a marked and significant reduction in nitrite production 18 hrs following LPS treatment compared to N9 microglia treated with LPS (100 ng/mL) alone (**fig. 7.6A**), which was accompanied by a similar decrease in iNOS protein expression (**fig. 7.6B**).

7.3.3.2 *Effect of forskolin on IL-6, IL-1 β and TNF α release in LPS-activated N9 microglia*

Simultaneous treatment of N9 microglia with forskolin and LPS (100 ng/mL) led to a robust enhancement of IL-6 release 18 hrs following LPS treatment compared to treatment with LPS alone (**fig. 7.7A**). Similarly, forskolin treatment led to an increase in IL-1 β release 18 hrs following LPS treatment, with only the highest forskolin concentration (100 μ M) reaching statistical significance with respect to control condition (**fig. 7.7B**). Conversely, forskolin treatment induced a marked decrease in LPS (100 ng/mL)-stimulated TNF α release 18 hrs following LPS treatment, which, at the highest concentrations (80 and 100 μ M), eliminated the statistical difference with respect to control treatment (**fig. 7.7C**). Importantly, forskolin treatment did not affect cellular viability above LPS treatment, as assessed by comparable LDH release (**fig. 7.7D**) and comparable cellular protein concentration (**fig. 7.7E**).

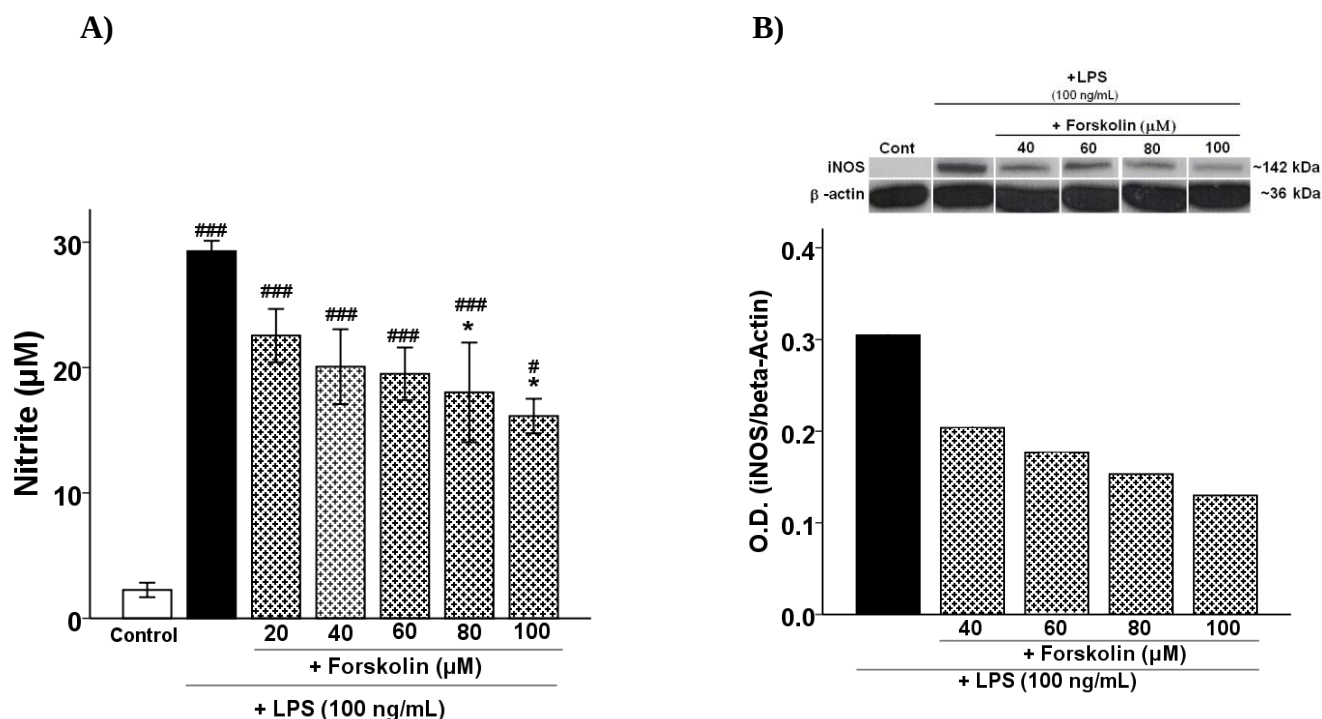


Figure 7.6 Effect of forskolin treatment on nitrite production and iNOS protein expression in LPS-activated N9 microglia. The adenylyl cyclase activator forskolin was employed to investigate the effect of cAMP increase on LPS-stimulated nitrite production and iNOS expression by N9 microglia. N9 microglia were simultaneously treated with forskolin and LPS (100 ng/mL) and nitrite levels in the culture medium quantified through the Griess assay at 18 hrs **(A)** following LPS treatment. The effect of forskolin on iNOS protein expression by N9 microglia at 18 hrs following LPS activation was assessed through immunolabelling of Western blots of microglial cell lysates from LPS (100 ng/mL)-activated N9 microglia co-treated with. To confirm equal protein loading, Western blots were labelled with anti-β-actin **(B)**. Results are displayed as mean $\pm$ SEM of three independent experiments measured in triplicate. Results in lower panel B are displayed as mean $\pm$ SEM of two independent experiments. Upper panel B is a representative image from two independent experiments. ### denotes $p < 0.005$ vs. control. *, ** denote $p < 0.05, 0.005$ vs. LPS treatment. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 12.95$ (A)

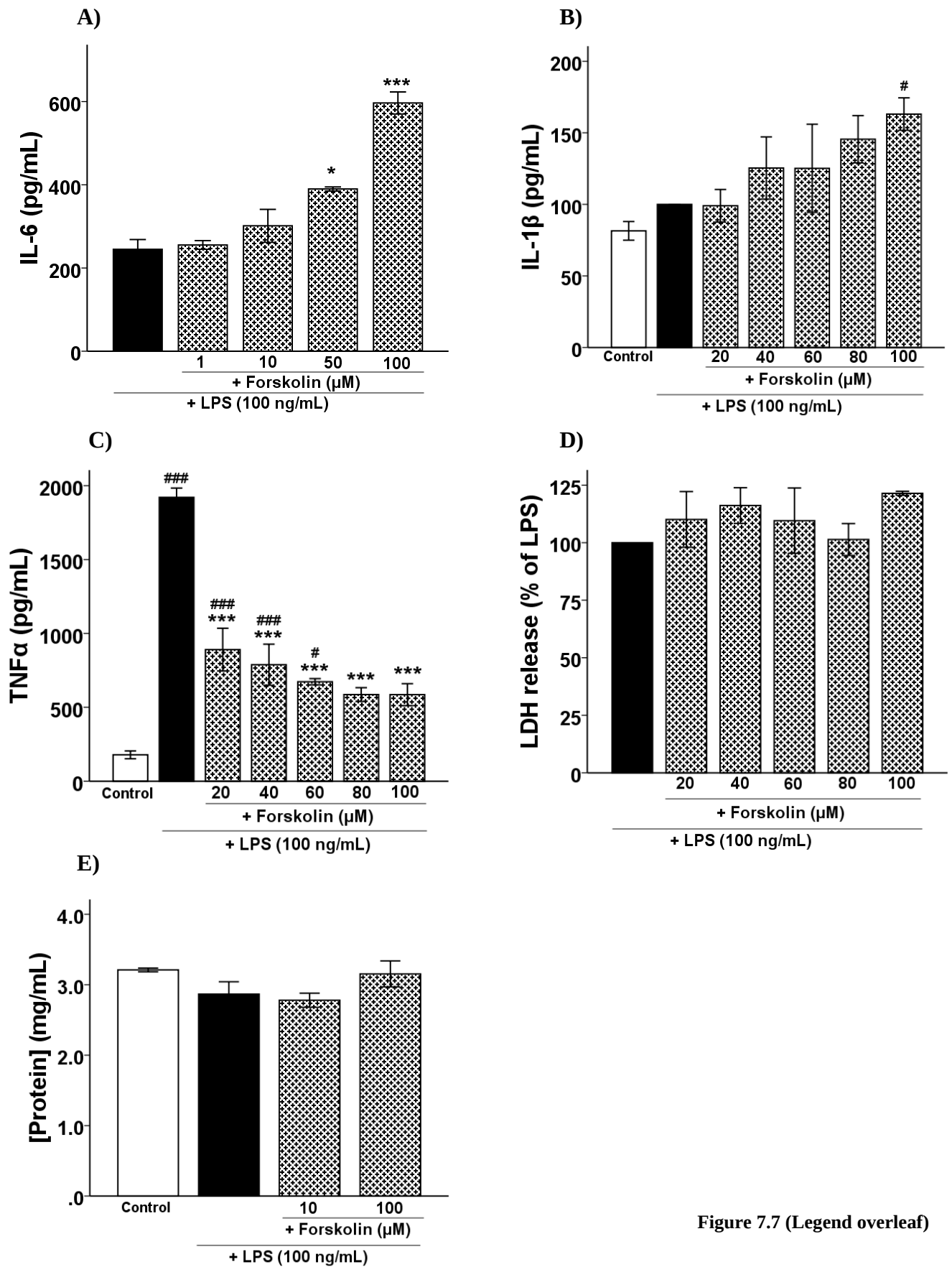


Figure 7.7 (Legend overleaf)

7.3.4 Effect of protein kinase-A or adenylyl cyclase inhibition on modulation of microglial activation by AMN082 or VU0155041

The role of adenylyl cyclase and PK-A on modulation of microglial reactivity by AMN082 was investigated with the inhibitors MDL-12330 and H-89, respectively. As reported in previous chapters, AMN082 treatment of N9 microglia 1 hr following LPS (500 ng/mL) activation led to a marked reduction in nitrite production 18 hr following LPS activation. However, the AMN082-stimulated reduction in nitrite production failed to be reversed either by H-89 treatment (**fig. 7.8A**) or MDL-12330 treatment (**fig. 7.8B**) 20 min prior to AMN082 treatment.

Similarly to AMN082, treatment of N9 microglia with VU0155041 one hour following LPS (500 ng/mL) activation stimulated a robust inhibition of nitrite production 18 hrs following LPS activation. Conversely however, the VU0155041-stimulated reduction in nitrite production was modestly but significantly reversed by both H-89 pre-treatment (**fig. 7.8C**) and MDL-12330 pre-treatment (**fig. 7.8D**). Treatment of LPS (500 ng/mL)-activated N9 microglia with H-89 treatment on its own had no effect on nitrite production 18 hrs following LPS activation (**fig. 7.8E**), while MDL-12330 on its own actually led to a decrease in nitrite production at the same time-point (**fig. 7.8F**).

Interestingly, H-89 stimulated an increase in LPS (500 ng/mL)-stimulated TNF α release from VU0155041-treated N9 microglia 18 hours following LPS (**fig. 7.9**).

Figure 7.7 Effect of forskolin treatment on IL-6, IL-1 β and TNF α release by LPS-activated N9 microglia. The adenylyl cyclase activator forskolin was employed to investigate the effect of cAMP increase on LPS-stimulated IL-6, IL-1 β and TNF α release by N9 microglia. N9 microglia were simultaneously treated with forskolin and LPS (100 ng/mL) and IL-6 (**A**), IL-1 β (**B**) and TNF α (**C**) levels in the culture medium quantified through an ELISA at 18 hrs following LPS activation. To assess forskolin toxicity, LDH release from N9 microglia co-treated with forskolin and LPS (100 ng/mL) was quantified at 18 hrs following LPS activation through an enzymatic reaction assay (**D**), as well as quantification of protein content in cell lysates from treated microglia through a Bradford assay (**E**). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. #, ### denote $p < 0.05$, 0.005 vs. control, respectively. *, *** denote $p < 0.05$, 0.005 vs. LPS treatment, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 35.7$ (A), 2.32 (B), 38.7 (C), 0.9 (D), 1.96 (E).

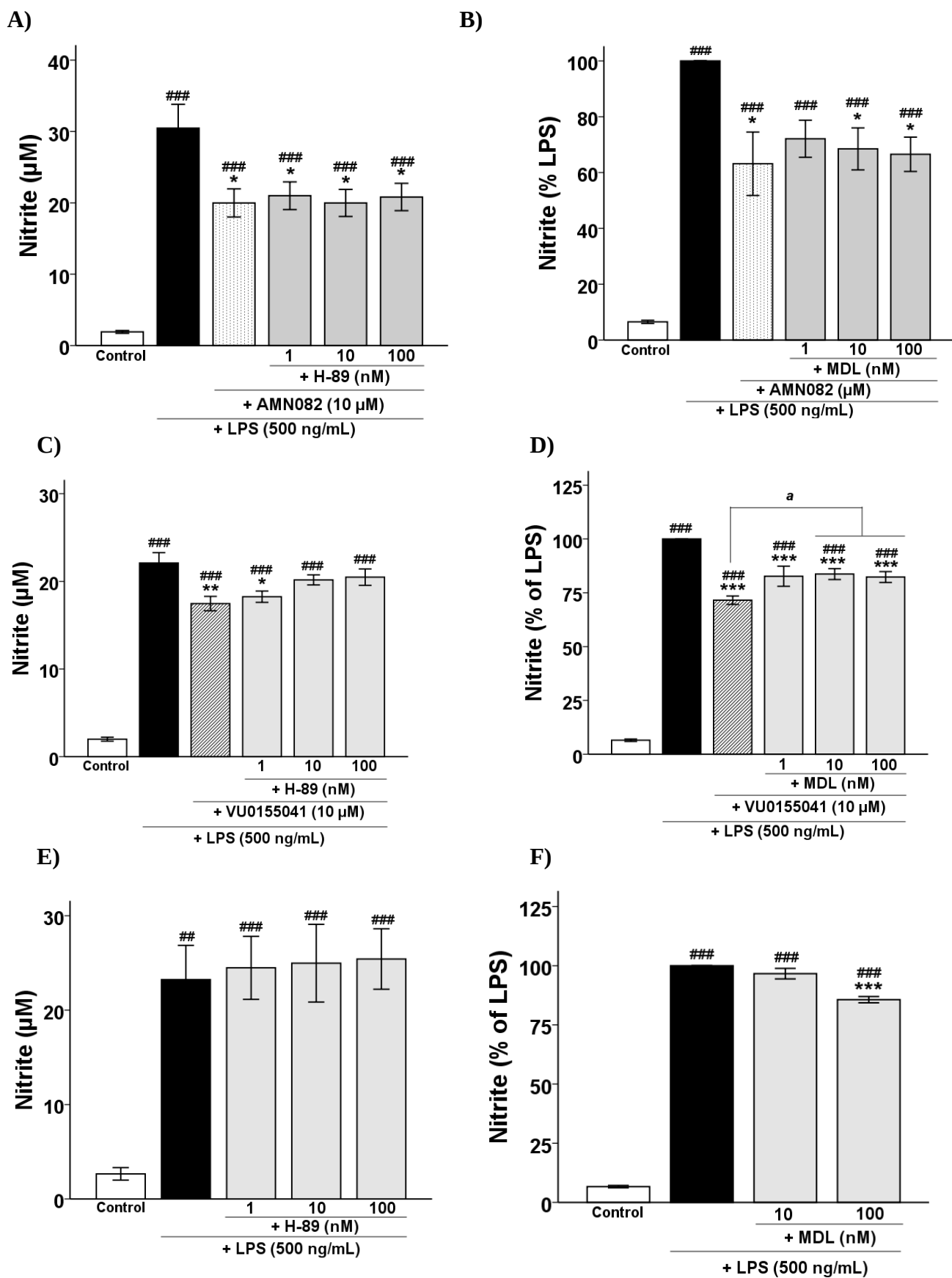


Figure 7.8 (Legend on following page)

Figure 7.8 Inhibition of protein kinase-A or adenylyl cyclase differentially modulates mGluR ligand-mediated reduction in nitrite production. To directly examine the involvement of the adenylyl cyclase/cAMP/PK-A signalling pathway in the reduction of LPS-stimulated nitrite production by mGlu4 or mGlu7 activation, LPS (500 ng/mL)-activated N9 microglia were treated with AMN082 (**A, B**) or VU01155041 (**C, D**) one hour following LPS activation with or without treatment (20 minutes before ligand treatment) with the protein kinase-A inhibitor H-89 (**A, C**) or the adenylyl cyclase inhibitor MDL-12230 (**B, D**). In addition, modulation of LPS-stimulated nitrite production by H-89 or MDL-12230 was examined in the absence of mGluR ligand treatment (**E, F**). Nitrite production was quantified through the Griess assay 18 hrs following LPS activation. Results are displayed as mean $\pm$ SEM of 5 (**A**), 4 (**C**) or 3 (**B, D-F**) independent experiments measured in triplicate. **##, ###** denote $p < 0.01, 0.005$ vs. control, respectively. ***, **, ***** denote $p < 0.05, 0.01, 0.005$ vs. LPS treatment, respectively. *a* denotes $p < 0.05$ between corresponding conditions. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 19.87$ (**A**), 18.9 (**B**), 87.6 (**C**), 244.2 (**D**), 9.29 (**E**), 1130.4 (**F**).

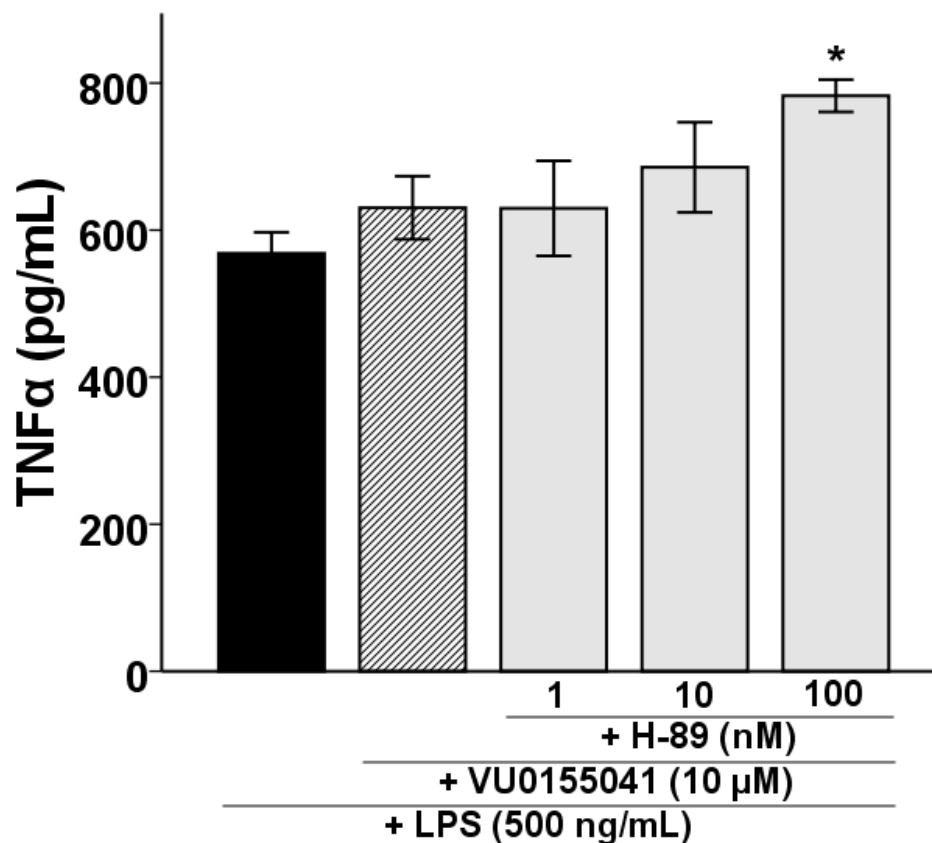


Figure 7.9 Protein kinase-A inhibition enhances TNF α release from LPS-activated VU0155041-treated N9 microglia. The effect of protein kinase-A inhibition on LPS-stimulated TNF α release from VU0155041-treated microglia was examined by treating LPS (500 ng/mL)-activated N9 microglia with VU0155041 one hour following LPS activation with or without H-89 pre-treatment (20 minutes before VU0155041 treatment). TNF α release into the cell culture medium was examined through an ELSIA at 18 hrs following LPS activation. Results are displayed as mean $\pm$ SEM of 3 independent experiments measured in duplicate. *denotes $p < 0.05$ vs. LPS. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 3.47$

7.4 Discussion

7.4.1 *Inhibition of NF κ B replicates certain aspects of mGluR-mediated reduction of microglial activation*

The results presented in this chapter demonstrate that inhibition of microglial reactivity by activation of individual group III mGluR is replicated by treatment with the NF κ B inhibitor APDC. The effects of AMN082 on microglial reactivity were completely replicated by APDC treatment, including inhibition of NO/iNOS synthesis and IL-6 release, as well as inhibition of TNF α release stimulated by 5 ng/mL but not 500 ng/mL LPS, suggesting activation of mGlu7 leads to reduction in iNOS, IL-6 and TNF α expression through inhibition of NF κ B activation. On the other hand, the fact that APDC did not replicate the effects of VU0155041 on IL-6 or TNF α release indicate that VU0155041 may also signal through an NF κ B-independent mechanism.

Interestingly, APDC treatment 1hr following LPS activation had an inverted bell-shaped dose-response curve for inhibition of nitrite production, iNOS expression and IL-6 release, indicating APDC lost efficacy at higher concentrations. The loss of efficacy of APDC could result from a feedback loop acting on NF κ B activity. Indeed, NF κ B activity is known to be partly self-regulated. Accordingly, activation of NF κ B not only promotes the transcription of pro-inflammatory genes, but in addition results in increased expression of its inhibitory protein I κ B, as well as decreased I κ B degradation (Vallabhapurapu and Karin, 2009; Sun *et al.*, 1993; Scott *et al.*, 1993). Hence, NF κ B is able to terminate its own activation by promoting binding of I κ B, leading to shuttling the NF κ B-I κ B complex out of the nucleus and increased cytosolic sequestration. Therefore, strong NF κ B inhibition, as might be the case during treatment with 10 μ M APDC, would lead to an excessive decrease of available I κ B proteins, resulting in reduced NF κ B cytosolic sequestration and increased inflammatory gene expression. However, treatment with 10 μ M APDC 20 minutes before LPS treatment did not result in loss of efficacy with respect to nitrite production inhibition, suggesting

treatment of N9 microglia with APDC before the onset of activation avoids the negative NFκB feedback loop.

Interestingly, treatment of N9 microglia with VU0155041 at high concentrations (100 μM) led to reduced inhibition of nitrite production similarly to APDC treatment. Furthermore, treatment with VU0155041 20 minutes before LPS treatment prevented loss of efficacy. Therefore, VU0155041 treatment at high concentrations might establish a negative NFκB feedback loop similarly to APDC treatment. Interestingly, VU0155041 did not lose efficacy at enhancing IL-6/1β release, again suggesting mGlu4 activation couples to NFκB-independent signalling pathways.

It is of interest that, while APDC treatment failed to modulate TNFα release stimulated by 500 ng/mL LPS, it successfully promoted inhibition of TNFα release stimulated by 5 ng/mL LPS. The TNFα promoter in monocytes/macrophages is known to contain binding sequences for several transcription factors, including NFκB, AP-1 and CREB (Liu *et al.*, 2000; Yao *et al.*, 1997; Shakhov *et al.*, 1990). The NFκB binding sites have been shown to be important for TNFα expression following LPS stimulation, as evidenced by a marked reduction in TNFα promoter activity following deletion of the NFκB binding site κB3 (Yao *et al.*, 1997) and robust decrease in LPS-stimulated TNFα release following transfection with a dominant negative form of p65 (Liu *et al.*, 2000). However, non-NFκB transcription factors have also been shown to be important for induction of TNFα expression in monocytes/macrophages following LPS stimulation, specially the transcription factor AP-1 (cJun/cFos). For instance, deletion of the AP-1 binding sequences within the TNFα promoter leads to a reduction in promoter activity following LPS treatment (Yao *et al.*, 1997); conversely, overexpression of c-Jun protein increases TNFα promoter activity (Liu *et al.*, 2000). In addition, LPS-stimulated TNFα release is significantly reduced by transfecting macrophages with a dominant negative form of c-Jun (Liu *et al.*, 2000). Therefore, in N9 microglia, an LPS concentration of 500 ng/mL may stimulate TNFα expression which is either mainly driven

by AP-1 activation or driven by redundant NF κ B and AP-1 signalling capable of maintaining LPS-stimulated TNF α expression upon NF κ B inhibition, thereby explaining the failure of APDC to inhibit 500 ng/mL LPS-stimulated TNF α release. On the other hand, 5 ng/mL LPS may stimulate a comparable level of TNF α expression which is mainly driven by NF κ B signalling or lacking a redundant AP-1 signalling input, thereby allowing the APDC-mediated NF κ B inhibition to translate to reduced TNF α expression. The lack of dose-response in TNF α release inhibition by APDC may point to an underlying submaximal AP-1 signalling activated by 5 ng/mL LPS not affected by APDC (Comalada *et al.*, 2003). The involvement of AP-1 in TNF α release stimulated by 500 ng/mL LPS in addition explains the lack of effect of AMN082 at this LPS dose, as AMN082 would only reduce the NF κ B-mediated TNF α release. It is interesting that even at 5 ng/mL LPS treatment with VU0155041 failed to inhibit TNF α release (see chapter V), suggesting mGlu4 activation may lead to an NF κ B-independent enhancement of TNF α release capable of counteracting the reduction of TNF α release expected to be brought about by NF κ B inhibition. Indeed, Iacovelli *et al.* (2004) have shown that in embryonic human kidney cells expressing mGlu4 (and presumably no other group III mGluR), treatment with L-AP4 leads to activation of a mitogen activated protein kinase signalling pathway involving phosphorylation of the protein extracellular signal-regulated kinase (ERK)-1, known to be able to stimulate TNF α expression and release in murine macrophages (Shi *et al.*, 2002; Rousseau *et al.*, 2008). Importantly, Iacovelli *et al.* demonstrated the phosphorylation of ERK-1 to be dependent on the β and γ subunits of mGlu4-associated G-proteins, therefore ERK-1 phosphorylation would be independent of adenylyl cyclase activity.

7.4.2 L-AP4, AMN082 and VU0155041 enhance forskolin-stimulated cAMP accumulation

Contrary to expectations, co-treatment of N9 microglia with forskolin and L-AP4, AMN082 or VU0155041 resulted in an enhanced cAMP accumulation response. Importantly, co-treatment with

the mGlu7-specific allosteric antagonist reverted the AMN082-stimulated increase in cAMP accumulation, confirming a receptor-mediated effect. Receptor mediation by VU0155041 was not examined due to lack of mGlu4-specific allosteric antagonists. In addition, treatment with the mGlu8-specific orthosteric agonist DCPG failed to stimulate a significant increase in cAMP accumulation, perhaps reflecting the failure to detect significant levels of mGlu8 expression in N9 microglia (see chapter IV), as well the lack of effect of DCPG treatment on nitrite production (see chapter V).

Potentially, the unexpected effects of group III mGluR activation on cAMP accumulation could be due to glutamate in the culture medium interfering with the cAMP assay through direct competition for receptor binding. Hence, cAMP accumulation experiments were repeated replacing cell culture media with Locke's buffer. However, even under these conditions a modest increase in forskolin-stimulated cAMP accumulation by L-AP4, AMN082 and VU0155041 treatment was detected. Furthermore, DCPG treatment again failed to modulate cAMP accumulation.

The discrepancy between the marked effect of mGluR ligands on microglial reactivity and the modest effect on cAMP accumulation could be attributed to the short incubation time of microglia with ligands before harvesting for cAMP ELISA.

Even though the majority of published literature indicates activation of group II and III mGluR decreases cAMP production through coupling to a G_i protein (*e.g.* Taylor *et al.*, 2003; Vernon *et al.*, 2011; Niswender and Conn, 2010), there are a number of studies, both *in vitro* and *ex vivo*, indicating that activation of group II or III mGluR may lead to accumulation of cAMP and augmentation of cAMP-mediated responses. Accordingly, *in vitro* treatment of hypothalamic neurons transfected with group II or group III mGluR with agonists selective for group II or group III mGluR stimulated an increase in forskolin-stimulated cAMP accumulation (Sortino *et al.*, 1996). In addition, *ex vivo* electrophysiological studies employing tissue slices derived from the rat cortex have shown administration of the group III-specific agonist ACPT-1 leads to facilitation of

excitatory post-synaptic currents (EPSC) (Evans *et al.*, 2001), an effect usually ascribed to group I mGluR positively coupled to AC. Interestingly, this facilitatory effect was mimicked by application of the AC activator forskolin. Furthermore, the effects of ACPT-1 on EPSC were abolished by prior application of either an AC inhibitor or a PKA inhibitor. Importantly, the authors state treatment with either inhibitor did not reduce basal EPSC, indicating the reduction in EPSC was specifically due to inhibition of ACPT-1. Therefore, this study suggests group III mGluR in cortical neurons are positively coupled to AC and signal through the canonical AC-cAMP-PKA pathway, echoing a previous electrophysiological study indicating activation of a pre-synaptic group II-like mGluR in the drosophila neuromuscular junction led to increased synaptic transmission which was mimicked by cAMP analogues and AC activators (Zhang *et al.*, 1999). Similarly, pharmacological *in vitro* studies have shown that activation of group II-like mGluR enhances the responses to cAMP (Winder and Conn, 1995). Furthermore, Moldrich *et al.* (2002) have shown that, while *in vitro* treatment of primary astrocytes with agonists specific for group II mGluR under calcium-free conditions leads to a decrease in forskolin-stimulated cAMP production, treatment with the same ligands in the presence of a physiological extracellular calcium concentration (1.8 mM) led to an increase in cAMP production which was able to be reversed by group II mGluR antagonists and pertussis toxin. Interestingly, this dual action of group II mGluR agonists on cAMP production was not seen in primary neurons, with a decrease in cAMP being elicited under conditions of calcium depletion as well as physiological calcium concentrations. The study by Moldrich *et al.* again points to the complexity of mGluR signalling and its possible cross-talk with other receptor systems, as well as the difference in signalling mechanisms between neurons and glial cells.

Therefore, taken together, the above studies indicate that under certain circumstances, activation of mGluR putatively coupled to AC inhibition may result in increased production of cAMP, leading to signalling through the canonical AC-cAMP-PKA pathway. However, the increase in cAMP production by group II/III mGluR might not result from direct coupling to G_s proteins capable of

increasing AC activity. More likely, it results from secondary activation of G_s-coupled receptors (*e.g.* adenosine receptors) following calcium-mediated transmitter release. The study by Moldrich *et al.* suggests the mGlu2/3-mediated calcium increase results from a Gi_{βγ}-mediated activation of phospholipase C, leading to production of IP3 and DAG and stimulation of calcium release from intracellular stores.

7.4.3 Forskolin treatment replicates certain aspects of mGluR-mediated reduction in microglial activation

Given the unexpected finding that group III mGluR ligands enhanced cAMP accumulation in N9 microglia, we hypothesised that mGluR ligand treatment resulted in reduced microglial reactivity through increased cAMP production and PK-A activation, leading to decreased NFκB-dependent gene expression. To test this hypothesis, N9 microglia were simultaneously treated with LPS (100 ng/mL) and forskolin to induce an increase in cAMP. As hypothesized, simultaneous LPS and forskolin treatment stimulated a marked reduction in nitrite production and iNOS protein expression compared to LPS treatment alone. In addition, as hypothesized, forskolin treatment stimulated a reduction in LPS-stimulated TNFα release 18 hrs following LPS treatment. Unexpectedly however, forskolin treatment stimulated a robust and dose-dependent increase in LPS-stimulated IL-6 release at the same time-point, as well as an increase in IL-1β release.

As the dual ability of PK-A to positively and negatively affect NFκB activity results largely from differences in signalling mechanisms within distinct cell types as well as inflammatory stimuli, the opposing effects of forskolin on IL-6/1β and iNOS/TNFα evidenced in this thesis more likely arise due to PK-A-independent signalling mechanisms engaged by cAMP. Indeed, cAMP is known to exert important inflammatory responses independently of PK-A through activation of ‘exchange protein activated by cAMP’ (EPAC)-1 (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998), a small guanine exchange factor which interacts specifically with the Ras-like GTPase Rap-1 to elicit downstream

signalling (reviewed by Tasken and Aandahl, 2004). Accordingly, treatment of primary murine fibroblasts with β -adrenergic receptor agonists capable of inducing a marked increase in cAMP production leads to a significant increase in IL-6 release which is ablated by co-treatment with EPAC-1 silencing RNA, indicating cAMP signals through an EPAC-1-dependent mechanisms to induce IL-6 release in primary fibroblasts (Chen *et al.*, 2011). More relevant to this thesis, an *in vitro* study employing murine macrophages (RAW 264.7 cell line) has demonstrated treatment with a β -adrenergic receptor agonist capable of stimulating cAMP production leads to an increase in IL-6 expression which is not affected by either PK-A inhibition or NF κ B inhibition. In addition, treatment with an EPAC-1-specific cAMP analogue (8-CPT-2-O-Me-cAMP) led to upregulation of IL-6 mRNA (Tan *et al.*, 2007). Furthermore, the same study demonstrated cAMP stimulates IL-1 β expression which is similarly not affected by PK-A or NF κ B inhibition.

Crucially, *in vitro* studies have in addition shown EPAC-1 activation in macrophages/ microglia does not affect either TNF α release or nitrite production. Accordingly, treatment of LPS-activated primary human macrophages with 8-CPT-2-O-Me-cAMP fails to modulate TNF α release, while TNF α release is significantly inhibited by a PK-A specific cAMP analogue (6-Bnz-cAMP) (Bryn *et al.*, 2006). Similarly, treatment of the murine microglial cell line BV-2 with 8-CPT-2-O-Me-cAMP fails to modulate LPS-stimulated nitrite production (Moon *et al.*, 2005).

In support of increased adenylyl cyclase and PK-A activation following mGlu4 activation, treatment of microglia with the adenylyl cyclase inhibitor MDL-12330 or the PK-A inhibitor H-89 successfully reverted the VU0155041-stimulated reduction in nitrite production. In addition, H-89 treatment enhanced TNF α release following LPS/VU0155041 treatment. The increase in TNF α release could be due to silencing of mGlu4-mediated PK-A activity responsible for decreased NF κ B activity. Hence, by silencing PK-A, H-89 would allow the effects of the mGlu4 alternative signalling pathway ($G_{\beta\gamma}$ -mediated ERK-1 activation) (Iacovelli *et al.*, 2004) to become unmasked, resulting in an overall increase in TNF α release.

Unexpectedly however, neither MDL-12330 or H-89 were successful at reversing the AMN082-stimulated decreases in nitrite production. Speculatively, these unexpected results could be explained by a nuclear compartmentalization of mGlu7 in N9 microglia, thereby preventing inhibitors from reaching target proteins efficiently. In support of this, immunocytochemical and immunohistochemical evidence presented in this thesis (chapter IV) indicate mGlu7 is able to localize to the nucleus both in N9 microglia and in dopaminergic neurons. In addition, mGlu7 nuclear localization has also been evidenced by independent research groups (Dr. A. Vernon, personal communication). Furthermore, nuclear localization of both adenylyl cyclase and PK-A have been established in cultured cells (Corredor *et al.*, 2012; Zippin *et al.*, 2010; Neary *et al.*, 2001 and 2004). However, since a previous single study has suggested nuclear adenylyl cyclase to be insensitive to G-protein activation (Braun *et al.*, 1977), a more straight forward explanation for lack of effect of H-89 or MDL-12330 on AMN082-stimulated nitrite reduction would be lack of specificity of the inhibitors for the appropriate isoforms of adenylyl cyclase and PK-A or lack of potency at such low (nanomolar) concentrations. Alternatively, the ability of MDL-12330 treatment on its own to further decrease nitrite production might mask any reversal effects it might have on AMN082-stimulated nitrite production decrease. The ability of MDL-12230 to decrease LPS-stimulated nitrite production might be explained by its inhibitory effect on phosphodiesterases, thereby reducing cAMP breakdown (Hunt and Evans, 1980).

7.5 Conclusion

The results presented in this chapter suggest that, while our hypothesis regarding decreased cAMP accumulation following group III mGluR activation was erroneous, our hypothesis regarding modulation of NF κ B was correct. In addition however, these results add to our hypothesis by indicating activation of mGlu4 or mGlu7 engages different cAMP-dependent signalling mechanisms, possibly PK-A and EPAC-1 dependent signalling mechanisms, of which one appears to

be NF κ B-independent (see **fig. 9.1**). Hence, AMN082-mediated mGlu7 activation leads to an increase in cAMP which induces effects mimicking NF κ B inhibition, resulting in an overall inhibition of iNOS, IL-6 and TNF α expression. On the other hand, VU0155041-mediated mGlu4 activation leads to an increase in cAMP which simultaneously might activate EPAC-1, resulting in an overall inhibition of iNOS expression, but an increase in IL-6 and IL-1 β expression. In addition, VU0155041 might not have affected TNF α release due to the opposing effects on TNF α expression/release by the G $_{\alpha}$ and G $_{\beta\gamma}$ subunits coupled to mGlu4.

Chapter VIII

Microglial activation by
dopaminergic neuronal factors

8.1 Introduction

A substantial body of *in vitro*, *in vivo*, and clinical evidence implicate neurons as key regulators of microglial activation. Neurons may maintain microglia in a quiescent state through production of ligand/receptor interactions, termed ‘off’ signals which, if disrupted, lead to increased microglial activation and neuronal damage (Wang *et al.*, 2011b; Zhang *et al.*, 2011b; Luo *et al.*, 2010; Pabon *et al.*, 2011) (see also *section 1.6.4.3.1*). Therefore, degeneration of neuronal cells will lead to reduced ‘off’ signalling, resulting in increased microglial activation.

In addition however, factors released by dying neurons, such as matrix metalloproteinase enzymes, ATP, and glutamate, have been shown to be capable of activating microglia (see *section 1.6.4.3.2* for a more in depth discussion). Of particular relevance to PD, factors released from degenerating dopaminergic neurons, such as α -synuclein, neuromelanin and have also been shown to lead to increased microglial activation *in vitro*, as determined by increased production of TNF α , IL-6/1 β , iNOS, nitric oxide and reactive oxygen species (Zhang *et al.*, 2005; Wilms *et al.*, 2003). Furthermore, this increased microglial activation leads to increased dopaminergic neuron degeneration. Similarly, dopaminergic neuron-related compounds are able to cause microglial reactivity and neuronal loss *in vivo* (Zhang *et al.*, 2011).

Therefore, by releasing factors capable of stimulating microglial activation, degenerating dopaminergic neurons are able to establish a feed-forward cycle leading to chronic microglial activation and progressive neuronal degeneration. Hence, for a pharmacological compound to be a feasible candidate to prevent the progressive neuronal loss seen in progressive neurodegenerative diseases such as PD, it needs to be able to reduce microglial activation elicited not only by bacterial compounds, but in addition by factors released from degenerating neuronal cells, as might be the case during ‘sterile’ microglial activation (Stewart *et al.*, 2010).

With that in mind, this chapter sets out to induce microglial activation by factors released from damaged dopaminergic neurons and in addition aims to examine whether group III mGluR ligands are capable of inhibiting neuronal-stimulated microglial activation.

- **Hypothesis:** N9 microglia are able to be activated towards a pro-inflammatory phenotype by incubation with cell culture medium derived from degenerating dopaminergic neurons. Furthermore, group III mGluR ligands are capable of reducing such microglial activation

8.2 Experimental design

Microglial activation by dopaminergic neuronal factors released from damaged neurons was attempted by incubating N9 microglia for 18 hrs with medium derived from dopaminergic N27 neurons (*i.e.* neuronal conditioned medium, NCM) damaged by either treatment with 6-OHDA, the ATP-competitive protein kinase inhibitor staurosporine or glutamine/serum starvation. NCM was not centrifuged prior to transfer to microglial cultures to ensure all possible neuronal factors capable of activating microglia were transferred to microglial cultures.

N27 neurons plated in complete N27 medium (see section 2.1.5) were treated with 6-OHDA for 24 hrs, after which time-point the NCM was transferred to N9 microglia (plated 24 hrs prior) and microglia incubated in NCM for a further 18 hrs. As 6-OHDA oxidation interfered with the nitrite quantification assay (see section 3.3.2.2), 6-OHDA had to be removed from the culture well prior to nitrite quantification. To this end, the original NCM was aspirated from N9 microglia following 18 hr incubation, the N9 microglia washed once with sterile PBS and 1 mL complete microglial medium (see section 2.1.3) replaced into the culture well. N9 microglia were further incubated in complete microglial medium for 6 hrs to allow nitrite accumulation, after which time-point the medium was subjected to Griess assay for nitrite quantification (**fig. 8.1**).

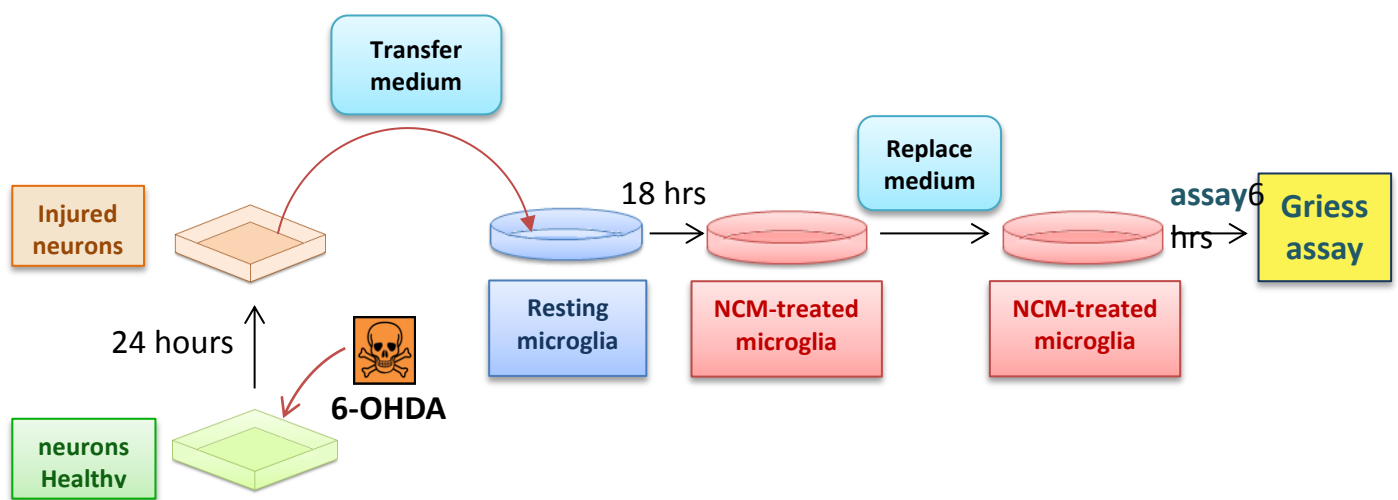


Figure 8.1 Treatment protocol for microglial activation by 6-OHDA-treated neurons. Following 24hrs incubation of neurons with 6-OHDA, neuronal conditioned medium (NCM) was transferred to microglial cultures. After 18hrs incubation in NCM, the medium was replaced to remove 6-OHDA and microglia cultured for a further 6 hrs, at which time-point nitrite levels in the culture medium were quantified through a Griess assay.

For staurosporine treatment of neurons, N27 neurons plated in complete N27 medium were treated with staurosporine for 4 hrs. Neurons were then thoroughly washed with sterile PBS (five washes with 2 mL sterile PBS with gentle shaking) to completely remove staurosporine from the culture well and 2 mL complete microglial medium replaced into the culture well. Neurons were incubated in staurosporine-free complete microglial medium for a further 6 hrs, after which time-point the NCM (1.5 mL) was transferred to N9 microglia (plated 24 hrs prior). Following an 18 hrs incubation with NCM, medium was collected and nitrite production, IL-6 and TNF α release measured through the Griess assay or ELISAs. In order to verify staurosporine did not induce nitrite production in neurons which might confound nitrite production quantification in N9 microglia, a Griess assay was carried out on the 0.5 mL of NCM left after transferring NCM to the microglia. In addition, NCM-treated microglia were lysed with RIPA buffer and the lysates subjected to protein quantification through the Bradford assay. The effect of mGlu7 activation on NCM-mediated microglial nitrite production was examined by treating N9 microglia with AMN082 immediately following NCM transfer and Griess assay carried out as above (**fig. 8.2**).

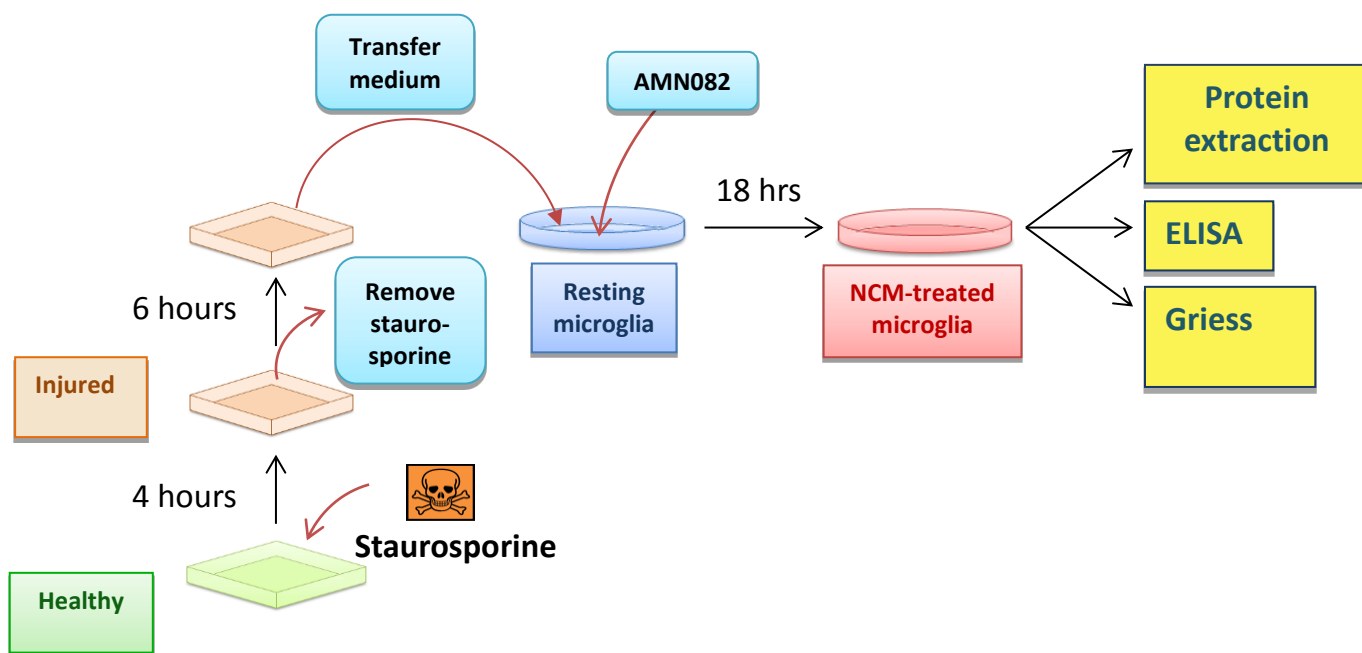


Figure 8.2 Treatment protocol for microglial activation by staurosporine-treated neurons. Following 4 hrs treatment with staurosporine, neuronal cultures were thoroughly washed to remove staurosporine and incubated in fresh medium for a further 6hrs. Neuronal conditioned medium (NCM) was then transferred to microglial cultures (with or without simultaneous treatment with AMN082) and the microglia incubated in NCM for a further 18hrs, at which time-point nitrite and cytokine levels in the culture medium were quantified through the Griess assay and ELISAs, respectively. Microglial viability was examined at the same time-point through quantification of intracellular protein.

For glutamine/serum starvation experiments, N27 neurons were incubated in D-MEM supplemented with penicillin/streptomycin in the absence of glutamine and foetal calf serum for 24 hrs, after which time-point the NCM was transferred to N9 microglial cultures. Immediately after transfer, the NCM was supplemented with 8 mM glutamine and 5% foetal calf serum. Microglial activation was assessed 18 hrs following NCM transfer through nitrite quantification by the Griess assay (**fig. 8.3**).

In order to verify N9 microglia were able to be activated by neuronal factors, N9 microglia were incubated with MMP-3 for 18 hrs, after which time-point IL-6 released into the culture medium was quantified through an ELISA in order to assess microglial activation. In addition, N9 microglia were treated with an MMP-3 inhibitor 10 minutes prior to MMP-3 treatment to verify any increase in IL-6 release was specifically due to MMP-3 activity.

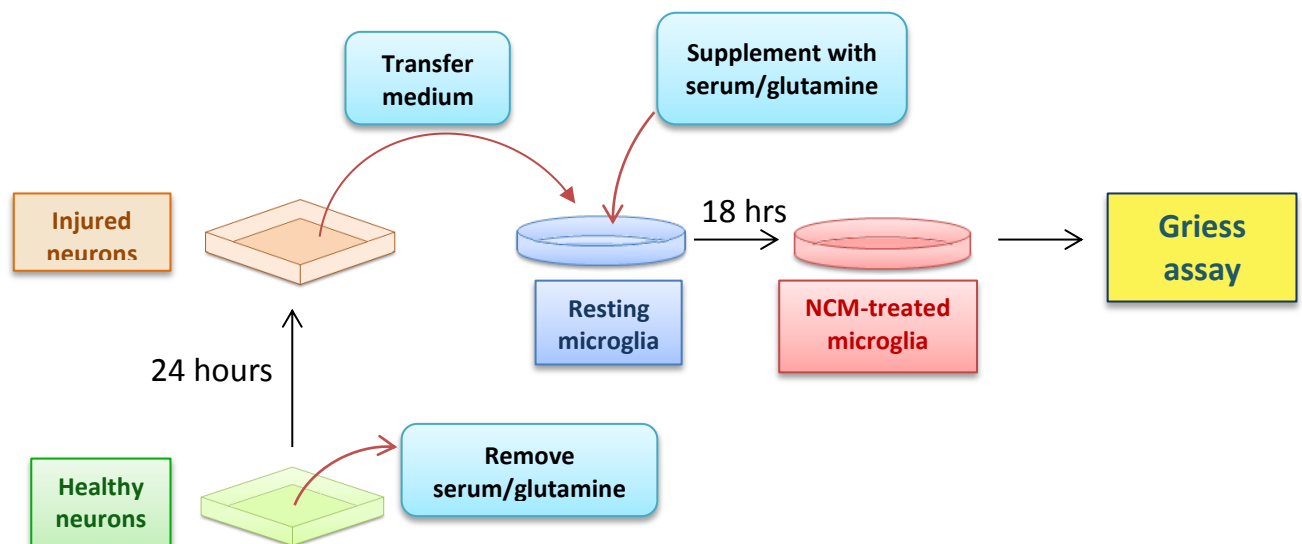


Figure 8.3 Treatment protocol for microglial activation by glutamine/serum-starved neurons. Following a 24hrs incubation of neurons with serum/glutamine free medium, the neuronal conditioned medium (NCM) was transferred to microglia and immediately supplemented with serum and glutamine. After a further 18hrs microglial incubation in supplemented NCM, nitrite levels in the culture medium were quantified through the Griess assay.

8.3 Results

8.3.1 *Effect of 6-OHDA-treated N27 neuronal medium on nitrite production by N9 microglia*

Treatment (24 hrs) of N27 dopaminergic neurons with increasing concentrations of 6-OHDA resulted in a steady decrease in cellular protein content (**fig. 8.4A**). However, incubation (18 hrs) of N9 microglia with neuronal conditioned medium (NCM) from 6-OHDA-treated neurons (6-OHDA-NCM) did not stimulate an increase in nitrite production in comparison to N9 microglia incubated with NCM from control N27 neurons (C-NCM) (**fig. 8.4B**).

8.3.2 *Effect of staurosporine-treated N27 medium on nitrite production by N9 microglia*

Treatment of N27 neurons with the protein kinase inhibitor staurosporine at 1 and 10 μM caused visible cell death at 4 hrs post-staurosporine treatment, characterized by a robust decrease in cell density and adoption of a condensed cell body morphology with spindly and nodulated processes (images not shown). Treatment with lower staurosporine concentrations (0.01 and 0.1 μM) did not result in a visible loss of cell density, but neurons shifted towards a morphology resembling that seen with higher staurosporine concentrations (images not shown). Incubation (18 hrs) of N9 microglia with NCM from staurosporine treated N27 neurons (S-NCM) following removal of staurosporine (see section 8.2) led to a modest increase in nitrite production compared to N9 microglia incubated with NCM from control N27 neurons (C-NCM), though this did not reach statistical significance (**fig. 8.5A**). Interestingly, NCM from N27 neurons treated with 1 and 10 μM staurosporine stimulated a marked increase in N9 microglial cell death, as evidenced by a marked reduction in cellular protein content in N9 microglia incubated with 1 and 10 μM S-NCM compared to N9 microglia incubated with C-NMC (**fig. 8.5B**).

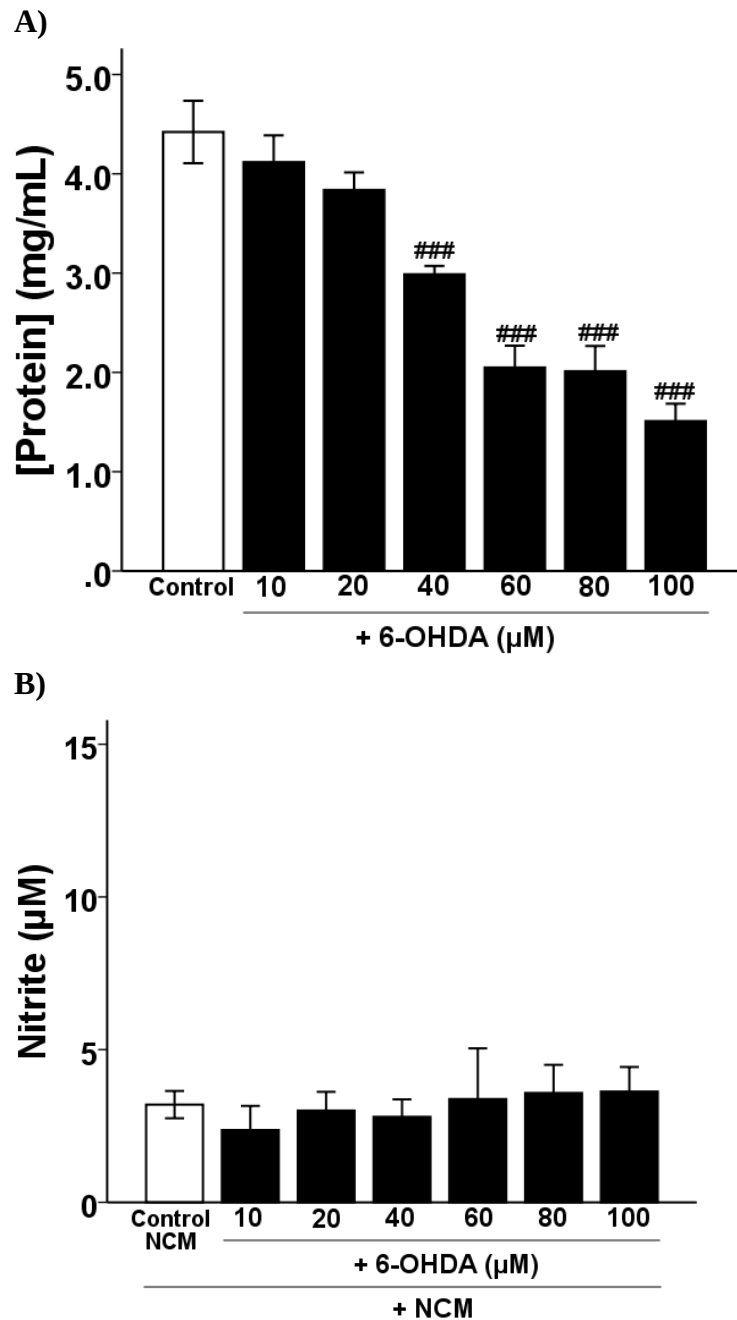


Figure 8.4 Viability of 6-OHDA-treated neuronal cultures and microglial activation by medium derived from 6-OHDA-treated neurons. Cellular viability was assessed after 24hrs incubation of neuronal cultures with the dopaminergic toxin 6-OHDA through quantification of neuronal protein by the Bradford assay (A). Microglial activation by incubation (18hrs) with neuronal conditioned medium (NCM) derived from 6-OHDA-treated neurons was assessed through quantification of nitrite production by the Griess assay (B). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate (A) or triplicate (B). ### denotes $p < 0.005$ vs. control. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 26.5$ (A), 0.25 (B).

Staurosporine did not induce nitrite production in N27 neurons, as evidenced by equal nitrite concentration in the NCM from control and staurosporine treated neurons before transfer to microglia (**fig. 8.5C**). Preliminary data indicated simultaneous treatment of N9 microglia with S-NCM and the mGlu7-specific allosteric agonist AMN082 led to an inhibition of NCM-stimulated nitrite production increase (**fig. 8.5D**).

8.3.3 Effect of staurosporine-treated N27 neurons on IL-6 and TNF α release by N9 microglia

Incubation (18 hrs) of N9 microglia with S-NCM following removal of staurosporine failed to stimulate IL-6 release (**fig. 8.6A**) and TNF α release (**fig. 8.6B**).

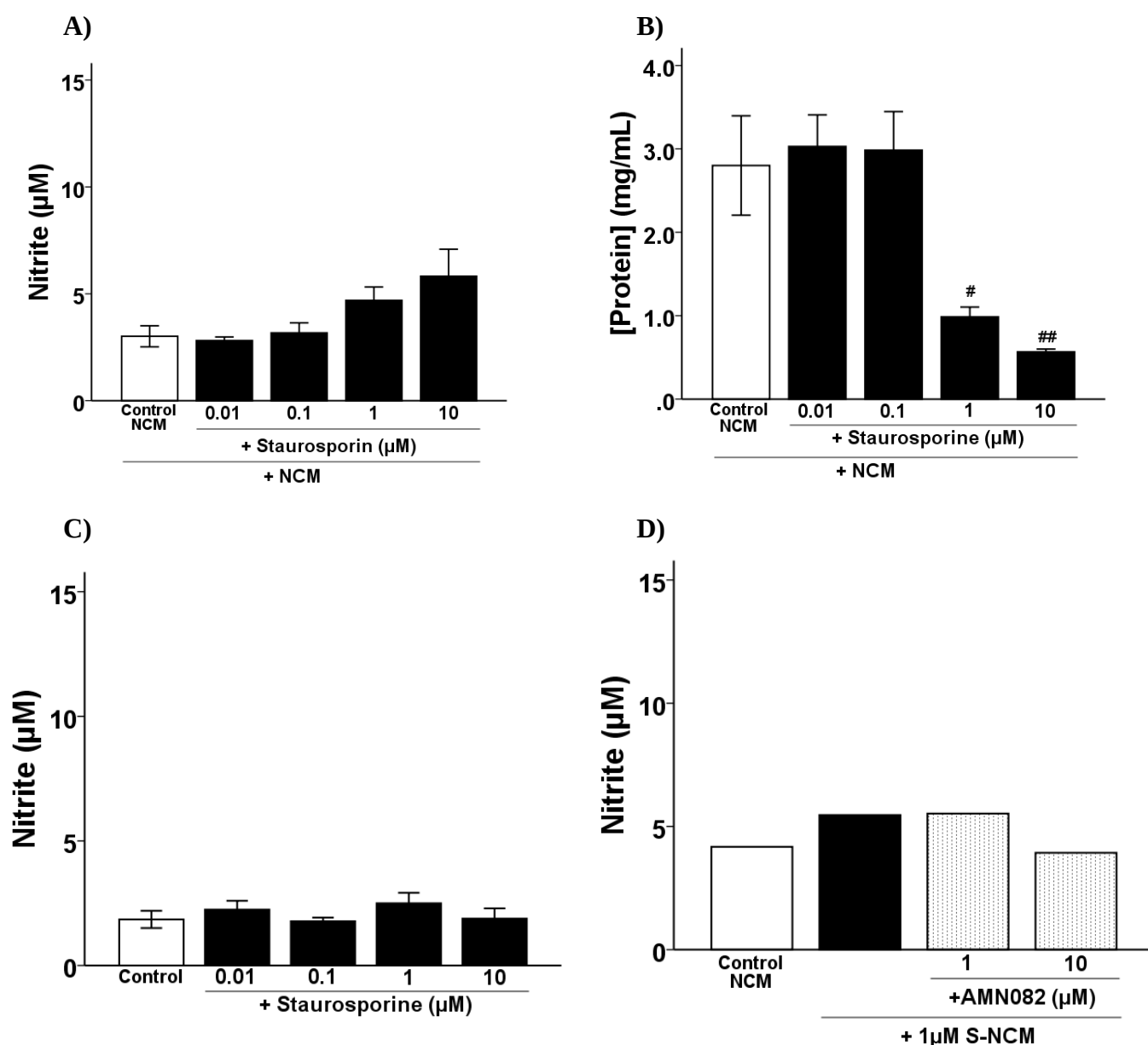


Figure 8.5 Nitrite production by N9 microglia incubated with neuronal conditioned medium (NCM) derived from staurosporine treated neurons. N9 microglial nitrite production and viability following incubation (18hrs) with neuronal conditioned medium (NCM) derived from neurons treated with increasing staurosporine concentration (S-NCM) was assessed through the Griess assay (A) and cellular protein content through the Bradford assay (B), respectively. Nitrite production by neurons treated with increasing staurosporine concentrations was also quantified through the Griess assay (C). The effect of AMN082 treatment on microglial reactivity stimulated by incubation with S-NCM (1 μM) was also assessed through nitrite quantification by the Griess assay (D). Results are displayed as mean $\pm$ SEM of three independent experiments (except D, which represents a single experiment) measured in triplicate (A, C-D) or duplicate (B). #, ### denote $p < 0.05$, 0.005 vs. control, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 2.93$ (A), 9.85 (B), 0.84 (C).

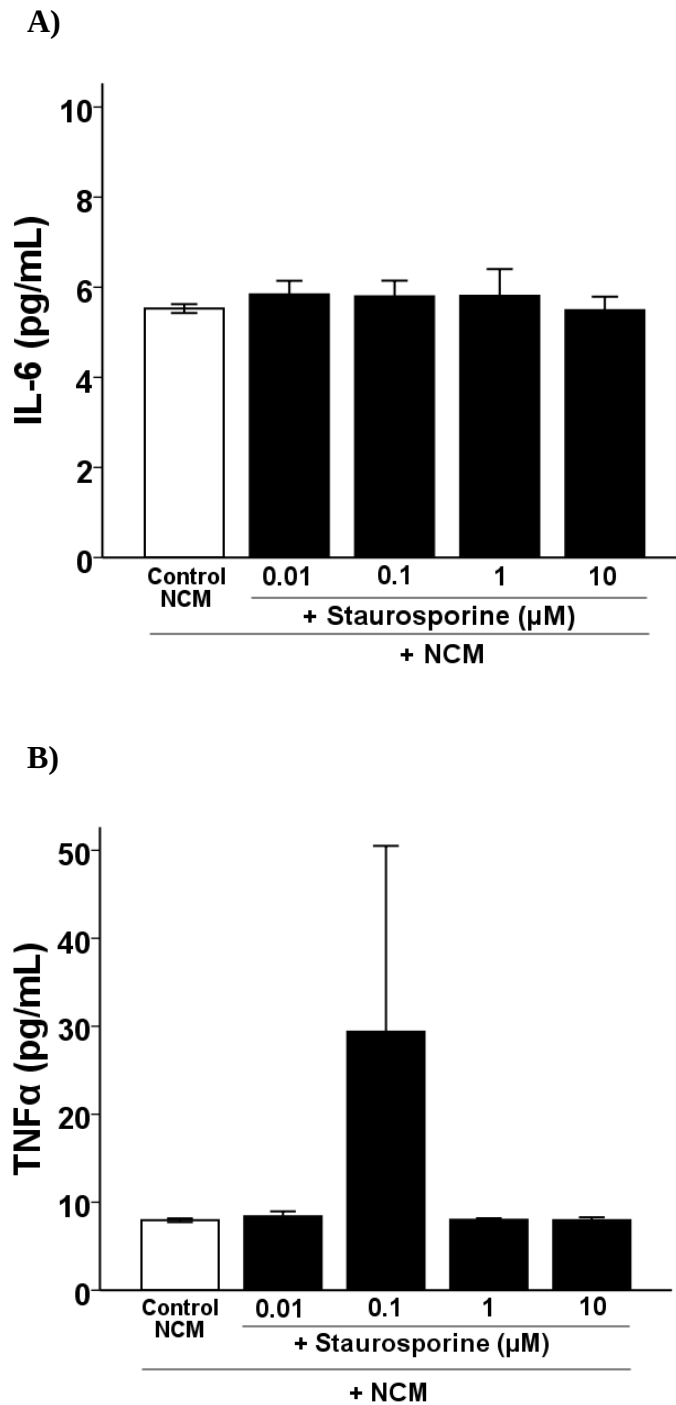


Figure 8.6 IL-6 and TNFα release by N9 microglia incubated with neuronal conditioned medium derived from staurosporine treated neurons. Release of IL-6 (A) and TNFα (B) by N9 microglia incubated (18hrs) with neuronal conditioned medium (NCM) derived from neurons treated with increasing staurosporine concentration (S-NCM) was assessed through ELISA assays. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 0.22$ (A), 17.5 (B).

8.3.4 *Effect of glutamine/serum-starved N27 neurons on nitrite production by N9 microglia*

In order to induce neuronal death, glutamine and serum were removed from the incubation medium and neuronal cultures incubated for 24hrs. By 24hrs incubation in glutamine/serum-free medium, there was a marked increase in cell death, as assessed by increased release of LDH into the culture medium (**fig. 8.7A**). However, incubation (18hrs) of N9 microglia with neuronal conditioned medium derived from glutamine/serum-starved neurons failed to stimulate an increase in nitrite production (**fig. 8.7B**).

8.3.5 *Effect of MMP-3 on IL-6 release by N9 microglia*

To confirm matrix metalloproteinase enzymes are capable of activating N9 microglia, N9 microglia were treated with MMP-3. An 18h incubation with MMP-3 led to a robust increase in IL-6 release (Control: 1.67 ± 0.41 pg/mL; MMP-3: 778.8 ± 33.6 , $p = 0.0005$ vs. control). Furthermore, addition of MMP-3 inhibiting peptides to the cell culture medium 10 minutes prior to MMP-3 treatment led to a significant and dose-dependent decrease in MMP-3-stimulated IL-6 release (5, 25 μ M MMP-3 inhibitor: 299.7 , 0.06 ± 0.4 , respectively; $p = 0.0005$ vs. MMP-3) (**fig. 8.8**).

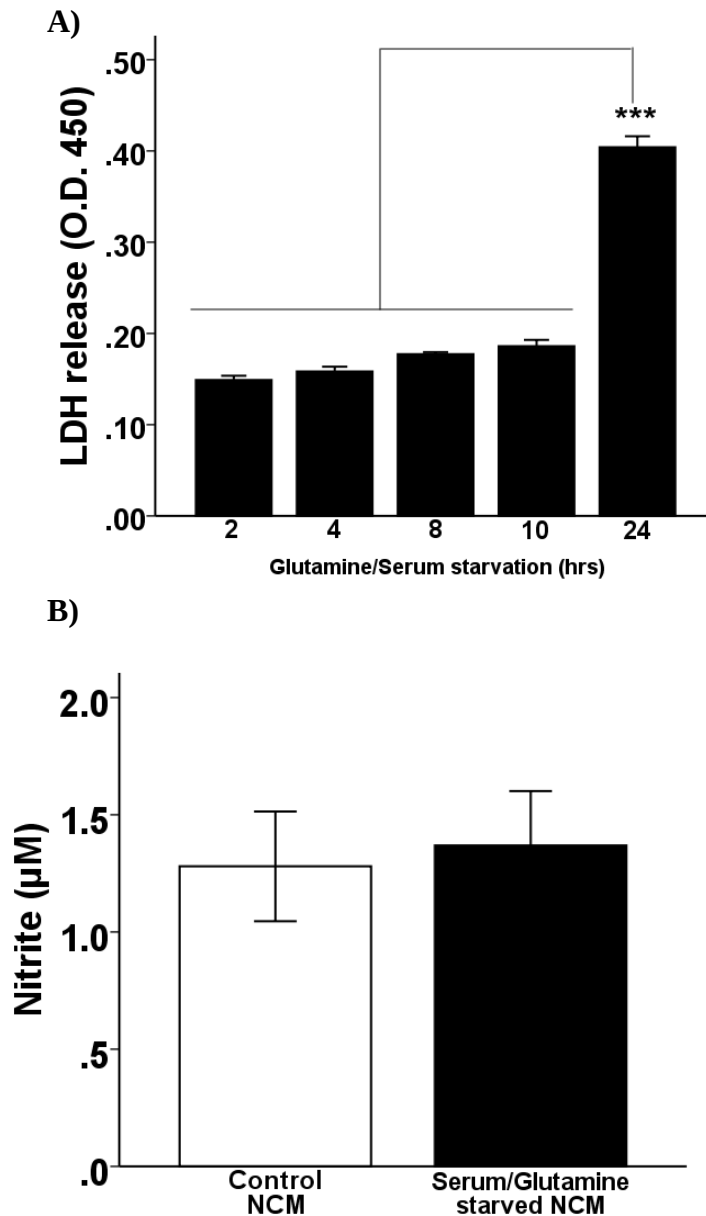


Figure 8.7 Neuronal viability following glutamine/serum starvation and induction of microglial nitrite production by incubation with serum/glutamine-starved neuronal conditioned medium. Neuronal viability following incubation with serum/glutamine-free medium at different time-points was assessed through quantification of LDH release (A). Microglial activation by incubation (18hrs) with neuronal conditioned medium (NCM) derived from serum/glutamine-starved neurons was assessed through quantification of nitrite production by the Griess assay (B). Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate (A) or triplicate (B). Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test (A) or independent samples t-test (B). *** denote $p < 0.005$ between corresponding conditions. $F = 167.6$ (A), 0.04 (B).

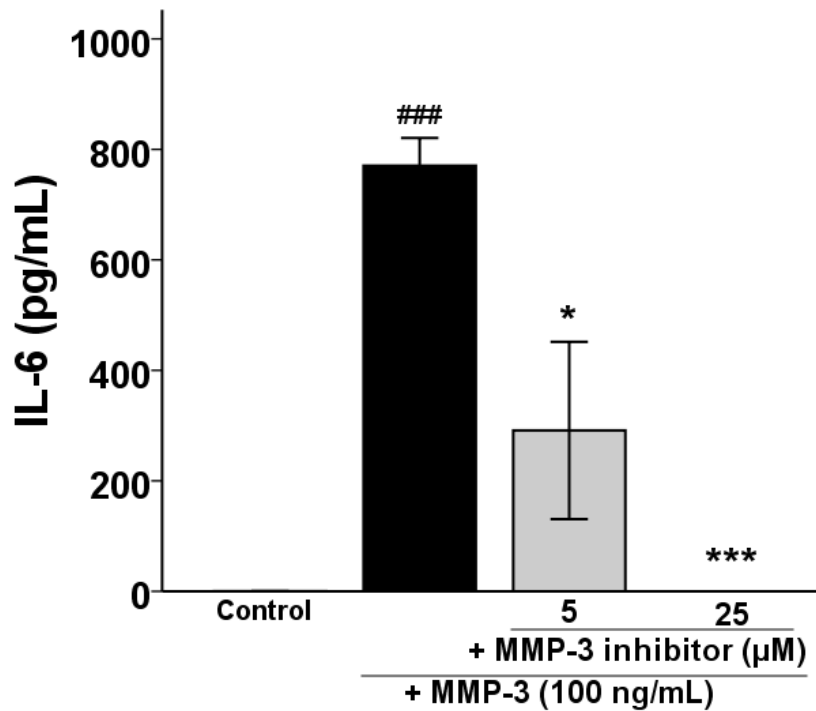


Figure 8.8 MMP-3 stimulates IL-6 release from N9 microglia. In order to verify matrix metalloproteinases are capable of activating microglia, N9 microglia were incubated with MMP-3 for 18h, after which time-point IL-6 release into the cell culture medium was quantified through an ELISA. To establish the direct effects of MMP-3 on IL-6 release, microglia were incubated with an MMP-3-inhibiting peptide 10 minutes prior to MMP-3 treatment. Results are displayed as mean $\pm$ SEM of three independent experiments measured in duplicate. ### denotes $p < 0.005$ vs. control, respectively. *, *** denote $p < 0.05$, 0.005 vs. MMP-3, respectively. Statistical significance was determined by one-way ANOVA with Tukey's *post-hoc* test. $F = 15.3$

8.4 Discussion

Although treatment of neurons with 6-OHDA stimulated robust neuronal cell death, NCM from 6-OHDA damaged neurons failed to induce microglial activation. However, 6-OHDA treatment led to a mode of cell death characterized by membrane disruption, increased cell debris and higher number of unattached floating cells. These observations led us to hypothesize 6-OHDA treatment principally led to cell death through necrotic mechanisms. Previous studies demonstrating microglial activation by NCM have induced neuronal death principally through apoptotic mechanisms (Kim *et al.*, 2005). To investigate whether the lack of microglial activation evidenced in our experiments was due to an inappropriate mode of cell death, we induced neuronal damage by treatment with staurosporine, a kinase inhibitor which is known to stimulate a strong apoptotic response (*e.g.* Jacobsen *et al.*, 1996). Similarly to 6-OHDA treatment, staurosporine led to marked neuronal cell death. However, the mode of cell death differed greatly to 6-OHDA-stimulated cell death. Staurosporine treatment stimulated the neuronal cell bodies to become condensed and their processes to become spindly with scattered nodes throughout the process length, a morphology more closely related with an apoptotic process. However, exposure of phosphatidylserine was not examined to confirm apoptosis.

Incubation of microglia with NCM from staurosporine-damaged neurons (S-NCM) led to a modest increase in nitric oxide synthesis. Importantly, the increase in nitric oxide was specifically microglial, as nitrite levels in the NCM prior to transfer to microglial cells was equal for all staurosporine concentrations. Furthermore, preliminary experiments suggested the S-NCM-stimulated nitrite synthesis increase can be reversed by AMN082 treatment similarly to reversal of LPS-stimulated nitrite synthesis, further highlighting the potential of AMN082 as a viable therapy in neurodegenerative diseases.

Although S-NCM led to a modest increase in nitrite production, there was no detectable increase in pro-inflammatory cytokine release. Interestingly however, despite repeated washings of neurons

to thoroughly remove staurosporine, microglia incubated with NCM from neurons treated with 1 and 10 μ M staurosporine underwent marked cell death characterized by marked loss of cell density and increased cellular debris. Therefore, the lack of increased release of pro-inflammatory cytokines could be due to decreased cellular density. .

Regardless whether microglial cell death was due to staurosporine carried over in the culture medium or to factors released from staurosporine-treated neurons, the evidenced microglial cell death confounded the measurements of microglial activation. Therefore, serum/glutamine deprivation was adopted to induce neuronal cell damage. However, despite marked cell death following 24h serum/glutamine deprivation, microglial activation was not detected following incubation with NCM.

In the first instance, these results would appear to suggest neuronal degeneration is incapable of inducing microglial activation, which would call into question the theory of feed-forward neurodegeneration/microgliosis/neurodegeneration cycle. However, the lack of microglial activation may not rest in true lack of microglial activation by neuronal factors, but in limitations in the experimental protocol. For instance, we were not able to measure either the levels of MMP in the collected NCM or their enzymatic activity. Therefore, the neuronal density, cell culture volume, and/or time-frame of experimentation might have led to the concentration of neuronal factors being too low to cause microglial activation. Furthermore, naïve N27 neurons have been shown to not express detectable levels of α -synuclein (Jin *et al.*, 2011). Therefore, N27 neurons might not have produced appropriate factors to induce microglial activation in the first instance. In order to overcome this limitation, the experimental protocols will have to be optimized to ensure the neuronal density, mode of degeneration, and neuronal phenotype employed are adequate to produce appropriate levels of neuronal factors in the cell culture medium. In addition, the mode of microglial treatment could be optimized (for instance, employing trans-well co-cultures instead of cell culture medium transfer).

8.5 Conclusion

Despite employing several methods to induce neuronal cell death, transfer of NCM to microglia failed to convincingly stimulate microglial activation. Although this negative result may invalidate our hypothesis regarding microglial activation by dopaminergic neuronal factors, it may also be due to inappropriate protocols to induce microglial activation, insufficient concentration of activating factors, or simply lack of expression of appropriate microglia-activating factors by N27 neurons. In support of this, treatment with MMP-3 induced a robust increase in release of pro-inflammatory cytokines. Therefore, future studies will need to optimize both the protocols and the phenotype of employed neurons to successfully test our hypothesis.

Chapter IX

General Discussion

9. General discussion

Neuroinflammation, characterized primarily by activation of the brain's resident immune cell, namely microglia, is thought to contribute to the progressive neuronal cell death seen in various neurodegenerative diseases, including Parkinson's disease (PD). At present, there is an unmet need to develop therapies which not only ameliorate PD symptoms, but in addition prevent, and possibly reverse, the progressive neuronal degeneration which characterizes these diseases. Therefore, the present thesis set out to investigate the effect of activating individual group III metabotropic glutamate receptor (mGluR) on microglial reactivity and microglia-mediated dopaminergic neuronal death in order to establish the potential of these receptors as therapeutic agents against PD.

9.1 Principal findings of the thesis and their implications on neurodegenerative diseases

The main novel contribution to scientific knowledge of the results presented in this thesis consist of demonstrating: 1) N9 and primary microglia express mGlu7 receptors, a fact previously overlooked probably due to limited detection of mGlu7 receptor splice variants; 2) activation of N9 microglial mGlu4 and mGlu7 by VU0155041 and AMN082, respectively, as well as general group III activation by L-AP4, leads to an overall increase in forskolin-mediated cAMP production, demonstrating important differences in neuronal and microglial mGluR signalling; 3) despite putatively sharing signalling mechanisms, activation of mGlu4 and mGlu7 by VU0155041 and AMN082, respectively, leads to differential modulation of microglial reactivity with respect to nitrite production, iNOS expression and pro-inflammatory cytokine release, which translates to differential modulation of microglia-mediated neurotoxicity towards dopaminergic neurons. Therefore, we present evidence indicating that, as has been shown previously for group I and group II mGluR, modulation of microglial reactivity and neurotoxicity by group III mGluR activation greatly depends on individual receptor identity (**fig. 9.1**).

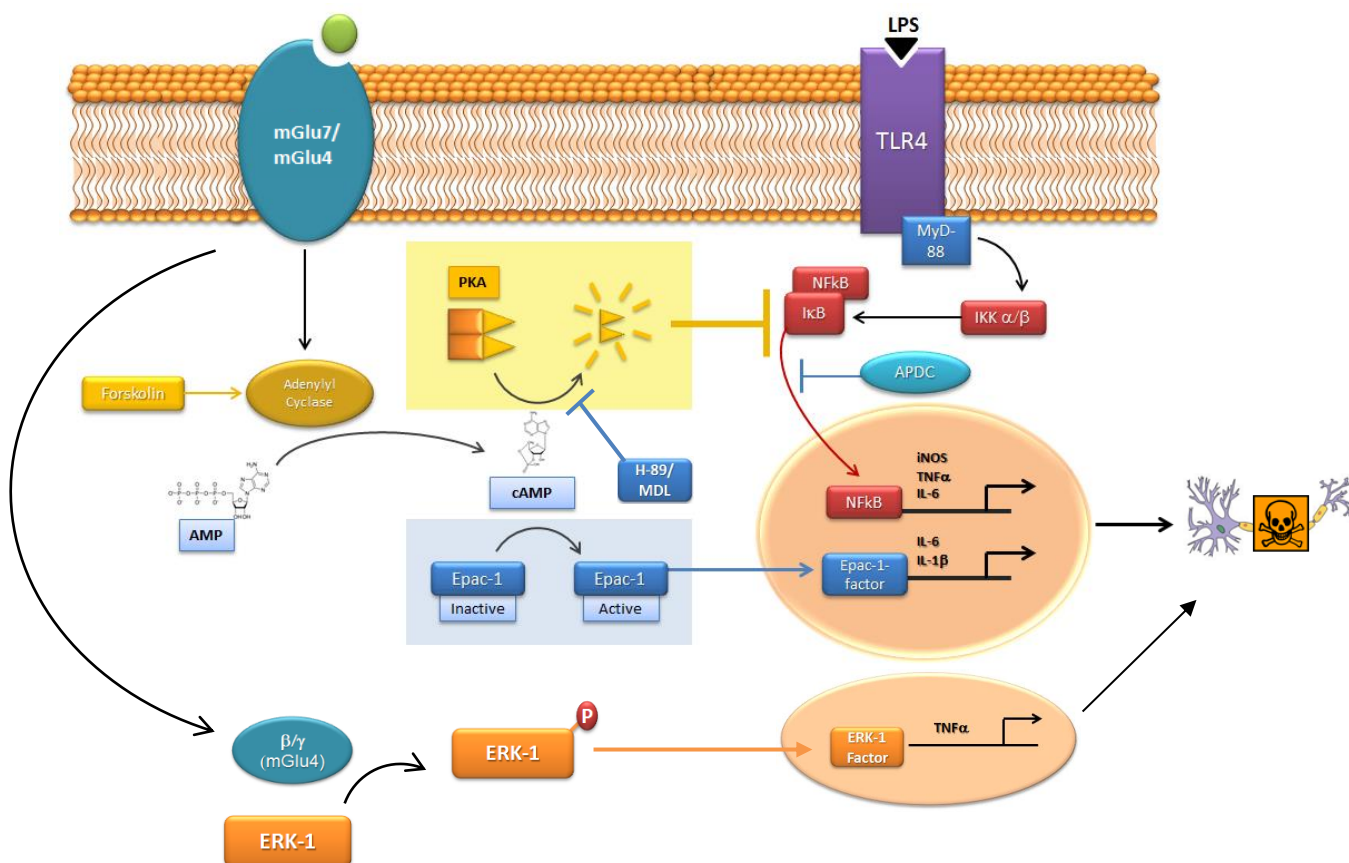


Figure 9.1 Possible signalling mechanisms underlying modulation of microglial reactivity and neurotoxicity by mGlu4 and mGlu7. LPS induces expression of pro-inflammatory proteins through activation of the transcription factor NFκB, an effect which can be counteracted by the NFκB inhibitor APDC. Similarly, cAMP reduces LPS-mediated inflammation by inhibiting NFκB through activation of PKA. However, cAMP may also activate the guanine exchange factor EPAC-1, leading to NFκB-independent expression of IL-6 and IL-1β. In N9 microglia, activation of mGlu7 leads to a decrease of inflammatory gene expression, possibly through an increase in cAMP which specifically activates PKA without affecting EPAC-1 activation. Conversely, mGlu4 activation in N9 microglia, as well as forskolin treatment, leads to a decrease in pro-inflammatory protein expression with a concomitant increase in IL-6/1β expression, possibly through an increase in cAMP which activates both PKA and EPAC-1. Furthermore, the βγ subunits of the G-protein coupled to mGlu4 is capable of phosphorylating ERK-1, potentially inducing an NFκB-independent increase in TNFα expression.

The principal discrepancy between this thesis and published literature concerns the increase in intracellular cAMP following group III mGluR activation. To the best of our knowledge, the only study examining the effect of microglial group III mGluR activation on cAMP production is the *in vitro* study by Taylor *et al.* (2003), which demonstrated a decrease in forskolin-stimulated cAMP accumulation following L-AP4 treatment. Interestingly, this study employed cell culture medium with levels of potassium chloride which were fivefold greater than in the culture medium employed in this thesis (this high potassium chloride concentration was not adopted in order to benefit microglial cells or to replicate microglial biology, but to allow proper survival of cerebellar neurons during incubation with microglial conditioned medium). As has been discussed in previous chapters, effect of activation of mGluR putatively coupled to G_i proteins on cAMP levels greatly depends on intracellular calcium concentration due to their ability to stimulate release of adenosine (Moldrich *et al.*, 2002). Hence, the relatively low potassium chloride concentration employed in this thesis may have had a significant impact on intracellular calcium leading to an increase in adenosine release. In order to test this, the cAMP assay will need to be carried out in the presence of adenosine receptor antagonists to verify the involvement of adenosine in the evidenced increase in cAMP.

The present findings have several implications for the field of neurodegenerative diseases and therapy. Firstly, although modulation of microglial reactivity will most probably not prevent the onset of PD, it would be particularly useful to prevent the progressive neurodegeneration typical of this disease. Although this would not represent a cure, it might reduce neuronal degeneration to such an extent as to allow current therapies (*e.g.* L-DOPA) to be employed for much longer periods which might maintain the quality of life of the patient.

In addition to PD, microglial activation has been shown to be associated with several different CNS disorders. For instance, microglial activation has been linked to damage of oligodendrocytes during multiple sclerosis (MS). In particular, peroxynitrite radicals, derived from nitric oxide synthesised by activated microglia, may be particularly damaging to oligodendrocytes and myelin

sheaths due to their abundance of lipid chains, capable of producing lipid alkyl radicals which propagate free-radical chain reactions (reviewed by Jack *et al.*, 2005). Hence, inhibiting nitric oxide synthesis by activating mGlu4 and/or mGlu7 may decelerate the rate of myelin damage in MS. However, due to the fact that mGlu4 activation leads to increased interleukin release, mGlu7 may be the most appropriate target for MS therapy.

Similarly, microglial reactivity has been implicated in the demise of motoneurons during amyotrophic lateral sclerosis (ALS). For instance, in ALS mouse models, there is a shift from an M2 microglial phenotype (characterized by expression of anti-inflammatory cytokines) to an M1 phenotype (with increasing expression of NADPH oxidase, TNF α and IL-1 β) as disease progression accelerates. Furthermore, systemic administration of LPS results in exacerbated disease progression and motor axon damage (reviewed by Henkel *et al.*, 2009). Presumably, given that a major risk factor for ALS is reduced protection against oxidative damage due to impaired superoxide dismutase activity, the major contribution of microglial reactivity to ALS progression is not release of pro-inflammatory cytokines, but synthesis of reactive molecular species. Therefore, as with MS, activating mGlu4 and/or mGlu7 may prevent motoneurons damage in ALS.

In addition, microglial reactivity has also been associated with Alzheimer's disease (AD). For instance, through PET imaging using the benzodiazepine receptor, whose expression is increased in activated microglia, Edison *et al.* (2008) have demonstrated an increase in microglial reactivity in AD brains compared to age-matched controls, which importantly correlates with cognitive decline. Furthermore, levels of proinflammatory cytokines capable of being secreted by microglia, such as IL-1 β /6 and TNF α , are elevated in AD brains compared to controls (Sastre *et al.*, 2006). Hence, while activation of mGlu4 may be counter indicative due to its enhancement of cytokine release, activating mGlu7 may promote neuronal survival in AD.

Therefore, developing a therapy capable of inhibiting microglial reactivity would not only benefit PD patients, but would have wide implications of the field of neurodegeneration as a whole.

Interestingly, in our hands, immunohistochemical labelling of mouse brain tissue failed to identify group III mGluR expression in resting Ox-42⁺ microglia. In line with this finding, previous studies have indicated microglia fail to express detectable levels of mGluR under resting conditions. However, following microglial activation due to pathology or injury, there is a marked expression of microglial mGluR (Byrnes *et al.*, 2012; Geurts *et al.*, 2005, 2003). The expression of mGluR specifically in reactive microglia presents a possibility for targeting solely microglia involved in pathologic events. This is highly desirable, as, under resting conditions, microglia play a crucial role in maintaining neuronal health (see below). Therefore, solely modifying the phenotype of reactive (and presumably neurotoxic) microglia by targeting mGluR would allow for the microglial neurotrophic support in healthy brain regions to go undisturbed.

Interestingly, microglial activation by LPS or myelin has been shown to increase glutamate release *in vitro* (Pinteaux-Jones *et al.*, 2008; Barger *et al.*, 2007), possibly through an atypical release mechanism regulated by oxidative stress. Furthermore, receptor affinity for glutamate is highest in group II mGluR, and lowest in group III mGluR (Cartmell *et al.*, 2000). Therefore, microglial reactivity may be perpetuated not only through paracrine mechanisms (*i.e.* damage of neurons leading to release of neuronal factors capable of activating microglia), but also through an autocrine mechanism whereby glutamate release by activated microglia preferentially activates microglial group I/II mGluR, leading to further activation and glutamate release. Therefore, activation of group III mGluR would allow for this vicious cycle to be inhibited.

However, though inhibiting microglial reactivity may promote neuronal survival through reduction of pro-inflammatory cytokine release and reactive molecular species synthesis, it may also have detrimental side-effects, as reduced microglial reactivity would in addition lead to reduced phagocytic activity. Indeed, phagocytic activity is at least partly regulated by the same mechanisms involved in inducing a neurotoxic microglial phenotype in this thesis, *i.e.* TLR-4 ligation. Accordingly, *in vitro* studies have demonstrated ligation of TLR-4 promotes microglial phagocytosis

(Ribes *et al.*, 2010). Furthermore, TLR-4 knock out in animal models of AD results in increased amyloid- β load (Tahara *et al.*, 2006), possibly through reduced microglial phagocytosis. Similarly, ablation of TLR-4 has been shown to lead to decreased clearance of α -synuclein *in vivo*, with a concomitant increased loss of nigrostriatal dopaminergic neurons (Stefanova *et al.*, 2011). Interestingly, the same authors demonstrated increased levels of TNF α in the midbrain following TLR-4 ablation, which were associated with increased activation of astrocytes. Therefore, decreased phagocytosis as a consequence of overall reduced microglial reactivity may decrease neuronal survival by failing to remove pathology-related compounds capable of directly damaging neurons or promoting pro-inflammatory factor release from astrocytes. Hence, a tailored therapy which specifically inhibits the pro-inflammatory/neurotoxic microglial phenotype without reducing phagocytic potential would be most advantageous. Interestingly, a recent *in vivo* study has shown that LPS treatment, in addition to inducing an M1 (pro-inflammatory) profile on microglia as discussed in previous chapters, can also induce an M2 phenotype, which translates to increased neuroprotection following injury (Chen *et al.*, 2012), possibly depending on the dose of LPS treatment. Therefore, the neuroprotective or neurotoxic fate of activated microglia may be determined through a single receptor, probably depending on the extent and/or time-frame of pathway activation.

In theory, it would be possible to identify a specific interplay between microglial signalling pathways which would decrease microglial neurotoxicity, as well as increase neuroprotection (*e.g.* through increased trophic factor support or phagocytic activity) which would not only prevent neuronal cell death, but in addition promote neuronal regeneration. Microglia are optimally poised to carry out this task, as not only are they capable of clearing the surrounding environment of axon-growth inhibiting components (*e.g.* myelin/myelin-associated proteins) and providing trophic support for neuronal survival, but in addition are capable of promoting axon growth and sprouting, as well as ensuring proper synaptic connection between emerging axons through synaptic pruning

(Mocchetti *et al.*, 2012; Schafer *et al.*, 2012). To achieve this, we will need to understand which signalling pathways control individual elements of microglial phenotype, the interplay between them, as well as their effects on acute and chronic microglial reactivity.

9.2 Limitations of the thesis

Although the present thesis does shed light on the biology of microglial group III metabotropic glutamate receptors and their impact on neurodegenerative diseases, the experimental protocol does place limitations on the potential clinical relevance of our findings.

The first possible limitation of this thesis is the use of the bacterial cell-wall component lipopolysaccharide (LPS) to induce microglial activation. LPS has been widely employed in inflammatory studies due to its robust and reliable activation of macrophages. However, employing LPS to model microglial activation during neurodegenerative diseases possibly leads to microglial activation through mechanisms that are irrelevant to the diseases of interest, *i.e.* the inflammatory signalling mechanisms engaged by LPS may not be activated in microglia during neurodegenerative diseases, therefore compounds able to reduce microglial activation by LPS will not necessarily succeed in reducing microglial activation induced during neurodegenerative diseases. In support of this caveat, even though interferon- γ stimulates a similar increase in nitric oxide and TNF α synthesis to LPS in microglia (Kato *et al.*, 2008), proteomic studies of human macrophages have shown a different pattern of protein expression in human macrophages activated with interferon- γ to macrophages activated with LPS (Brown *et al.*, 2009). Furthermore, exposure of human macrophages to interferon- γ leads to an upregulation of CD200 receptor, an effect which is not seen in LPS activated macrophages (Pietila *et al.*, 2012). Therefore, interferon- γ treated macrophages may be more amenable to inhibition of activation than LPS treated macrophages. These

discrepancies indicate microglial activation occurs through various mechanisms which engage specific signalling proteins resulting in different activated phenotypes.

However, previous studies have shown that the mechanisms employed by LPS to induce microglial inflammation, namely TLR-4 ligation and NF κ B activation, are also engaged by compounds relevant to neurodegenerative diseases, as well as neuronal factors released from damaged neurons. Accordingly, ablation of TLR-4 expression in microglia has been shown to stimulate a marked reduction in amyloid- β -stimulated IL-6, TNF α and nitric oxide synthesis *in vitro*, with a subsequent reduction in neurotoxicity towards hippocampal neurons (Walter *et al.*, 2007). The role of TLR-4 in amyloid- β -induced inflammation has also been demonstrated *in vivo*, where upregulation of pro-inflammatory cytokines in mouse models of Alzheimer's disease is dependent on TLR-4 expression (Jin *et al.*, 2008b). Similarly, compounds relevant to Parkinson's disease are known to engage pathways relevant to LPS signalling to induce microglial activation. For instance, *in vitro* treatment of microglial cultures with α -synuclein or with medium derived from neurons overexpressing α -synuclein stimulated an increase in expression of NF κ B-dependent proteins, such as TNF α and NADPH oxidase (Alvarez-Erviti *et al.*, 2011; Zhang *et al.*, 2005). Although it has been reported that this effect may rely on TLR-2 over TLR-4, a recent preliminary study has suggested α -synuclein leads to astroglial activation and NO and IL-6 release in a TLR-4-dependent fashion (Fellner *et al.*, 2012), therefore a similar mechanism may be present in microglial cells. Interestingly, α -synuclein has been associated with an increase in TLR-4 expression, though the exact significance of this observation remains unclear (Beraud *et al.*, 2011). Furthermore, neuromelanin, a component released from damaged dopaminergic neurons, induces microglial expression of NO, IL-6 and TNF α *in vitro* in an NF κ B-dependent manner, an effect which translates to dopaminergic neurotoxicity *in vivo* similarly to LPS administration (Zecca *et al.*, 2008; Wilms *et al.*, 2003).

The above studies therefore provide support for LPS as a valid microglial activator to investigate the therapeutic potential of pharmacological agents against PD and AD. However, an optimal experimental protocol would be to induce microglial activation with factors released from damaged dopaminergic neurons, as was attempted in chapter VIII. In addition to addressing the possible lack of relevance to pathologic mechanism, activation of microglia by degenerating neurons would also address the limitation of inducing microglial activation by a single activating factor. Given that in the clinical setting degenerating neurons release various factors capable of activating microglia (*e.g.* α -synuclein, neuromelanin and matrix metalloproteinases), microglial activation in pathologic conditions would be induced through activation of various receptors/signalling pathways, possibly leading to synergism and/or modification of cellular responses which would not be elicited by activating microglia with a single activating factor.

A further limiting factor of the present thesis is the focus on cytotoxic/pro-inflammatory agents following microglial activation. Although microglia are known to execute inflammatory and cytotoxic actions in order to clear invading pathogens, leading to collateral neuronal damage, they are also able to provide neuronal support through release of trophic factors such as glial-derived neurotrophic factor (GDNF). GDNF is of special interest in PD, as its effects have been shown to be essential for survival of adult dopaminergic neurons (Pascual *et al.*, 2008), and shown to be decreased in dopaminergic neurons from PD brains (Chauhan *et al.*, 2001). Interestingly, activation of microglia with LPS leads to a reduction in GDNF release (Matsushita *et al.*, 2008), despite an increase in GDNF gene expression (Tanaka *et al.*, 2008). Furthermore, glutamate has been shown to be able to induce expression of GDNF in cultured resting microglia (Liang *et al.*, 2010). Therefore, AMN082 and/or VU0155041 treatment may have had important effects on GDNF secretion which would have a significant impact on the ability of microglia to prevent dopaminergic neuronal death. In order to assess this, the levels of available GDNF following LPS activation and AMN082/VU0155041 treatment will need to be quantified. As discussed above, solely quantifying

intracellular GDNF levels may lead to inaccurate measurement of available GDNF, as expression and release are differentially regulated. Therefore, in order to accurately quantify available GDNF, concentration of GDNF in the microglial conditioned medium will have to be quantified possibly through enzyme-linked immunosorbent assays. Furthermore, the effect of microglial GDNF on neuronal viability could be examined by incubating neurons with microglial conditioned medium under conditions of GDNF signalling disruption (*e.g.* employing GDNF neutralizing antibodies).

Furthermore, the ability of mGluR to induce an increase in trophic factor release would be crucial for these receptors to be able to promote neuronal survival and regeneration in addition to decreasing neurotoxicity.

A final limiting factor in this thesis is the employment of microglial cell lines. Even though we have taken care to demonstrate N9 immortalized microglia reliably replicate primary rat microglia behaviour with respect to a ramified morphology and low levels of nitric oxide and pro-inflammatory cytokine production under resting conditions, as well as a shift towards a hypertrophied morphology and increased synthesis of nitric oxide and pro-inflammatory conditions, other biological aspects not examined in this thesis might differ between N9 microglia and primary microglia. For instance, it has been shown that N9 microglia and primary microglia differentially regulate glutathione synthesis following activation (Fry, 2009). However, even employing primary microglia derived from rats may pose a limitation on clinical relevance, as there are important biological differences between cells isolated from rodents and from humans (*e.g.* Kotokorpi *et al.*, 2007; Aronsson *et al.*, 2000). In addition, microglia isolation protocols routinely employ rat pup brains, therefore do not reliably represent the changes induced by ageing. Importantly, given the discussed reliance of mGluR on interaction with secondary signalling proteins to elicit specific cellular responses, and how this interaction can be modulated by receptor conformation, mGluR expressed on primary microglia derived from young rodents may have intrinsic biological differences to human (and specially aged human) mGluR, despite the high level of homology

between certain human and rat mGluR (Varney *et al.*, 1999 and references therein). However, even employing human microglia cell lines may not be a completely reliable *in vitro* model for human microglial biology, as the process of immortalization (*v-myc* oncogene transformation) has been shown to lead to differences in pro-inflammatory cytokine expression (IL-6, IL-1 β and TNF α) both under control conditions and following exposure to LPS or amyloid- β (Nagai *et al.*, 2001). Therefore, ultimately, if microglia biology related to human neurodegenerative disease is to be studied *in vitro* it may be necessary to employ primary microglia acutely isolated from aged human brains, and optimally from pathologically affected individuals.

9.3 Future work stemming from this thesis

Despite the limiting factors discussed above, the present findings bring into focus important future experiments to be carried out to move microglial modulation by mGluR activation forward as therapeutic strategies.

Firstly, given that neuronal survival in this thesis was examined through transfer of microglial conditioned medium to neuronal cultures, the evidenced neurotoxicity was most probably due to stable soluble factors, such as pro-inflammatory cytokines, more than to the actions of reactive molecular species (due to their relatively short half-lives). Therefore, it will be important to establish the effect of reactive molecular species through co-culture studies (*e.g.* transwell incubation). In theory, given that both AMN082 and VU0155041 led to a reduction in iNOS expression and nitric oxide synthesis, these experiments would lead to a greater neuroprotective effect of mGlu4/7 activation than seen in the present experiments due to decreased nitrosative damage to neurons. However, an elegant *in vitro* study (Neher *et al.*, 2011) has shown that nitric oxide synthesis in addition promotes microglial clearance of neuronal cells, regardless of their viability status, *i.e.* peroxynitrite derived from nitric oxide synthesized by activated microglia tags neuronal cells for phagocytic clearance through exposure of phosphatidylserine. Crucially, inhibition

of phagocytosis allowed reversal of phosphatidylserine exposure and survival of neurons. Hence, production of nitric oxide may lead to neuronal demise not only through increased cellular stress, but in addition through stimulation of phagocytic clearance of otherwise healthy neurons. Therefore, in order to evaluate the complete neuroprotective effect of reduced nitric oxide synthesis, a cell culture system permissible of neuronal-microglial physical contact would need to be employed in addition to trans-well studies. Furthermore, a cell culture system permitting neuronal-microglial physical contact would allow for important neuronal ‘off’ signals towards microglial reactivity to be examined. For instance, under normal circumstances, interaction of the microglial CD200 receptor with its neuronal ligand would maintain microglia in a quiescent state. Therefore, allowing this interaction in contact-permissible culture studies would allow for the increase in microglial reactivity due to decreased neuronal contact to be examined.

Secondly, it will be important to establish the effect of microglial activation on microglial expression of all group III mGluR in models of neurodegeneration (*e.g.* MPTP) in order to examine the possible extent of involvement of each receptor on modulation of microglial reactivity. Furthermore, it will be necessary to establish group III mGluR expression in both resting microglia and activated microglia in brains from PD patients in order to establish the prominence of each receptor in pathologic environments. As a speculative point, if no mGluR expression is found on resting or activated microglial cells during PD, this may open up the possibility of utilizing gene therapy as a neuroprotective mechanism by inducing mGluR expression in reactive microglia. The feasibility of gene delivery to microglial cells is supported by studies demonstrating systemically injected lentiviral vectors have the capacity to deliver targeted genes to the brain parenchyma in animal models (*e.g.* Huang *et al.*, 2007). Importantly, microglial cells could be targeted over non-myeloid cells as well as macrophages by employing a keratin sulphate proteoglycan (detected by the antibody 5-D-4) uniquely expressed in resting and activated microglia (Wang *et al.*, 2005; Wilms *et al.*, 1999). Furthermore, a unique variation of mGluR could be engineered which, upon activation,

would lead to interaction with specific secondary signalling proteins which elicit only desirable effects on microglial reactivity.

Thirdly, in order to establish whether in fact the detrimental effects of VU0155041 outlined in this thesis hold true *in vivo*, experiments untangling the effects of neuronal and microglial mGlu4 activation need be carried out. Potentially, this could be achieved through genetic manipulation of animals to insert the suicidal gene herpes simplex virus 1 thymidine kinase (HSV-1 TK) into a genome region controlled by a microglia-specific promoter such as the CD11b promoter (Gowing *et al.*, 2006). If mGlu4 is indeed neuroprotective when activated in neurons, but neurotoxic when activated in microglia, treatment of HSV-1 TK animals with a neurotoxin (*e.g.* 6-OHDA) and VU0155041 should lead to increased neuroprotection compared to wild-type animals. However, global ablation of microglia might confound the neuroprotective results, as there would be no background microglial activation (leading to possible further neurotoxicity) elicited by neuronal damage. Therefore, a more attractive genetic manipulation approach would be to employ Cre-Lox technology, where LoxP sites are targeted to span the mGlu4 gene and the Cre gene is under the control of a microglia specific promoter, such as CD11b. Hence, expression of the Cre enzyme is only elicited in cells expressing CD11b, where it functions to excise the genetic information flanked by LoxP sites. Therefore, this technology would allow for the effects of microglial mGlu4 in VU0155041 neuroprotection experiments to be elucidated without affecting the normal microglial contribution to neuronal loss.

Although several studies have examined the symptomatic effects of AMN082 in PD animal models, to the best of our knowledge, there are currently no studies examining the neuroprotective effect of AMN082 in PD animal models. Therefore, it will be interesting to examine whether the *in vitro* inhibitory effects of AMN082 on microglial reactivity translate to neuroprotection *in vivo*. Although the same genetic manipulation models outlined above could be employed to elucidate the microglial effect of AMN082, a more simple approach would be to employ the endotoxin model of

dopaminergic neurodegeneration. In this animal model, an LPS injection into the substantia nigra leads to marked and specific degeneration of dopaminergic neurons. Crucially, microglial activation precedes dopaminergic cell death and motor symptom emergence (Tufekci *et al.*, 2011), implicating microglial activation, and not excitotoxicity due to subthalamic overactivity, as the responsible mechanism for primary dopaminergic degeneration. Therefore, if AMN082 protects neurons from early degeneration in this animal model, it would indicate a microglia-mediated mechanism.

Given the evidence presented indicating individual mGluR differentially affect microglial neurotoxicity despite belonging to the same receptor group, future studies will have to be carried out to establish either a therapeutic strategy that targets mGluR in specific cell types (*e.g.* activation of neuronal mGlu4, and inhibition of microglial mGlu4), or identify an mGluR which has beneficial effects when activated in all cell types. In order to fulfil the former, structural differences between microglial and neuronal mGluR may be exploited. Allosteric ligands, targeting the highly variable transmembrane receptor region (see above), are prime candidates to take advantage of these differences. Structural differences may not necessarily reside in the transcriptional machinery, but may arise from post-translational modification (*e.g.* palmytolation) (Zheng *et al.*, 2012), giving rise to receptors with modified structure or variability in dimerizing dynamics.

9.4 Final conclusion

Taken together, the results presented within this thesis further support the neurotoxic role of microglial reactivity during neurodegenerative diseases. Furthermore, our neurotoxicity experiments demonstrate microglia-mediated toxicity does not rely solely on oxidative/nitrosative damage and phagocytosis, but in addition may cause neuronal damage through stable soluble factors.

Most importantly, our findings support our original hypothesis with regard to modulation of microglial reactivity and neurotoxicity by ligands targeting individual group III mGluR. In addition however, these results add to our original hypothesis and demonstrate that activation of receptors

classified within a single group due to sequence homology may still elicit different effects on cellular physiology, further highlighting the need to carefully select which mGluR to target to develop as a therapeutic strategy. Hence, our results indicate targeting mGlu4 as a therapeutic strategy against PD may fail to provide full neuroprotection due to increased microglia-mediated inflammatory damage. Conversely, targeting mGlu7 may prove to be the most efficient receptor target.

A salient point of the thesis is that, contrary to our hypothesis, mGluR expression by microglia differed *in vitro* and *in vivo*, perhaps reflecting a discrepancy in phenotype between extracted/plated microglia and microglia in their physiological environment. However, as discussed previously, far from precluding the use of *in vitro* microglia to study microglial biology, this discrepancy might open up possibilities for more targeted pharmacological intervention with respect to therapies against neurodegenerative diseases.

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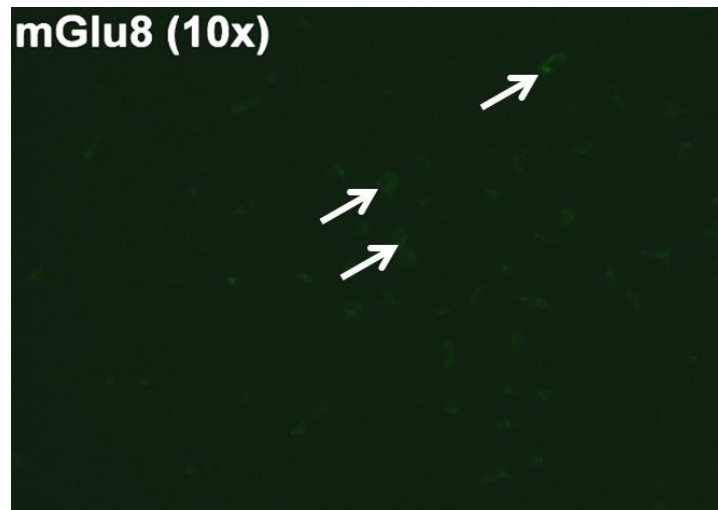
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Appendix 1



mGlu8 expression in primary microglia. Primary microglia were plated onto glass coverslips (20,000 cells per well) and fixed with 4% PFA. Cells were then incubated overnight with a primary anti-mGlu8 antibody and tagged with secondary Alexa 488-conjugated. Images were captured using a Nikon E1000M eclipse fluorescence microscope equipped with a Q-imaging Q-CAM camera and are representative of two independent experiments.