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Determining factors in the differential activation of microglia

by

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DEDICATION

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ABSTRACT

Microglia, the resident immune cells of the central nervous system (CNS), become activated in response to danger signals given out by other cells when homeostasis has been disturbed. Microglial activation is a multifaceted phenomenon that includes numerous distinct phenotypes. The type of activation often influences the survival of surrounding CNS tissue, and thus gaining a better understanding of how microglial activation is regulated has important therapeutic implications. Currently, it is known that the phenotype of activated microglia depends on both the type of CNS insult and the specific activating agent. The aim of this thesis was to investigate the potential involvement of other determining factors. Extrinsic regulators of microglial activation, including the severity of CNS insult and the stimulation strength of activating agents, were examined. Intrinsic differences among different microglial populations, namely differences in region of origin and age of origin, were also investigated. To study microglial behavior without interference from other cells, rat primary cultures were used as the system of study. With regard to extrinsic factors, it was found that different severities of hypoxic neuronal injury induced distinct microglial phenotypes. Among the activating agents released by injured neurons, adenosine

5’-triphosphate (ATP) was studied in isolation and was found to induce trophic and toxic effectors in microglia depending on the strength of ATP stimulation. In regards to intrinsic factors, it was found that microglia derived from different regions of the brain had distinct responses to activators, with cortical and hippocampal microglia generating more toxic responses than brainstem, striatal, and thalamic microglia. Microglia derived from various ages of origin also responded differentially to activators, with neonatal and aged microglia being more reactive than microglia derived from other age groups. Together, the results here present several novel concepts, that the phenotype of activated microglia are dependent not only on the type of activating stimulus, but the strength of that stimulus, and that in addition to stimuli from other cells, the regional and age differences among microglia themselves are also crucial in determining their activation phenotype.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
Aβ	amyloid beta
AD	Alzheimer's disease
ADO	adenosine
ADP	adenosine 5'-diphosphate
AG	aminoguanidine
ALS	amyotrophic lateral sclerosis
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
ASN	asparagine
ASP	aspartate
ATP	adenosine 5'-triphosphate
BDNF	brain-derived neurotrophic factor
BBB	blood-brain barrier
BBG	Brilliant Blue G
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
Blk	blank media
Brs	brainstem
BSA	bovine serum albumin
CCA	common carotid artery
CCI	controlled cortical impact
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CGS-19543	9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5--amine
CM	conditioned media
CNS	central nervous system
CREB	cAMP response element binding protein
CSF	cerebrospinal fluid
Ctl	control
Ctx	cortex
DAB	diaminobenzidine

DHK	dihydrokainic acid
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle's media
DMEM/F12	Dulbecco's modified Eagle's media with Ham's F12
DMSO	dimethyl sufoxide
DOPAC	3,4-dihydroxyphenylacetic acid
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
EAE	experimental autoimmune encephalitis
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Em	embryonic
ERK	extracellular signa-regulated kinase
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GDNF	glia-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLN	glutamine
GLT-1	glutamate transporter-1
GLU	glutamate
GSH	glutathione
gp120	glycoprotein 120
HBSS	Hank's balanced salt solution
Hip	hippocampus
HIV-1	human immunodeficiency virus type 1
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HVA	homovanillic
Hyp	hypoxia
Iba1	ionized calcium binding adaptor molecule 1
IBLC	isobutyryl-L-cysteine
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor-1
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
inhib	inhibitor

JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
μglia	microglia
M-CSF	macrophage colony-simulating factor
MAP	mitogen-activated protein
MAP-2	microtubule-associated protein-2
MCA	middle cerebral artery
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MS	Multiple Sclerosis
MTT	Thiazolyl Blue tetrazolium bromide
NADPH	reduced β-nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue
NCM	conditioned media from injured neurons
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Neo	neonatal
NGF	nerve growth factor
NO	nitric oxide
NOS2 (iNOS)	inducible nitric oxide synthase
NSE	neuron-specific enolase
OPA	o-phthaldialdehyde
OxATP	adenosine 5'-triphosphate-2',3'-dialdehyde
P1	purinergic type 1
P2	purinergic type 2
PBS	phosphate-buffered saline
PD	Parkinson's disease
PD 98059	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PMSF	phenylmethanesulphonylfluoride
PrP	prion protein
PrP^{c}	endogenous form of prion protein
PrP^{TSE}	mutated form of prion protein
PVDF	polyvinylidene fluoride
R	receptor
RB2	Reactive Blue 2

SB 202190	4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H--imidazole
SDS	sodium dodecyl sulphate
SER	serine
SOD	superoxide dismutase
SP600125	1,9-Pyrazoloanthrone
STAT	signal transducers and activator of transcription
Str	striatum
Tat	transactivating regulatory protein
TAU	taurine
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TGF-β	transforming growth factor-beta
THF	tetrahydrofuran
Thl	thalamus
TMB	tetramethylbenzidine
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
tPA	tissue-type plasminogen
UDP	uridine 5'-diphosphate

h (hr)	hours
M	molar
mg	milligrams
min	minutes
mL	millilitres
mM	millimolar
mm	millimetres
mo	months
µCi	microCuries
µg	micrograms
µM	micromolar
µm	micrometres
N	normal
nm	nanometres
rpm	rotations per minute

U	enzymatic units
^{3}H	tritium
HCl	hydrochloric acid
MeOH	methanol
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
NaOH	sodium hydroxide

CHAPTER 1

Introduction to microglia: Physiological, pathological, and controversial roles

1.1 Introduction and historical perspectives

The central nervous system (CNS) consists of two primary cell types, namely neurons and glia. Neurons are excitable cells that transmit electrochemical signals and make up less than half of the CNS cell population (Azevedo et al., 2009). Glial cells constitute the remainder of CNS cells and perform a variety of functions that support and protect the neurons (Azevedo et al., 2009). There are several types of glial cells. Macroglia include cells such as astrocytes and oligodendrocytes which are essential for maintaining CNS homeostasis and neuronal functions. The other glial cell type are microglia, which constitute about 20% of the total glia population (Kreutzberg, 1995).

The term ‘microglia’ was coined by the Spanish neuroanatomist del Rio-Hortega, who made the first detailed description of these cells in 1932 (del Rio-Hortega, 1932). He observed that microglia possess elongated bipolar cell bodies with spine-like processes that branch out perpendicularly (del Rio-Hortega, 1932). This is indeed the morphology exhibited by the majority of the microglial population when the CNS is in an undisturbed state. This morphological state of microglia with spiny protrusions is now commonly referred to as the ‘ramified’ or ‘resting’

state. When the CNS is disturbed, resting microglia can often sense the disturbances and rapidly shift to an activated state. The typical microglial activation response includes migration to the site of disturbance, proliferation, and often phagocytosis of debris if debris is left behind by the initial insult (Hanisch and Kettenmann, 2007). This activation response has long been regarded as the primary function of microglia. One of the most commonly used synonyms of microglia in the literature is the term 'brain phagocytes'. Moreover, because microglia often upregulate cytotoxic effectors during activation, they are traditionally regarded as detrimental when activated.

During the past decade, however, the classical views of microglial functions have shifted. These cells are no longer regarded as merely phagocytes, as they have been shown to be essential players in CNS development and physiology. Moreover, the conventional classification of microglia into ramified and activated is now considered by many as an over-generalization. Several recent reviews on microglia point out that microglia activation is multifaceted, and the type of activation depends very much on the type of disturbance (Hanisch and Kettenmann, 2007; Lynch, 2009; Ransohoff and Perry, 2009). The objective of

this general introduction is to summarize the key findings that have influenced our views of microglial functions, in both the normal and the disturbed CNS. This chapter will also discuss the controversial topics surrounding microglia that remain unresolved, and outline the specific issues that this thesis addresses.

1.2 Microglia in the normal CNS

1.2.1 Microglia in development

One of the oldest debates regarding microglia is regarding their developmental origin. The primary germ layers post-gastrulation consist of ectoderm, mesoderm, and endoderm; the ectodermal layer gives rise to the majority of CNS cells (Sasai and De Robertis, 1997). However, unlike neurons and other types of glia, microglia, despite having the term 'glia' as part of their name, are generally believed not to be of ectodermal origin. Some still claim that at least part of the microglial population are derived from neuroectodermal matrix cells along with other types of glia (Ling and Wong et al., 1993; Soulet and Rivest, 2008), and yet more evidence supports the idea that microglia are derived from the bone-marrow. Bone-marrow is mesodermal in origin and gives rise to circulating blood cells including monocytes, the precursor of tissue macrophages. The cell surface

markers of monocytes and microglia are very similar and often indistinguishable, suggesting that they are derived from the same precursors (Perry et al., 1985; Ling et al., 1990; Cuadros et al., 1992). The question then arises as to how the precursors of microglia, originating from the periphery, form the CNS microglial population. An immunohistochemical study by Andjelkovic et al. (1998) found that in human fetuses, microglia-like cells populate the CNS parenchyma as early as 5.5 weeks gestation. They also showed that these cells are negative for the monocyte marker CD14. These findings led them to hypothesize that microglia could have a dual origin, first derived during embryonic development, possibly from yolk sac macrophages, then a second population forms during postnatal stages from the infiltration of circulating monocytes. The neonatal invasion of monocytes from the periphery is well demonstrated in several labeling studies, where pre-labeled monocytes were injected during perinatal development and appeared to have migrated from the circulation to the CNS (Ling, 1979; Kaur et al., 1987; Leong and Ling, 1992). In addition, it has also been suggested that a population of microglia originates from pericytes, a type of small undifferentiated cell associated with small blood vessels (Ling and Wong, 1993).

The role of infiltrating monocytes during perinatal development has been proposed as a mechanism to aid neurogenesis and synaptic pruning, which are highly active processes during this developmental stage. Microglia in the neonatal brain have a 'fried egg'-like amoeboid appearance that is distinct from the ramified morphology seen in the adult brain (Soulet and Rivest, 2008). It is generally assumed that their amoeboid shape indicates their phagocytic activity which would help to remove the debris resulting from pruning. Whether or not this process actually takes place remains an uncertainty. Some have also suggested that not only do microglia phagocytose debris, they actively induce apoptosis of developing neurons as part of pruning (Marin-Teva et al., 2004). In addition, microglia are known to secrete neurotrophic factors *in vitro* (Miwa et al., 1997; Lu et al., 2005), leading some to believe that they have a role in neurogenesis and synaptogenesis. The presence of microglia along the white matter tracts also raises the possibility that they promote the development of oligodendrocytes and the progression of myelination (Rezaie and Male, 1999). Many of these speculations, however, have yet to be corroborated by *in vivo* evidence.

1.2.2 The role of microglia in CNS homeostasis

As previously mentioned, in the undisturbed adult CNS, microglia exhibit a ramified morphology that presumably reflects their quiescent state. This assumption has been challenged by recent *in vivo* imaging studies of resting microglia. Using fluorescent time-lapse imaging, these studies showed that microglial processes are not quiescent, but highly mobile; in addition, withdrawal and rebuilding of processes occur continuously (Nimmerjahn et al., 2005; Davalos et al., 2005; Haynes et al., 2006). This dynamic movement of processes suggests that microglia are constantly scanning and monitoring their surroundings rather than passively receiving potential danger signals. As reported in these studies, when a lesion is induced, microglial processes quickly shift from a state of random movement to a targeted movement towards the lesion site. Some investigators have suggested that henceforth the term 'resting microglia' should be renamed 'surveying microglia', which reflects their functions more accurately (Hanisch and Kettenmann, 2007).

In addition to surveying for danger, circumstantial evidence implicates several other functions of microglia in brain homeostasis. *In vitro*, microglia express the glutamate transporter (GLT)-1 and have been shown to both take up and release

the neurotransmitter glutamate (Patrizio and Levi, 1994; Persson et al., 2005; Persson et al., 2006). Glutamate is the main excitatory neurotransmitter in the CNS and its levels in the extracellular space are tightly regulated, mostly by astrocytic activity. The fact that microglia have the potential to regulate glutamate levels suggests that they participate in regulating synaptic activities and plasticity. Indeed, the microglial cytokines interleukin (IL)-1β and tumor-necrosis factor-α (TNF-α) have been shown to modulate the expression, activity, and trafficking of neuronal glutamate receptors, which are integral components of synaptic strength (Beattie et al., 2002; Viviani et al., 2003; Lai et al., 2006). However, normally microglia do not release detectable levels of these cytokines in culture conditions without the presence of activating agents. Thus it remains unclear whether microglia *in vivo* are modulators of synaptic activity.

1.3 Microglia in the disturbed CNS

1.3.1 Danger signals sensed by microglia

Relative to the scarce knowledge of microglias' physiological functions, the role of these cells in the perturbed brain has been studied more extensively. As mentioned, microglial processes actively scan their surroundings, and microglia

are usually the first cells to respond to danger. Microglia possess a wide variety of surface receptors that can recognize different types of danger signals. Foreign substances such as pathogens are naturally harmful to the brain. Microglia are thus equipped with receptors that sense molecules of foreign origin. The most classic example of these are the toll-like receptors (TLRs), a family of receptors that recognize pathogen-derived components such as bacterial lipopolysaccharide (LPS), bacterial cell wall proteoglycans, and nucleic acids from viruses (van Noort and Bsibsi, 2009). The inflammatory responses that result from the activation of TLRs induce microglia to release inflammatory cytokines and chemokines such as interleukins and macrophage inflammatory proteins (MIPs). These molecules are also detected by their respective receptors on microglia as danger signals (Lynch, 2009).

In addition to foreign substances, microglia are also capable of sensing disturbance signals resulting from injuries and disease states. One of the direct results of tissue injury is leakage of cellular contents. Potassium ions, for example, are normally in high concentration intracellularly and low extracellularly. Abnormally high levels of extracellular potassium ions are rapidly detected by

microglia as danger signals (Chang et al., 2000). Adenosine 5'-triphophate (ATP) is another molecule released by injured cells into the extracellular space. ATP can leak from injured neurons and astrocytes; microglia express purinergic receptors that sense the released ATP as danger signals (Verderio and Matteoli, 2001; Hansson and Ronnback, 2003; Bianco et al., 2005). Similar to potassium and ATP, intracellular glutamate is spilled into the extracellular space during energy failure; microglia express both ionotropic and metabotropic glutamate receptors that can detect glutamate (Hansson and Ronnback, 2003; D'Antoni et al., 2008).

In addition to leakage of cellular content, injury to the CNS often results in inflammation, which consequently compromises the integrity of the blood-brain barrier (BBB) to allow infiltration of peripheral immune cells. Leakage of the BBB introduces plasma proteins into the CNS parenchyma that is otherwise free of plasma components. Plasma proteins are thus also recognized by microglia as signals of injury (Moller et al., 2006). Interestingly, some of these plasma proteins, such as thrombin and fibronectin, are recognized by the same TLRs that recognize pathogens, albeit associated with different signaling cascades (Moller et al., 2006; del Zoppo et al., 2007).

Endogenous danger signals not only originate from tissue injury, but also from CNS disease states. Microglial cells are also sensitive to signals of disease states, the more studied examples being prion protein (PrP) and the various forms of the amyloid-beta peptide/aggregate (Veerhuis et al., 2005). However, it is so far unclear which specific receptors on microglia recognize these molecules.

1.3.2 Microglial responses triggered by danger signals

Upon binding to their respective receptors on microglia, molecules that signal danger can activate the intracellular second messenger pathways, subsequently resulting in the translocation of several transcription factors. Signal transducers and activators of transcription (STAT), cAMP response element binding protein (CREB), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are microglial transcription factors that are often activated upon stimulation by danger signals (Lai and Todd, 2006; Potucek et al., 2006). The genes that are transcribed can define the function of activated microglia. In the classical definition, microglial activation consists of four main functions: 1) migration to the site of danger, 2) proliferation, 3) coordination of

neuroinflammation by release of cytokines, and 4) phagocytosis of dangerous substances or debris left from an insult. This is a very loose definition that no longer accurately describes how microglia respond to danger. It is now known that microglia have the potential to do more than just phagocytose at the site of disturbance. For example, they can produce neurotrophins that promote the survival of surrounding cells (Miwa et al., 1997; Lu et al., 2005). It is also evident that while the main functions of microglial cytokines are to recruit immune cells and propagate neuroinflammation, they also have a variety of other functions including the coordination of regenerative responses (Ekdahl et al., 2009).

1.3.3 Released effectors of microglia

Activated microglia produce molecules that regulate the functions and survival of other cells. These molecules are called effectors. Microglial effectors can be roughly classified into released effectors and cell-mediated effectors. Released effectors of microglia in response to danger include a long list of cytokines, neurotrophins, neurotransmitters, and non-organic compounds. Although some of these molecules have defined roles, the majority can be either toxic or trophic to the surrounding target cells depending on the context. This is where the concept of

microglial activation begins to gather controversy. For example, under arginine-depriving conditions, microglial inducible nitric oxide synthase (iNOS) produces superoxide anions, which are free radicals that harm many components of a cell (Gibson et al., 2005). The same enzyme also generates nitric oxide (NO). NO, like superoxide, can cause cell death; such is the case when NO is converted to the reactive oxidant peroxynitrite. However, NO is also neuroprotective in that it is a potent vasodilator, which is key to rescuing of brain tissue in an event of obstructed blood flow (Gibson et al., 2005). The pro-inflammatory cytokine TNF-α is another classic example: The target cells of TNF-α can express different ratios of the two TNF-α receptors TNFR1 and TNFR2, the former linked to a cell death-favoring pathway while the latter, is linked to a cell survival-favoring pathway (McCoy and Tansey, 2008). It is rather erroneous and over-simplifying to classify released effectors of microglia based on cytotoxicity. Nevertheless, they can be roughly grouped based on their systemic functions with respect to other immune cells (see **Table 1-1** for a comprehensive list). The pro-inflammatory effectors, when released systemically, promote the inflammatory process which includes recruitment of immune cells to the site of inflammation, focal edema, fever, and production of pathogen-killing compounds. The anti-inflammatory

effectors inhibit the inflammatory process. Since the brain contains a very low number of non-microglial immune cells when the BBB is intact, systemic inflammation and neuroinflammation are coordinated differently. The cytotoxic nature of pro-inflammatory cytokines in the systemic context, as illustrated in the above examples of TNF-α and NO, is not always transferable to the CNS setting.

1.3.4 Non-released (cell-mediated) effectors of microglia

The two-tiered nature of released microglial effectors is also observed in several cell-mediated effectors of microglia (see **Table 1-1** for a comprehensive list). A classic example is microglial phagocytosis: While engulfment of cellular debris and cytotoxic substances such as amyloid plaques can prevent the spread of tissue death, it could potentially impair the function of these microglia, and could lead to their death (Magnus et al., 2001; Hanisch and Kettenmann, 2007). Another cell-mediated effector that has both toxic and trophic functions is the glutamate transporting machinery in microglia, mainly mediated by the glutamate transporter GLT-1. Microglial GLT-1 transports extracellular glutamate into the cell and through this activity prevents excess glutamate from causing excitotoxic injury to neurons; on the other hand, GLT-1 can also transport glutamate out of

microglia to promote excitoxicity (Patrizio and Levi, 1994; Persson et al., 2005; Persson et al., 2006). GLT-1 has been shown to be closely associated with the sodium-potassium ATP pump (Rose et al., 2009). Therefore, under energy-depriving conditions, GLT-1 has the tendency to switch from uptake to release. Other than phagocytic and glutamate-uptake machineries, microglia express several intracellular and membrane-bound enzymes that modulate the toxicity level of the surrounding milieu. As shown in **Table 1-1**, microglia can release several compounds that increase oxidative stress in the surrounding tissue, including superoxide, hydrogen peroxide, and peroxynitrite (Dringen, 2005). However, they also possess enzymes such as superoxide dismutase (SOD) and catalase that degrade these compounds to lower the oxidative stress (Dringen, 2005). Another group of enzymes expressed by activated microglia is the nucleotide- and nucleoside-hydrolyzing enzymes such as ectoapyrase and ecto-5'-nucleotidase, which are responsible for degrading the highly neurotoxic ATP to its less bioactive metabolites adenosine monophosphate (AMP) and adenosine (Braun et al., 1998; Farber et al., 2008).

1.3.5 Different activating agents yield specific microglial responses

Not only are microglial effectors multi-functional in nature, the manner by which these effectors are regulated is also multi-faceted. The microglial effectors listed above are never simultaneously upregulated in response to any given danger signal. More often than not, different sets of effectors are upregulated in response to different danger signals, resulting in a variety of activation phenotypes and adding another layer of complexity to evaluating the precise role of activated microglia. For example, in ATP-stimulated microglia, the release of interleukin (IL)-6 has a more delayed time-course compared to its pro-inflammatory companion TNF-α (Inoue, 2002). In another study, Nakajima et al. (2002) showed that in response to ceramide, microglia can release the neurotrophin brain-derived neurotrophic factor (BDNF) without releasing the pro-inflammatory mediators TNF-α, IL-1β, and NO. LPS on the other hand stimulated the release of all four microglial effectors (Nakajima et al., 2002). The authors further demonstrated that ceramide and LPS stimulated the phosphorylation of different kinases, providing a mechanistic explanation for the differential upregulation of microglial effectors (Nakajima et al., 2002). To categorize the phenotypes of activated microglia, some researchers have used the conventional grouping of activated tissue macrophages, which includes ‘classically-’ and ‘alternatively-’ activated macrophages. Classical

activation of macrophages, referred to as M1 activation, results from stimulation with pro-inflammatory activators such as LPS and interferon-γ (IFN-γ), while alterative, or M2, activation of macrophages results from stimulation with a combination of anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor-β (TGF-β) (Ransohoff and Perry, 2009). Macrophages produce pro-inflammatory mediators from M1 activation and anti-inflammatory and proliferative factors from M2 activation (Ransohoff and Perry, 2009). The microglial version of M1 and M2 activation generally follow the same patterns, but the specific effector profile is distinct from that of macrophages. For instance, IFN-γ alone has little effects on macrophage phagocytosis, but significantly increases microglial phagocytic activity (Smith et al., 1998).

1.3.6 Microglia in acute injuries to the CNS

Most of our current knowledge regarding microglial activation comes from *in vitro* studies, where microglia are grown in isolation from other brain cell types. This arrangement allows microglial effectors to be measured without contributions or interference from other cell types, yet it also creates the caveat of collecting data from an artificial environment. Production of toxic or trophic

factors by microglia *in vitro* does not necessarily translate into toxicity or protection *in vivo*. Nonetheless, the multi-dimensional nature of microglial activation is also observed in animal models. The role that activated microglia play in *in vivo* models of CNS disturbance is quite variable, often dependant on the specific disease or insult. This correlates with the aforementioned principle that distinct danger signals yield specific activation phenotypes. However, in some cases, even in the same model, experimental manipulations of microglial activation can yield opposite outcomes, generating more controversy to an already complex issue. There are several examples in experimental models of acute CNS injury, such as cerebral ischemia. Cerebral ischemia, or ischemic stroke, is the restriction of blood flow to an area in the brain. The energy deprivation from decreased blood flow results in metabolic injury and excitotoxicity, which induce nearby microglial activation (Dirnagl et al., 1999). There is debate as to whether activated microglia are detrimental or beneficial to the stroke outcome. Treatment of stroke animals with anti-inflammatory drugs such as minocycline and doxycycline promotes tissue survival post-stroke (Yrjanheikki et al., 1998; Jantzie et al., 2005). Similarly, knockout or pharmacological inhibition of microglial pro-inflammatory cytokines such as IL-1β and TNF-α also improves the outcome

of the stroke (Wang and Shuaib, 2002). This evidence would suggest a detrimental role for microglia in cerebral ischemia. However, selective elimination of the microglial population using the drug ganciclovir exacerbates post-ischemic tissue damage (Lalancette-Hebert et al., 2007), while injection of exogenous microglia into the ischemic brain, both in rats and gerbils, confers neuroprotection (Kitamura et al., 2004; Hayashi et al., 2006; Imai et al., 2007). These results would then point to a protective role for microglia. The possibility exists such that moderate microglial activation eliminates the toxic debris and compounds resulting from the initial ischemic insult, but if left without intervention such as anti-inflammatory drugs, prolonged microglial activation can cause cytotoxicity. Regardless, the role of microglia in ischemia remains relatively undefined.

The outcome of manipulating microglial activation is slightly different in models of non-metabolic mechanical injury. In a mouse model of hypoglossal nerve axonomy, ablation of proliferating microglia with ganciclovir does not affect the survival of hypoglossal motor neurons (Gowing et al., 2006). Treatment with anti-inflammatory drugs, however, is neuroprotective in the controlled cortical impact (CCI) model (Clausen et al, 2009; Li et al., 2009). Interestingly, while

TNF-α knockouts in ischemia models show improved outcome, TNF-α knockouts in the CCI model show deficits in axonal regeneration and behavioral recovery, suggesting that the role of microglia may be dependent on the recovery stage of the injury (Oshima et al., 2009).

1.3.7 Microglia in neurodegenerative disorders

The multifaceted nature of microglial activation is a theme that is also present in more chronic disturbances to the CNS. In Alzheimer's disease (AD), multiple hypotheses have formed regarding the role of microglia. A hallmark of AD is the presence of amyloid beta (Aβ) aggregates; a finding that implicates a role for microglia in AD is the observation that soluble and oligomeric Aβ can activate microglia in culture (Maccioni et al., 2009). Microglia activated by soluble Aβ have a pro-inflammatory phenotype, upregulating TNF-α and iNOS (Rao et al., 2006). In mouse models of familial AD (plaque-generating mice strains commonly referred to as AD mice), genetically knocking down iNOS can alleviate amyloid plaque aggregation and premature mortality (Nathan et al., 2005). Treating AD mice with the anti-inflammatory drug minocycline also improves the disease outcome (Fan et al., 2007). While these *in vitro* and *in vivo*

observations point to a harmful role for microglia in AD, more evidence suggests that microglia are essential in controlling the disease progression. For example, activation of microglia by intracranial injection of LPS results in a decrease in amyloid plaque load (Herber et al., 2004). Injection of exogenous microglia into the AD mouse brain has similar effects (Takata et al., 2007), while inhibition of microglial migration by impairing the function of the microglial chemokine receptor CCR2 increases plaque load and worsens the outcome of the disease (El Khoury et al., 2007). In sporadic AD, the role of amyloid plaques is unclear because plaque load does not correlate with disease severity (Maccioni et al., 2009). The phagocytosis of amyloid plaques by microglia, though intuitively serving a neuroprotective purpose, is in reality a poorly understood phenomenon. There have been suggestions that formation of plaques by neurons is a self-protective mechanism against the more toxic fibrillar and oligomeric forms of Aβ. Phagocytosis of amyloid plaques by microglia then becomes of less significance. Additionally, microglia are actually inefficient phagocytes compared to tissue macrophages (Majumdar et al., 2008). Although their phagocytic activity can be increased by activating agents such as LPS or macrophage colony stimulating factor (M-CSF) (Majumdar et al., 2007), it is highly probable that they

protect neurons during AD via non-phagocytic mechanisms. In the sporadic forms of AD, where no animal models are available, the study of microglial activation relies on postmortem human samples. While microglia show an activated morphology in postmortem AD brains, there is also an increase in the number of microglia with a dysmorphic appearance (Miller and Streit, 2007). Streit and colleagues (2007) argued that telomere shortening due to aging, especially in locally replenished cell populations like microglia, eventually renders the cells senescent. They proposed that the development of AD is partly due to the loss of normal function in the senescent microglia (Miller and Streit, 2007), adding yet another dimension to the role of microglia in AD.

In the case of another degenerative disorder, amyotrophic lateral sclerosis (ALS), the role of microglia is more one dimensional. In animal models of familial ALS, the anti-oxidative enzyme superoxide dismutase-1 (SOD1) is mutated to yield ALS-like symptoms of upper and lower motor neuron degeneration. Microglial cultures derived from mice with mutant SOD1 produce significantly more superoxide and NO when activated with LPS (Beers et al., 2006), which is not surprising considering the anti-oxidative function of SOD1. To elucidate the

precise role of microglia in ALS *in vivo*, Beers et al. (2006) developed mutant SOD1 mice that are also deficient in the transcription factor PU.1, rendering them unable to produce cells of myeloid and lymphoid origin. Transplantation of bone marrow-derived cells into these mice alleviated the ALS symptoms, while transplantation of the same cells from mutant SOD1 mice had no effect (Beers et al., 2006). In another study, mutant SOD1 mice were crossed with a CD11b-specific Cre-recombinase mice, resulting in the selective removal of the mutant SOD1 protein from the microglial population; these mice showed fewer ALS symptoms than the mutant SOD1 mice (Boillee et al., 2006). Notably, in both of these mice studies, it is the latter stages of the disease that were ameliorated, suggesting that microglia may not be involved in the initiation of ALS, but rather the promotion of disease progression. Interestingly, a recent study observed that in mutant SOD1 mice, ablation of proliferating microglia with ganciclovir did not affect motor neuron degeneration (Gowing et al., 2008). While this finding may suggest that microglia's role in ALS is limited, one has to consider the differences in the experimental paradigms. While the studies by Beers et al. and Boillee et al. employ transgenics to remove microglial mutant SOD1, the study by Gowing et al., removes the whole cell of microglia. In

summary, the role of microglia in ALS is likely detrimental, but the precise mechanisms are still unclear.

Similar experiments that manipulate the microglial population with cell type specificity have not been performed in Parkinson's Disease (PD). The role microglia play in PD is thus more ambiguous. PD involves the selective degeneration of dopaminergic neurons in the substantia nigra. Whereas AD brains have extracellular plaques, degenerating neurons in PD contain cytoplasmic protein aggregates. Although direct evidence is lacking, microglia are generally thought to be involved in the development of PD. Firstly, the substantia nigra contains a higher microglia density than most other brain regions (Kim et al., 2000). Intranigral injection of LPS induces death of dopaminergic neurons while not affecting other neuronal cell types, a pattern similar to that observed in PD (Castano et al., 1998). Treatment of animal models of PD with anti-inflammatory compounds can partially rescue the loss of dopaminergic neurons (Lee et al., 2009). Epidemiological studies corroborate this observation, showing an inverse correlation between the use of anti-inflammatory drugs and the risk of PD (Lee et al., 2009). Furthermore, two genetic mutations involved in familial PD,

α-synuclein and parkin, are both linked to microglial functions. Mutated forms of α-synuclein itself induce hyper-secretion of cytotoxic pro-inflammatory cytokines from microglia (Klegeris et al., 2008). Microglial cultures from α-synuclein knockout mice are also hyper-reactive and appear morphologically dysfunctional (Austin et al., 2006). The parkin story is even more intriguing: While parkin knockout mice do not exhibit nigral degeneration, chronic intraperitoneal administration of LPS triggers degeneration in these mice without affecting the wild type mice (Frank-Canon et al., 2008). These data collectively suggest a detrimental role for microglia in PD, and unlike in ALS, microglia in PD seem to be involved in the triggering and initiation of the disease.

With regard to etiology, AD, as well as ALS and PD for the most part involve innate immunity and not adaptive immunity. In Multiple Sclerosis (MS), however, the interactions between microglia and T cells come into play. MS is generally thought to be an acquired inflammatory disorder, where T cells come to recognize myelin as foreign and attack it in an autoimmune manner. Most of what is known about microglia in MS comes from the animal model of MS, known as experimental autoimmune encephalitis (EAE). EAE is achieved either by systemic

immunization with myelin proteins or by transplantation of primed T cells from an immunized animal (Ransohoff and Perry, 2009). The latter method, known as adoptive-transfer EAE, cannot be achieved by the presence of microglia alone and requires the presence of perivascular macrophages that reside at the BBB interface (Hickey and Kimura, 1988, Greter et al., 2005). This is not surprising as microglia are relatively poor antigen presenters and do not constitutively express all classes of major histocompatibility complex (MHC) proteins (Perry et al., 2007). While microglia may not be directly involved in the development of MS, ablation of the microglial population with ganciclovir does reduce the severity of EAE (Heppner et al., 2005). This implies that microglia may act as amplifiers of T cell function in EAE and MS. Furthermore, inhibition of microglial glutamate release using connexin hemichannel blockers (Shijie et al., 2009), as well as inhibition of overall microglial activation by anti-inflammatory drugs (Maier et al., 2007; Luhder and Reichardt, 2009), can both alleviate EAE symptoms, further supporting a detrimental role for microglia in EAE and MS.

1.3.8 Microglia in neuropathic pain

Another chronic CNS disease that has gathered much attention in the field of

inflammation is neuropathic pain. The disease is unique in that it also contains an acute component, where a mechanical or ischemic injury to peripheral or central nerves results in abnormal pain sensations even after the injury has healed. Several different hypotheses have been formed with regard to the role of microglia in neuropathic pain. One school of thought proposes that the formation of glial scars and gliosis by astrocytes and microglia in the area of injury may cause abnormal regeneration and sprouting of nerves, leading to impaired pain processing (Cavaliere et al., 2007). This may explain why treatment with anti-inflammatory drugs can decrease pain symptoms in mouse models (Milligan and Watkins, 2009). However, inhibition of glial proliferation by fluorocitrate did not alleviate neuropathic pain, suggesting that the analgesic effects of anti-inflammatory drugs are likely not due to inhibition of glial scar formation (Mika et al., 2009). An alternative hypothesis for the role of microglia in neuropathic pain proposes that microglial cytokines, upregulated in response to the initial injury, may modulate neuronal activity and cause uncontrolled firing of excitatory neurons. The most convincing evidence to support this hypothesis comes from a seminal paper by Coull et al. (2004): They show that the neurotrophic factor BDNF is released from microglia when activated by ATP; by

injecting ATP-treated microglia into the spinal cord, they show that microglial BDNF is not neurotrophic, but rather induces a reversal of inhibitory current in neurons that results in neuropathic pain. Ulmann et al. (2008) later demonstrated that this effect is mediated by the microglial purinergic receptor P2XR4, providing further support for a detrimental role for microglia in neuropathic pain.

1.3.9 Microglia in infectious diseases

In most neurological disorders, there is persisting debate as to whether microglia actively participate in disease development or merely play a secondary reactionary role. In the case of CNS disturbances that involve the invasion of foreign pathogens, microglia, being the primary immune cells in the CNS, have a more defined role of combating and eliminating the pathogenic substances. Microglia express all of the main TLR subtypes, which can recognize pathogens ranging from bacteria to fungi (Mariani and Kielian, 2009). TLR activation elicits anti-pathogen effectors in microglia, such as free radicals and phagocytic activity, as well as pro-inflammatory mediators to recruit peripheral immune cells into the brain (Mariani and Kielian, 2009). Interestingly, some species of bacteria are known to induce apoptosis of microglia via TLR activation (Lehnardt et al., 2007).

This is thought to be a mechanism to prevent prolonged activation of microglia and the potential of secondary inflammatory damage. Nevertheless, the double-edged sword of microglial activation has led some to hypothesize that neuronal death during CNS infections is partly contributed by microglial activation. For instance, patients suffering from bacterial meningitis have an elevated level of fibronectin, an endogenous TLR ligand, in their cerebrospinal fluid (CSF) (Goos et al., 2007). The persisting activation of TLRs even after the infections have been cleared makes it possible for microglia to induce secondary inflammatory damage.

One of the more unique pathogens that infect the CNS is human immunodeficiency virus type 1 (HIV-1). While most viral infections of the brain target neurons, HIV-1 replicates productively only in macrophages and microglia (Yadav and Collman, 2009). In addition to harboring the virus, several lines of evidence suggest that microglia and infiltrating macrophages contribute to the neuronal death and cognitive deficits associated with HIV-1 infection. CSF viral load of HIV-1 patients correlates closely with level of monocyte chemotactic protein-1 (MCP-1), a chemokine produced by activated microglia (Kelder et al.,

1998). Human macrophage cultures infected by HIV-1 or its components, such as the viral proteins gp120 and Tat, release several pro-inflammatory mediators as well as toxic compounds such as quinolinic acid and glutamate (Yadav and Collman, 2009). The viral proteins, however, can themselves cause toxicity when in contact with neurons (Yadav and Collman, 2009), creating a caveat in discriminating the relative contribution of inflammation-mediated cell death. Another confounding factor is that due to the inability of HIV-1 to infect mouse or rat microglia, most of the *in vitro* data rely on human macrophages isolated from blood, which may have different activation responses than microglia. Nonetheless, in the rhesus monkey model of HIV-1 infection, the anti-inflammatory drug minocycline alleviated the severity of neuronal death and reduced the level of pro-inflammatory mediators (Zink et al., 2005), supporting the involvement of neuroinflammation in the disease.

Like HIV-1 infection, another CNS infectious disease that has several unique properties is prion disease. Here, the infectious agent is a mutated form (PrP^{TSE}) of the endogenous prion protein (PrP^{c}) (Kovacs and Budka, 2008). Thus far, it is unclear how PrP^{TSE} induces neurotoxicity; suggested mechanisms include the loss

of neuroprotective functions of PrP^c and formation of toxic prion protein aggregates (Kovacs and Budka, 2008). The relative contribution from microglia to the overall neurotoxicity is also undefined. However, unlike HIV-1 infection, prion disease is similar across most mammalian models, allowing for studies specific to microglia rather than tissue macrophages. The toxic domain of the prion protein can trigger the release of pro-inflammatory cytokines from microglial cultures (Brown et al., 1996; Yang et al., 2008). However, in knockout mice deficient of either TNF-α or IL-6, the disease progression is unaltered (Mabbott et al., 2000). Furthermore, in a study by Kercher et al. (2007), two mouse strains, one expressing the prion protein in all cell types and one only in neurons, when infected with PrP^{TSE}, showed similar profiles of inflammatory markers, suggesting a lack of glial involvement. In another study, Siskova et al. (2009) found that the loss of synapses, an early neurological symptom of prion disease, is not physically associated with microglial processes, casting further doubt on microglial involvement in the initiation of the disease.

1.3.10 Microglia in psychiatric disorders

A less investigated area of microglia biology is their role in CNS diseases that

have no apparent pathological features. For example, the majority of psychiatric disorders is normally not associated with significant neurodegeneration. In addition to the absence of anatomical features, studying psychiatric disorders can be difficult due to the lack of animal models that have behavioral symptoms resembling that of human subjects. Nevertheless, evidence from *in vitro* studies and scattered *in vivo* data do implicate the involvement of microglia in psychiatric disorders. Treatment of microglial cultures with anti-depressants or anti-psychotics can attenuate their release of pro-inflammatory cytokines (Lai et al., 2009). Unpublished data from our lab also show that anti-depressants can prevent the release of glutamate and D-serine, both mediators of excitotoxicity, from LPS-stimulated microglia, and at the same time augment the production of BDNF (Dhami et al., 2008; Dhami et al., 2009). In human subjects, anti-inflammatory agents have anti-depressive effects (McNally et al., 2008). Conversely, patients that have been treated with pro-inflammatory cytokines to combat infection often develop depressive symptoms as side effects (McNally et al., 2008). The role of microglial activation in depression thus appears to be detrimental, yet it remains unclear how inflammation can influence short-term depression which likely does not involve neurodegeneration. One hypothesis is

the modulation of synaptic plasticity by microglial cytokines such as TNF-α and IL-1β, which have been shown to affect glutamate receptor trafficking in neuronal cultures (Lai et al., 2009).

1.3.11 Systemic inflammation can switch microglial activation types

One of the caveats in the interpretation of *in vivo* studies is the observation that activation of peripheral immune cells can sometimes change the outcome of CNS diseases. For example, in the early stages of prion disease, microglia express TGF-β but no detectable levels of pro-inflammatory mediators (Cunningham et al., 2002). After peripheral LPS administration, however, microglia transform into a pro-inflammatory phenotype, and the disease severity is worsened (Cunningham et al., 2005; Cunningham et al., 2009). Similar phenotype switching of microglia is also observed in AD models, where a primarily phagocytic and anti-inflammatory phenotype is switched to pro-inflammatory by systemic LPS treatment (Perry et al., 2007). While this switch of microglial phenotype is detrimental in neurodegenerative diseases, similar systemic LPS treatment at a low dose prior to experimental stroke actually reduces post-ischemic inflammation and infarct volume (Lin et al., 2009). Interestingly, high dose of

LPS produced the opposite effect (Lin et al., 2009). The mechanism of how peripheral inflammation switches microglial activation is uncertain. Nonetheless, this phenomenon introduces challenges to interpretating data in studies that employ peripheral LPS treatment as a model of neuroinflammation.

1.4 Thesis objectives – remaining questions surrounding the controversial role of microglia

A general concluding statement that can be drawn from the discussion presented above is that our understanding of the physiology and pathobiology of microglia has shifted greatly in recent years. The term 'microglial activation' is no longer used to describe one particular microglial phenotype, but is rather a convenient way to describe a spectrum of different responses to danger. We now know that the type of microglial activation is determined by a combination of several factors including: The presence or absence of inflammation in the periphery, the scale of that inflammation, the type of CNS disturbance inflicted, the initial cell type that sends out the danger signals, and the specific signal sensed by microglia. Other than these factors, however, there are other determinants that may influence microglial activation type and have not been studied in depth. The aim of my

thesis, then, is to identify these determining factors and investigate how they regulate microglial activation (see **Figure 1-1** for schematics).

1.4.1 Microglias' interpretation of different signal strengths

The first half of the thesis investigates the possibility of the same danger signal inducing different microglial activation types due to the variation in signal strength. Signal strength- or dose-dependence is a common theme in all biological systems, and every ligand-receptor interaction has a particular range of effective dosage. However, within that effective dosage lie several possibilities. An example is the aforementioned systemic priming of LPS prior to stroke induction, where a high LPS dose exacerbated ischemic injury and a low dose attenuated the damage (Lin et al., 2009). This implies that the phenotypic changes in microglia within the effective dosage of a danger signal may not be linear, but instead bi-directional. Do similar bi-directional effects occur from endogenous CNS danger signals? One can almost certainly state that microglia have the potential to both protect and destroy. Thus intuitively, an evolutionarily favorable response for microglia would be to perform rescue functions while the insult is mild and rescuable, and when the insult is severe, to perform 'damage control' or

detrimental functions to eliminate injured cells that are harmful to their neighbouring tissue. Do microglia actually behave in this manner? If they do indeed have a polarized response to different injury severities, what effects does this polarized response have on the injured tissue? Do different severities of insult generate different combinations of danger signals, or just different amounts of the same signals? If different amounts of the same signals can induce polarized responses from microglia, what are the specific danger signals responsible for this phenomenon and through what microglial receptors do these signals exert their effects? These questions will be investigated by the first two experimental chapters of the thesis.

1.4.2 Intrinsic determinants of microglial activation

Another topic that has received relatively little attention is the intrinsic variations among different microglial populations. Conventionally, microglia are treated as a homogeneous entity and are thought to express different phenotypes only when activated by different stimuli. As a result, *in vitro* models of microglia have also been isolated as one population from the whole brain. In some ways this is quite surprising since we have long known that microglia, as mentioned earlier, have a

bone marrow origin, yet by the end of development have surface markers and morphological features distinct from that of other bone marrow-derived immune cells such as tissue macrophages or even perivascular macrophages. By the same reasoning then, one would expect microglia that have developed in different neurochemical backgrounds in the various brain regions to have distinct phenotypes. Several studies have shown this to be true in the healthy undisturbed CNS (Hanisch and Kettenmann, 2007), but how microglia from various regions respond to danger signals has yet to be investigated.

The neurochemistry of the brain not only varies across the different regions; anagolous to regional variations are the changes in the brain environment with respect to developmental age (Mora et al., 2007). The changing neurochemical environment from aging can potentially influence microglial phenotype. Whether or not this is actually the case remains uncertain. Additionally, the aging process within microglial themselves may affect their activation responses. The possibility exists such that microglia grow progressively senescent with increasing age, a theory proposed by Streit and colleagues as mentioned previously (Miller and Streit, 2007). There is region-selectivity as well as age-selectivity in most CNS

disorders. While this selectivity may be a result of variations in neurons alone, the importance of microglial contribution, especially considering their impact on neuronal survival, should not be overlooked. The last two results chapters of the thesis will investigate whether intrinsic variations and changes in microglia affect their activation responses. Microglia from different regions of origin, as well as microglia of various age groups, will both be examined.

Microglial effector	Conventional classification	Effector form	Effector functions in the CNS	References
IL-1β	pro-inflammatory	humoral	neurotoxicity, promotion of neuroinflammation, stress response, repair	Hanisch, 2002
IL-1 receptor antagonist (IL-1ra)	anti-inflammatory	humoral	endogenous antagonist of IL-1β	Hanisch, 2002
IL-6	pro-inflammatory	humoral	promotion of neuroinflammation	Hanisch, 2002
IL-10	anti-inflammatory	humoral	inhibition of neuroinflammation	Hanisch, 2002
TNF-α	pro-inflammatory	humoral	neurotoxicity, promotion of neuroinflammation, neuroprotection	Hanisch, 2002
Fas	pro-inflammatory	cell-mediated	neurotoxicity	Lee et al., 2000
CD40	pro-inflammatory	cell-mediated	neurotoxicity, modulation of immune cells	Matyszak et al., 1999; Ke et al., 2005
TGF-β	anti-inflammatory, fibrogenic	humoral	neuroprotection, neurogenesis, inhibition of neuroinflammation	Hanisch, 2002
insulin-like growth factor (IGF)-1	trophic	humoral	neuroprotection, neurogenesis	Butovsky et al., 2006
basic fibroblast growth factor (bFGF)	trophic, fibrogenic	humoral	cell proliferation	Araujo and Cotman, 1992
BDNF	trophic	humoral	neuroprotection, neurogenesis, modulation of synaptic transmission	Nakajima et al., 2001; Coull et al., 2005
nerve growth factor (NGF)	trophic	humoral	neuroprotection, neurogenesis	Heese et al., 1997
glia-derived neurotrophic factor (GDNF)	trophic	humoral	neuroprotection, neurogenesis	Lu et al., 2005; Matsushita et al., 2008
M-CSF	pro-inflammatory	humoral	proliferation of leukocytes	Hanisch, 2002
MCP-1	pro-inflammatory,	humoral	chemoattraction of	Hanisch, 2002

	chemotactic		leukocytes	
macrophage inflammatory protein (MIP)-1, 2, 3	pro-inflammatory, chemotactic	humoral	chemoattraction of leukocytes	Hanisch, 2002
prostanoids	pro-inflammatory	humoral	promotion of neuroinflammation, stress response	Minghetti et al., 1996
peroxynitrite	pro-inflammatory, oxidative	humoral	neurotoxicity	Gibson et al., 2005
hydrogen peroxide	pro-inflammatory, oxidative	humoral	neurotoxicity	Gibson et al., 2005
superoxide	pro-inflammatory, oxidative	humoral	neurotoxicity	Gibson et al., 2005
nitric oxide	pro-inflammatory, oxidative	humoral	neurotoxicity, neuroprotection, vascular regulation, promotion of neuroinflammation	Gibson et al., 2005
glutathione	anti-oxidative	humoral, cell mediated	neuroprotection	Dringen, 2005; unpublished observations from Todd lab
catalase	anti-oxidative	humoral, cell mediated	neuroprotection	Dringen, 2005; unpublished observations from Todd lab
superoxide dismutase	anti-oxidative	humoral, cell mediated	neuroprotection	Dringen, 2005; unpublished observations from Todd lab
tissue-type plaminogen (tPA)		humoral	neurotoxicity, vascular regulation, tissue remodeling, cell migration	Tsirka et al., 1995; Siao et al., 2003
matrix metalloproteinases (MMPs)		humoral	neurotoxicity, vascular regulation, tissue remodeling, cell migration	Gottschall et al., 1995; Chan et al., 2006

glutamate transporter (GLT)-1		cell mediated	neuroprotection, synaptic modulation	Persson et al., 2005; Nakajima et al., 2008
glutamate		humoral	neurotoxicity, neurogenesis, synaptic modulation	Patrizio and Levi, 1994; Takeuchi et al., 2006
ectonucleotidases		cell mediated	neuroprotection, microglial migration	Braun et al., 1998; Farber et al., 2008
phagocytosis machinery		cell mediated	neuroprotection	Napoli and Neumann, 2009
quinolinic acid		humoral	neurotoxicity	Heyes et al., 1996; Espey et al., 1997

Table 1-1 – List of released and cell-mediated effectors of microglia.

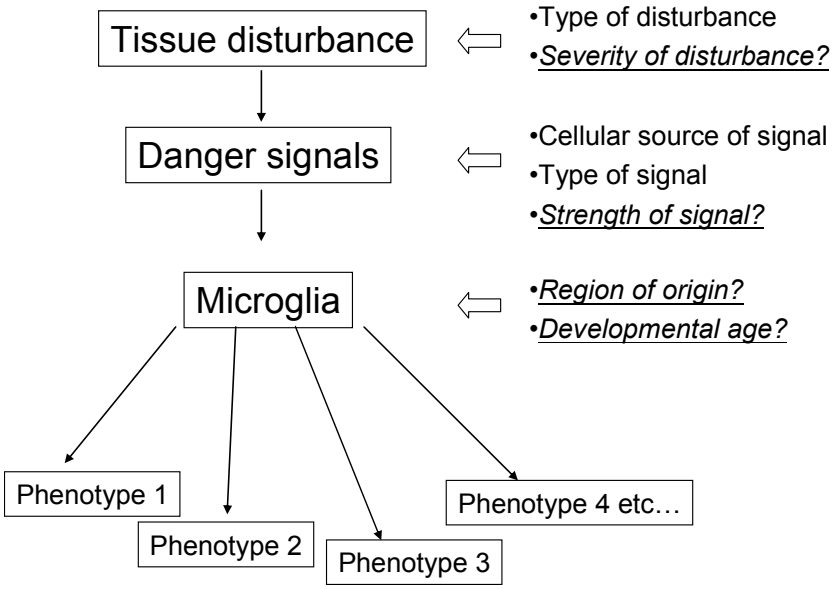

Figure 1-1 – Schematics of the thesis objectives. The process of activating microglia into various phenotypes can be regulated at different levels both extrinsic and intrinsic. The underlined and italicized regulating factors are the proposed objectives of the thesis.

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CHAPTER 2

Severity of neuronal injury as a determinant of microglial activation phenotype

*A version of this chapter appears as a published manuscript in *Glia* (Lai AY and Todd KG. 2008. Differential regulation of trophic and pro-inflammatory effectors in microglia is dependent on severity of neuronal injury. Glia 56(3):259-70.).*

2.1 Introduction

Microglia, the resident immune cells of the brain, normally exist in a ramified, or 'resting' state. In the event of brain injury, such as cerebral ischemia or trauma, resting microglia can detect signals from the site of injury, transform into an 'activated' state, and rapidly migrate towards the injury (Hansson and Ronnback, 2003; Raivich, 2005). Microglia are key determinants of neuronal survival. Activated microglia are capable of producing trophic biomolecules, such as neurotrophins, glutamate transporters, and antioxidants, but also effectors that can potentially be neurotoxic, such as nitric oxide and pro-inflammatory cytokines (Nakajima et al., 2002; Nakajima and Kohsaka, 2004; Persson et al., 2005; Lai and Todd, 2006b). Because of this dual nature, the role of microglia in the survival of injured neurons has remained controversial. Several lines of evidence suggest that activated microglia are detrimental, as post-ischemic inhibition of microglial activation and pro-inflammatory cytokine expression reduce neuronal death (Yrjanheikki et al., 1998; Wang and Shuaib, 2002; Jantzie et al., 2005). In contradiction with these studies, others have demonstrated a protective role for microglia. For example, intracerebral injection of exogenous cultured microglia was reported to promote neuronal survival after ischemia (Kitamura et al., 2004;

Hayashi et al., 2006; Imai et al., 2007). In a recent study by Lalancette-Hebert et al. (2007), ablation of proliferating microglia was found to actually exacerbate ischemic damage, further supporting a neuroprotective role for these cells. These seemingly contradicting results point to the possibility that microglial activation is not an all-or-none phenomenon, and that several different 'activation states' exist in which microglia may selectively upregulate neuroprotective and/or neurotoxic effectors. As mentioned in the previous chapter, this phenomenon was demonstrated in various studies and was found to be dependent on the activation environment, including the cell type that activates microglia and the specific activating molecule (Nakajima et al., 2002; Nakajima and Kohsaka, 2004; Shaked et al., 2005; Nakajima et al., 2006).

An additional mechanism that has not been investigated is how microglia respond to signals given out by the different degrees of neuronal injury. It is well known that upon acute insult, damaged neurons release a variety of biomolecules which under normal circumstances are tightly regulated and contained within the neuronal cell. The release of several of these molecules, including neurotransmitters, free ions, neucleotides, and extracellular proteinases, can

regulate microglial responses (Hansson and Ronnback, 2003; Siao et al., 2003; Kim et al., 2005). As the amount and composition of these signaling molecules change depending on the severity of the injury, it is possible that the microglial responses also vary accordingly, either to 'rescue' mildly-injured cells or to 'destroy' severely-damaged cells that are non-salvageable. The aim of this chapter was to address the following hypothesis: Activated microglia regulate their 'toxic' and 'trophic' effectors differentially depending on the amount and composition of signals released from injured neurons, which is dependent on the degree of injury. To distinguish between inputs from neurons and microglia, an *in vitro* injury model employing primary cultures of neurons and microglia was chosen as the system of study.

2.2 Materials and methods

2.2.1 Primary cortical neuronal cultures

Neuronal cultures were prepared from cortices of embryonic day 18 Sprague-Dawley rats based on previous methods (Brewer et al., 1993; Lai and Todd, 2006a). In brief, dissected cortices were dissociated by 0.25 % trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Gibco) digestion for 15

minutes (min), followed by mechanical titrations in Dulbecco's Modified Eagle's Media with F-12 (DMEM/F-12, Gibco) containing 10% fetal bovine serum (FBS, Gibco). Dissociated neurons were plated at a density of 2 x 10^5 cells per well in Neurobasal Media supplemented with 2% B-27, 25/25 penicillin-streptomycin, and 0.5 mM L-glutamine (all from Gibco) on 24-well plates pre-coated overnight with 5 μg/mL poly-L-lysine (Sigma). Contaminating glial cells were eliminated by treatment with 1 μM cytosine arabinoside (Sigma), which kills proliferative cells, from days *in vitro* (DIV) 3 to DIV 6. The purity of the cultures was verified to be > 98% neurons using the neuron-specific marker microtubule-associated protein-2 (MAP-2) and the astrocyte marker glial fibrillary acidic protein (GFAP). Experiments were performed on cultures that were 10-13 DIV.

2.2.2 Primary microglial cultures

Mixed brain cultures were prepared from whole brains of postnatal day 1 Sprague-Dawley rats. Dissected tissues were dissociated by 15 min enzymatic digestion with 0.25% trypsin-EDTA, mechanically titrated in DMEM/F-12 with 10% FBS, then plated on poly-L-lysine-coated 6-well or 24-well plates at a density of 4 x 10^6 cells and 1 x 10^6 per well respectively. After 10-14 DIV,

microglial cells were isolated from the mixed cultures based on the method of Siao and Tsirka (2002) by treatment of 15 mM lidocaine with 20 min shaking at 100 rpm. Purified microglia were plated in serum-free DMEM/F-12 at 1 x 10^5 cells on poly-L-lysine-coated plates. Non-adherent cells were removed after 45 min by washing. The remaining adherent cells were verified to be > 95% microglia using the microglia-specific marker ionized calcium-binding adaptor molecule 1 (Iba1) and the astrocyte marker GFAP. Microglial cultures were used for experiments 1 DIV after isolation.

2.2.3 Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma. Concentrations used for dihydrokainic acid (DHK, 100 μM) and MK-801 (1 μM) were based on previous *in vitro* studies (Zeevalk et al., 2000; Persson et al., 2005). The apyrase concentration used (0.1 U/mL) was calculated based on adenosine 5'-triphosphate (ATP) concentrations in the neuron-conditioned media.

2.2.4 *In vitro* hypoxia

Graded hypoxic injury was achieved by exposing neuronal cultures to hypoxia (<

1% oxygen) for 30 min, 2 hours (hr, h), 4 hr, or 6 hr, by means of incubating in an oxygen-sensitive chamber (PROOX model 110, BioSpherix). Following hypoxia, the cultures were re-oxygenated under normoxic conditions for 24 hr before they were collected for assays. In experiments where microglial cultures were also exposed to hypoxia, the same incubation times were applied.

2.2.5 Immunocytochemistry

Antibodies against inducible nitric oxide synthase (iNOS/NOS-2) (1:500) and glutamate transporter (GLT)-1 (1:500) were obtained commercially from Santa Cruz. Anti ED-1 and anti-neuron-specific enolase (NSE, 1:100) were obtained from Serotec. Anti- MAP-2 (1:500), GFAP (1:1000), and Iba1 (1:1000) were purchased from Sigma, Dako, and Wako respectively. Fluorescence-conjugated secondary antibodies (1:500) were obtained from Jackson ImmunoResearch. Biotinylated secondary antibodies (1:500) and horseradish peroxidase (HRP)-conjugated strepavidin (1:500) were purchased from DAKO.

Immunocytochemistry was performed using methods described in Lai and Todd (2006a). Cultures were first fixed in 2% paraformaldehyde for 10 min. After 1 hr

blocking and permeablization with 10% horse serum and 0.25% Triton, they were labeled with primary antibodies for 1 hr, then with the appropriate fluorescence-conjugated secondary antibodies for 30 min. For chromogenic detection using DAB (diaminobenzidine, Biomeda), the appropriate biotinylated secondary antibodies were used (30 min incubation), followed by 30 min incubation of the HRP-conjugated strepavidin. All of the procedures above were carried out at room temperature and had three washes of phosphate-buffered saline (PBS) in between each step.

The fluorescent staining intensities were quantified using Image-Pro Express 4.0 (Media Cybernetcis) as an estimation of the total protein expression. To ensure objectivity, the fields selected for analysis were randomly assigned by a bystander. A mean of 3-4 images were analyzed per culture well. The exposure time for each set of experiments was fixed to minimize changes to image intensity related to optics, and for each image taken, an area of background was selected as an internal control and subtracted from the total intensity value.

2.2.6 Measurement of cell viability

For neuronal or microglial mono-cultures, cell survival was measured by MTT (Thiazolyl Blue tetrazolium bromide) assay, an assay that measures mitochondrial activity. Cells were incubated with 0.5 mg/mL MTT for 1 hr, solubilized with dimethyl sulfoxide (DMSO), then read on a plate reader (PowerWave X, Bio-Tek) at 540 nm.

For the measurement of neurotoxicity in mixed cultures of neurons and microglia, the neuron-specific marker MAP-2 was used to distinguish neurons from microglia. The immunoreactivity of MAP-2 can approximate neuronal survival post-hypoxia comparably with other viability methods such as MTT (**Figure 2-1**). MAP-2 immunochemistry was carried out as previously mentioned. The cells were visualized by DAB, which was then solubilized by DMSO and manual stirring. The amount of DAB was measured by a plate reader at 492 nm. To control for the possibility of filament proteins such as MAP-2 remaining intact long after degeneration, the immunoreactivity of neuron-specific enolase (NSE) was also examined as a comparison (**Figure 2-1**).

2.2.7 Nitric oxide (NO) assay

NO was measured indirectly via its stable metabolite nitrite. The assay is based on the Griess reaction. The collected culture media was first incubated with sulphanilamide (in 3 M HCl stock, final concentration 0.25%) for 5 min. N-Naphthylethylenediamine (final concentration 0.005%) was then added to the mixture and incubated for 5 min. The final mixture was read on a plate reader at 540 nm. All of the above procedures were carried out at room temperature.

2.2.8 Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits for glia-derived neurotrophic factor (GDNF), interleukin-1-beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) were purchased from BioSource (Cytoset). The brain-derived neurotrophic factor (BDNF) kit was purchased from Promega (E_{max}). All procedures were carried out according to the manufacturer protocols. Briefly, 96-well plates were coated with the appropriate capture antibody and were allowed to stand overnight in the cold room. They were then blocked with the ELISA diluent [1% bovine serum albumin (BSA)] for 1 hr. After blocking, the samples and standards diluted in ELISA diluent were added and incubated for 2 hr. The biotinylated detection antibody were then added and incubated for 1 hr, followed by incubation with

HRP-conjugated strepavidin (in ELISA diluent) for half an hour. Finally, the chromagen tetramethylbenzidine (TMB, BioSource) was added to develop color. Upon development of the chromagen, 1.8 N sulphuric acid was added to stop the reaction, and the plates were read on a plate reader at 450 nm. All procedures were carried out at room temperature unless otherwise specified. All steps prior to chromagen addition had three PBS washes in between each step.

2.2.9 Glutamate uptake assay

Glutamate uptake assays were performed based on the method of Persson et al. (2005). Specifically, the cultures were incubated with Hank's balanced salt solution (HBSS) containing ^{3}H-L-glutamate (1 μCi/mL, Amersham) and 100 μM glutamate for 30 min, washed 3 times with cold saline, then lysed with 1% Triton. Separate aliquots were taken for protein determination and for measurements of radioactivity by liquid scintillation counting. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay. A reaction mixture containing 50:1 BCA (Fisher):copper II sulphate (Fisher) was added to the cell lysates and BSA standards at 19:1. After 30 min incubation at 37 ˚C, the plates were read at 562 nm on a plate reader. The samples for measurement of

radioactivity were transferred to vials containing 5 ml of aqueous scintillation mixture (Beckman-Coulter) and counted using a Beckman LS 6000 liquid scintillation counter.

2.2.10 High performance liquid chromatography (HPLC)

The amount of amino acids released from neurons was determined by HPLC using the conditions described in Jantzie et al. (2006). In brief, a C-18 column (Waters Symmetry, 5 μm, 4.6 x 150 mm) with a Sentry guard column was used in a Waters 2695 separation module. After inducing hypoxic injury to neuronal cultures, the media was collected; the proteins in the media were precipitated out by methanol (2:1 volume methanol:sample); the remaining supernatants were then subjected to fluorescence derivatization by o-phthaldialdehyde prior to running through mobile phases. Mobile phase A, adjusted to pH 6.2 with NaOH, contained 900 ml 0.08 M NaH_2PO_4, 240 ml methanol, 20 ml acetonitrile, and 10 ml tetrahydofuran (THF). Mobile phase B, adjusted to pH 6.2 with NaOH, contained 1340 ml 0.04 M NaH_2PO_4, 1110 ml MeOH, and 60 ml THF. The mobile phase gradient started at 95% A:5% B and shifted to 100% B over 13 min. The total runtime for each sample was 40 min, with all compounds eluting by 21 min. The

fluorescent-derivatives were detected using a Shidmazu RF10A detector, excitation at 260 nm and emission at 455 nm.

2.2.11 Measurement of ATP

The level of ATP released from neurons was measured using the Enliten bioluminescent kit (Promega) according to the manufacturer protocol. Briefly, the rL/L reagent was reconstituted 1:12 in the provided buffer, and the reconstituted buffer was mixed 1:1 with the samples and standards. After 30 min, the plate was read on a luminescence plate reader (Gemini EM, Bio-tek).

2.2.12 Western Blot

Immediately after the treatment, the cultures were harvested and lysed in ice-cold lysis buffer containing 50 mM Tris, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 150 mM NaCl, 0.8% Triton X-100, 0.2% sodium dodecyl sulphate (SDS), and 1 mM protease inhibitor phenylmethanesulphonylfluoride (PMSF). The lysates were boiled for 5 min in sample buffer [62.5 mM Tris-HCl (pH 6.8), 7.5% glycerol, 2% SDS, 15 nM Bromophenol blue, 1% β-mercaptoethanol], then subjected to

SDS-electrophoresis. To ensure total protein levels were equal in all samples, loading volumes were adjusted according to their measured protein concentrations. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (BioRad). After transfer, the membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween (TBS-T) for 1 hr. The membranes were then gently rocked in a primary antibody solution [1:200 anti-GLT-1 (Santa Cruz) or 1:5000 anti-actin (Sigma) in TBS-T containing 1% BSA and 0.02% sodium azide] overnight in 4 ˚C. After three 10 min washes with TBS-T, the membranes were labeled with the secondary antibody solution (HRP-conjugated immunoglobulin, Santa Cruz, diluted 1:2000 in TBS-T containing 5% milk) for 1 hr, then washed three times for 10 min in TBS-T, and exposed to film after enhanced chemiluminescence treatment (Amersham). All procedures were carried out at room temperature unless otherwise mentioned. Scanned images of the developed blots were quantified using densitometry functions in Image-Pro Express 4.0 (Media Cybernetcis).

2.2.13 Statistical analysis

The levels of significance were calculated by analysis of variance (ANOVA)

followed by Newman-Keuls *post hoc* test, or if unequal variances existed, by Kruskal-Wallis test followed by Dunn's *post hoc* test. The denotation * represents $p < 0.05$. Each n represents a single well of a culture plate.

2.3 Results

2.3.1 Regulation of microglial trophic factors by NCM

The first aim of this chapter was to generate a gradient of neuronal injury, and this was achieved using the *in vitro* hypoxia model. By varying the length of hypoxia, mild (< 20% death, 30 min hypoxia), moderate (40-60% death, 2-4 hr hypoxia), and severe (> 70% death, 6 hr hypoxia) injuries to neurons were produced (**Figure 2-1**). Following hypoxia and re-oxygenation, conditioned media from the injured neurons (NCM) was added to microglial cultures (1:1 volume) and incubated for 24 hr (see **Figure 2-2A** for schematics). Two major trophic molecules expressed in microglia are BDNF and GDNF (Nakajima and Kohsaka, 2004). ELISA showed that after incubation with NCM, microglial release of both BDNF and GDNF was upregulated, and the level of upregulation did not differ significantly between different severities of neuronal injury (**Figure 2-3)**. There were no detectable levels of either BDNF or GDNF in the NCM before incubation with

microglia (data not shown), suggesting that the upregulation of these trophic factors was contributed by microglia.

2.3.2 Regulation of microglial pro-inflammatory factors by NCM

Next, I tested the effect of the same NCM on microglial effectors that are classically regarded as neurotoxic, namely the pro-inflammatory mediators IL-1β, TNF-α, and NO (Nakajima and Kohsaka, 2004). NCM from mild injuries (30 min hypoxia) upregulated all of microglial IL-1β, TNF-α, and NO (**Figure 2-4**), but in contrast to the pattern seen with the trophic factors, NCM from moderate (2-4 hr hypoxia) and severe (6 hr hypoxia) injuries had no effect on these pro-inflammatory effectors (**Figure 2-4**). Only a negligible amount of these molecules in the NCM was detected (data not shown), and thus the majority of the measured IL-1β, TNF-α, and NO was of microglial origin. Interestingly, the microglial antigen ED-1, which is typically increased during microglial activation (Lai and Todd, 2006a), was upregulated in all injury conditions (**Figure 2-5A**), suggesting that all the injury conditions can induce some degree of microglial activation. Furthermore, the viability of microglia did not change in response to NCM (**Figure 2-5B**), suggesting that the observed suppressive effects were not

due to decreased microglial survival.

2.3.3 Regulation of microglia-induced neurotoxicity by NCM

The subsequent experiments addressed how the balance between trophic and pro-inflammatory effectors in microglia affected neuronal survival. Here, the microglial cultures that had been incubated 24h with NCM were detached and co-cultured with another set of injured neurons (see **Figure 2-2B** for schematics). Under this setup, untreated microglia provided a basal level of neuroprotection in the co-cultures (**Figure 2-6**). As expected, this neuroprotection was diminished when microglia were treated with NCM from mildly injured neurons (**Figure 2-6**). As the pro-inflammatory effectors were suppressed in moderate and severe injuries, one would expect more neuronal survival as a result. While neuroprotection did increase in the moderate group, NCM from severely injured neurons surprisingly abolished the neuroprotective effects induced by microglia (**Figure 2-6**), suggesting that there were other key contributing microglial effectors. As shown in a previous study (Persson et al., 2005), microglia possess the capacity to take up excess glutamate via the GLT-1 transporter, which can alleviate excitotoxicity. To identify whether microglial transport of glutamate

played a role in the toxic response, glutamate uptake in microglia incubated with NCM was determined. The results showed a reduced microglial uptake of glutamate in response to NCM (**Figure 2-7A**). Specifically, NCM obtained from severely injured neurons produced the largest down-regulation (**Figure 2-7A**), which may account for the lack of neuroprotection under severe injury conditions. To show direct involvement of the microglial glutamate transporter, microglial cultures were treated with 100 μM dihydrokainic acid (DHK), a GLT-1 inhibitor, along with NCM. The results showed that DHK treatment restored the microglia-induced neuroprotection in the severe injuries (**Figure 2-7B**).

2.3.4 Varying degrees of hypoxic treatment directly on microglia

The results thus far have shown that neuronal input is a determinant of microglial activation type. In addition to neuronal signals, it is possible that the autocrine signals generated by microglia themselves may have contributions of their own. Previous studies showed that microglia can be activated upon hypoxic insult without the presence of other cell types (Suk, 2004; Lai and Todd 2006a, 2006b; Wang and Wang, 2007), so varying degrees of hypoxic insult to microglia may induce differential auto-signals similar to that in the NCM. To investigate this,

microglia were exposed to varying lengths of hypoxia as carried out in neuronal cultures. In contrast to neurons, microglial viability was not significantly compromised in response to hypoxia (data not shown). More importantly, while hypoxia alone upregulated both trophic and pro-inflammatory effectors in microglia, in the absence of NCM, the activation levels did not change as a function of hypoxia length (**Figure 2-8A – E**), suggesting that autocrine signaling is not involved in the differential regulation of microglial effectors. Moreover, in co-cultures, without exposure to NCM, hypoxic microglia did not adopt a neuroprotective phenotype regardless of the hypoxia severity (**Figure 2-8F**).

2.3.5 Involvement of the neuronal signaling molecules glutamate and ATP

Since autocrine signals of microglia are not involved, the next set of experiments then focused on isolating the signaling molecules in NCM that are responsible for the differential regulation of microglial effectors. Neurons, when injured, release a wide variety of molecules capable of modulating microglia function, the most abundant being the neurotransmitters. HPLC measurement of amino acids in the NCM showed a severity-dependent increase in glutamate, aspartate, and GABA (**Figure 2-9A – C**), while taurine, asparagine, serine, glycine, and alanine levels

were not significantly different between the injury conditions (data not shown). NCM also had a severity-dependent increase of ATP (**Figure 2-9D)**. Out of these neurotransmitters, glutamate and ATP are known to have activating effects on microglia (Farber and Kettenmann, 2005, 2006). To demonstrate the involvement of these molecules, microglia were treated with MK-801, an antagonist against N-methyl-D-aspartate receptors, and apyrase, an extracellular ATP-degradating enzyme, to block the signaling of glutamate and ATP respectively. The results showed that in the presence of either apyrase (0.1 U/mL, **Figure 2-10A**) or MK-801 (1 μM, **Figure 2-10B**), microglia treated with NCM from severely injured neurons induced neuroprotection, while microglia treated with the same NCM in the absence of the drugs did not (**Figure 2-10A – B**). In the case of microglia stimulated with NCM from moderately injured neurons, which were shown to induce neuroprotection (**Figure 2-10A – B**), neither drug influenced this neuroprotective effect (**Figure 2-10A – B**), suggesting that both glutamate and ATP may be involved only when the injuries are severe. The next step then was to determine whether the effects of blocking glutamate and ATP signaling are related to modulations in microglial cytokine production. The release of microglial BDNF and TNF-α in response to NCM from moderately and severely injured

neurons, in the presence of MK-801 and apyrase, was measured. The results show that neither drug had a modulating effect on BDNF and TNF-α release from microglia (**Figures 2-10C – D**).

2.4 Discussion

The aim of this chapter was to define the role of neuronal injury severity on microglial activation phenotype. The results showed that injured neurons release different concentrations of danger signals depending on the injury severity. The neuronal signals then induce microglia into either a trophic or toxic phenotype depending on the severity of neuronal injury. This provides a mechanism for microglia to mount the most 'appropriate' response to an injury. It has been suggested by several researchers (Nakajima and Kohsaka, 2004; Perry et al., 2007) that microglial activation exists in more than one activated state, which may be pro-inflammatory, anti-inflammatory, or pro-trophic. In this study, at least two distinct activation states have been observed, one being pro-inflammatory, the other being pro-trophic/pro-repair.

One key concept that this study demonstrates is the importance of neuronal input,

and how it can directly determine microglial phenotype without the presence of astrocytes or T-cells, the more commonly known determinants of microglial activation (Hansson and Ronnback 2003; Schwartz et al., 2006). The finding that direct hypoxic insult to microglia in the absence of NCM could not yield a trophic phenotype further illustrates the importance of neuronal signals. Interestingly, although one would intuitively expect the most mild injury to yield a trophic rescuing phenotype, it was the moderate injured neurons that mediated this response. A possible explanation is that the microglial effectors produced during mild injury are not targeting neurons, but rather other cell populations such as astrocytes (which are absent in this study), perhaps upregulating their trophic machinery. A question that was raised in the previous chapter is whether the composition of activators or the abundance/signal strength of a specific activator plays the more crucial role in differential activation of microglia. While the composition of the microglial activators definitely influences microglial phenotype (Schwartz et al., 2006), results from this study indicate that signal strength is just as likely to sway microglia from toxicity to trophism. All of the neuronal signals measured in this study showed the same pattern of release in response to injury, suggesting that NCM from severely injured neurons released

the same activators as the moderately injured neurons, just in different amounts. How, then, do microglia translate different signal strengths into distinct activation types? One possible mechanism is through receptor subtypes. A number of microglial receptor systems do have receptor subtypes with differential ligand sensitivity, one example being the ATP purinergic receptors which contain both ionotropic and metabotropic subtypes that respond to different strengths of ATP stimulation (Inoue, 2002; McLarnon, 2005). An evolutionary advantage of using signal strength as a regulator is that it contains self-regulatory feedback mechanisms: Microglia possess machinery that can remove 'excess' signals, such as the ATP-degradating nucleotidases and the glutamate transporter GLT-1, both of which are upregulated upon microglial activation (Braun et al., 1998; Persson et al., 2005). It remains intriguing whether differential ligand sensitivity is a common theme among microglial receptors, or do most receptor systems feature the 'all-or-none' response.

An apparent limitation of this study is its significance in relation to *in vivo* circumstances. In metabolic injuries including stroke, the initial insult affects all cell types which are in close proximity with each other. The *in vitro* system

employed here is more comparable to the outer perimeter of the initial site of insult, where microglia themselves are not exposed to hypoxia, but only to the neuronal signals from the site of injury. More importantly, in an *in vivo* context, signaling inputs from other cell types need to be considered. Astrocytes, for example, are a major source of both glutamate and ATP release (Hansson and Ronnback, 2003; Schousboe and Waagepetersen, 2005). Whether this severity-dependent phenomenon is specific to hypoxic injury is also unclear. I have attempted to stimulate microglia with NCM from glutamate- or hydrogen peroxide-induced insults, and despite evident neuronal injury, neither NCM elicited a noticeable microglial response (data not shown). However, other metabolic injury models such as serum deprivation and thiamine deficiency have yet to be ruled out.

A novel finding of this study that is somewhat surprising is the loss of glutamate transport when microglia are stimulated by severely injured neurons (**Figure 2-7A**). This decreased ability of microglia to take up glutamate is accompanied by an upregulation of the GLT-1 protein (**Figure 2-7B**), suggesting that the loss of GLT-1 activity is not due to degradation of the transporter. Astrocytic GLT-1 is

known to 'reverse' its direction of glutamate transport under stress (Fellin et al., 2006), but this phenomenon is not as well documented in microglia. Nevertheless, the reversal of glutamate transport resulting in glutamate release has been observed in microglia by others (Noda et al., 1999). This would explain why blocking microglial GLT-1 with DHK would actually be neuroprotective (**Figure 2-7C**).

Another perplexing finding concerns the role of ATP and glutamate. While both are responsible for the lack of neuroprotection by microglia in severe hypoxia (**Figures 2-10A – B**), blocking either molecule (with apyrase or MK-801) had no effects on the microglial release of BDNF and TNF-α (**Figures 2-10C – D**). This suggests that despite ATP's known role of regulating microglial cytokines (Inoue, 2002), the actions of ATP and glutamate here involve other mechanisms. ATP and glutamate likely trigger different signaling pathways in microglia, and yet in this case, blocking the signaling of either molecule in severe hypoxia resembles the effects observed when blocking GLT-1 with DHK (**Figures 2-7C, 2-10A – B)**. It is a possibility that both ATP and glutamate exert their effects via modulation of GLT-1 activity.

It is worth noting that hypoxic activation is relatively subtle compared to other more potent microglial activators such as lipopolysaccharide, which may explain why in the co-culture experiments, none of the injury conditions exacerbated the injury. While using conditioned media to stimulate microglia prevents 'over-activation', due to the presence of microglia-inhibiting molecules such as γ-aminobutyric acid (GABA) and noradrenaline (Dello et al., 2004; Kuhn et al., 2004), it does present difficulties in isolating the specific signaling messengers involved in the differential activation of microglia. The list of known microglial activators present in neurons continues to expand: In addition to a wide range of neurotransmitters, there are chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and fractalkine, and proteinases, such as tissue plasminogen activator (tPA) and matrix metalloproteinases (Gravanis and Tsirka, 2005; Kim et al., 2005; Lai and Todd, 2006b). It is important for future experiments to study these molecules not only in isolation, but also in the context of the conditioned media.

Altogether, this study provides evidence of yet another dimension to the growing

list of determining factors that influence the microglial phenotype. As mentioned in the previous chapter, the type of injury and the composition of activating agents are two critical elements that influence microglial activation. The results presented in this chapter show that the severity of injury and the signal strength of activating agents are also important. In conclusion, microglia can be differentially activated into distinct phenotypes depending on the severity of neuronal injury and the amount of neurotransmitter signals released by injured neurons.

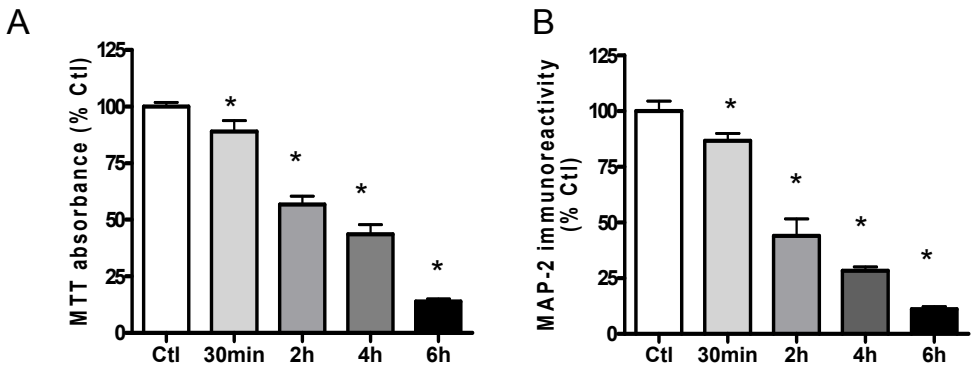

Figure 2-1 – Gradient of neuronal injury generated by varying the severity of *in vitro* hypoxia. The amount of surviving neurons is measured by **A.** MTT assay (n = 60; results represent six pooled experiments) and **B.** MAP-2 immunoreactivity (n = 20; results represent three pooled experiments). Significance levels are relative to control (Ctl). Error bars represent standard errors for all figures.

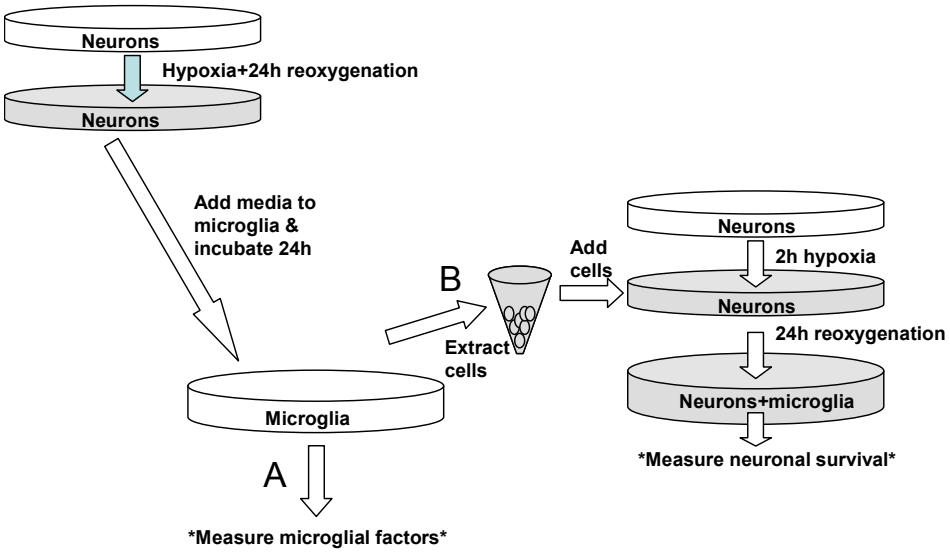

Figure 2-2 – Schematics of the experimental setup. A. Microglia were treated with conditioned media from injured neurons for 24 h, then the microglial effectors were measured. **B.** To measure the amount of microglia-induced neurotoxicity/neuroprotection, 24 h after the microglia have been treated with conditioned media from injured neurons, they were manually scraped off and re-plated on a new set of injured neurons. The re-plated microglia were allowed 30 min to settle; then the medium in the co-culture was refreshed. The amount of neuronal survival was measured 24 h later.

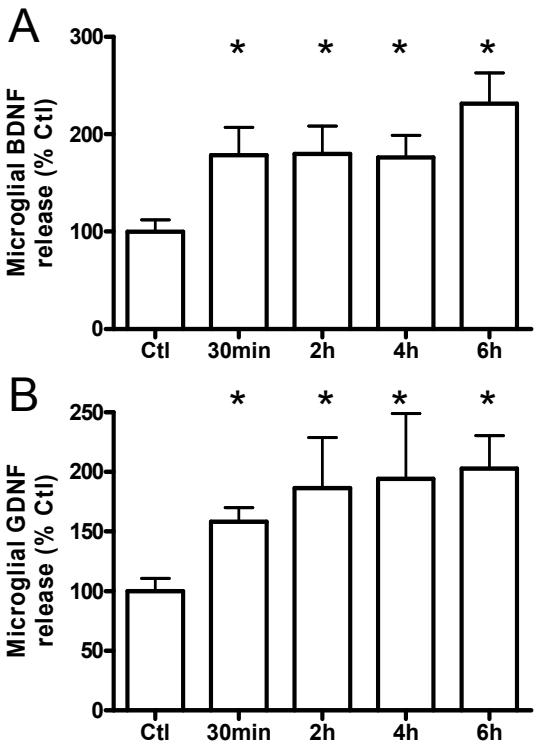

Figure 2-3 – ELISA of trophic factors in microglia exposed to NCM.
Microglial release of **A.** BDNF and **B.** GDNF are upregulated in response to media from injured neurons, and injury severity is not a factor. Results represent two experiments pooled (n = 16). Significance levels are relative to Ctl.

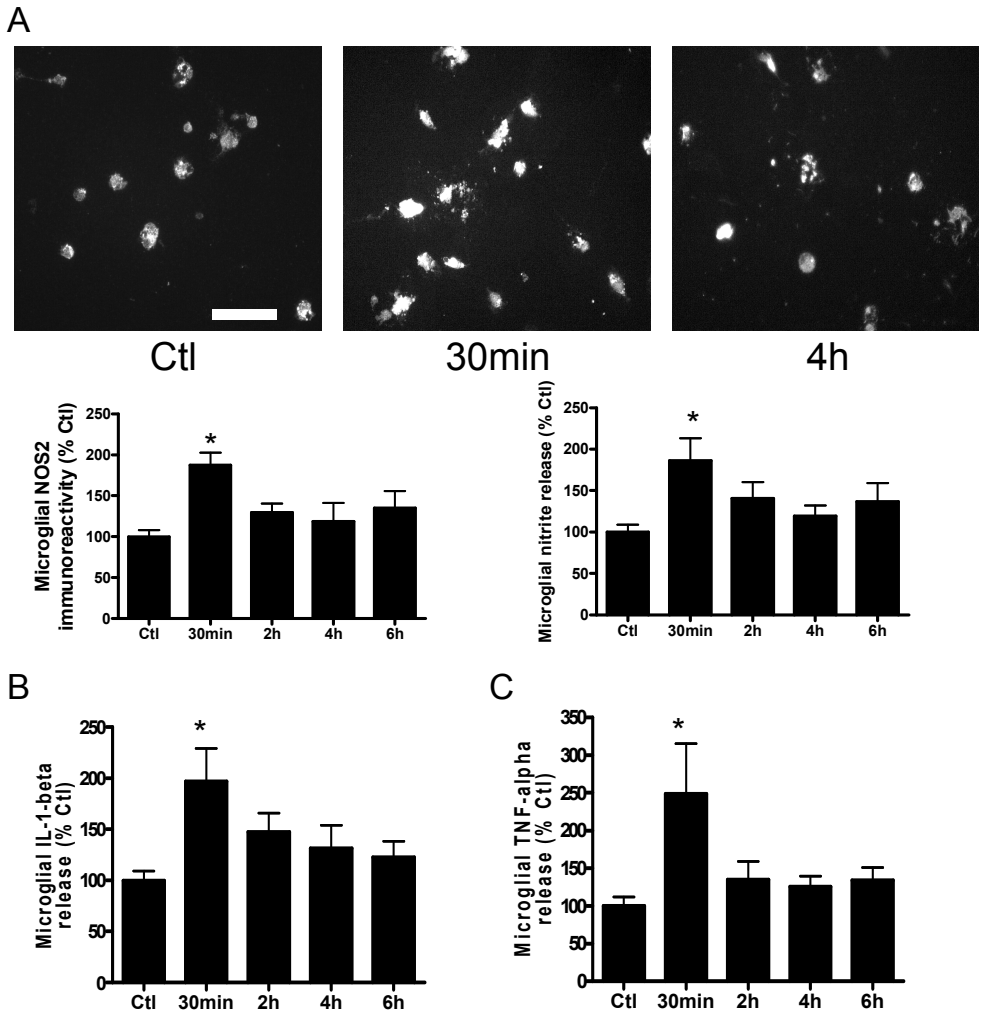

Figure 2-4 – Microglial release of pro-inflammatory effectors after exposure to NCM. All are upregulated in response to media from mildly injured neurons, but not other severity conditions: **A.** Representative micrographs (top, scale bar = 100 μm, 400X magnification) and quantification of NOS2 (iNOS) immunoreactivity (bottom left, n = 16 pooled from three experiments) and nitrite release (bottom right, n = 18 pooled from six experiments). **B.** ELISA of released IL-1β (n = 20 pooled from four experiments) and **C.** TNF-α (n = 19 pooled from four experiments). Significance levels are relative to Ctl.

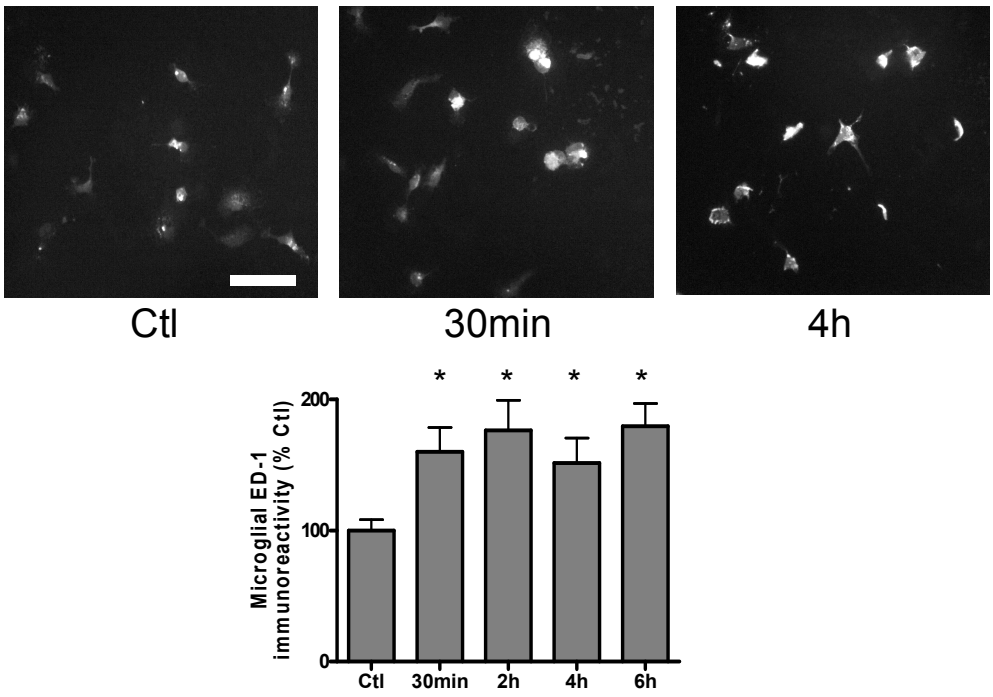

Figure 2-5 – Immunoreactivity of microglial ED-1 after NCM treatment. Representative micrographs (top, scale bar = 100 μm, 400X magnification) and quantified immunoreactivity of the microglial activation marker ED-1 (bottom), which is upregulated in response to media from injured neurons in a severity-independent manner (n = 12 from two pooled experiments). Significance levels are relative to Ctl.

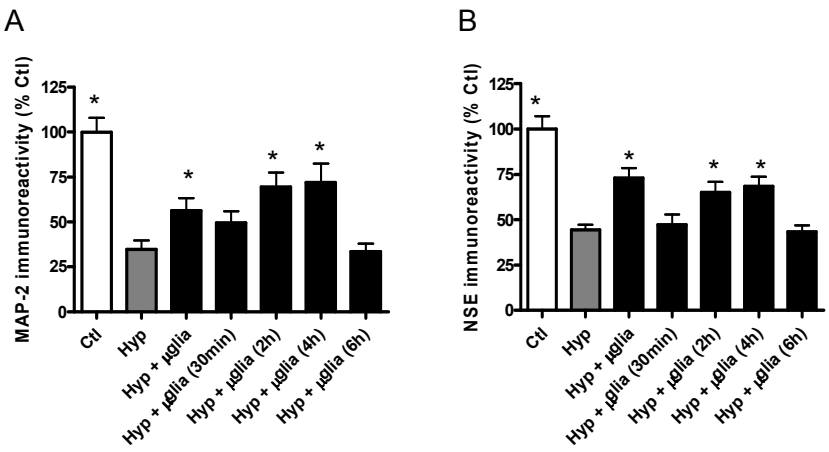

Figure 2-6 – Microglia treated with media from injured neurons are co-cultured with a new set of neurons to determine the overall neurotoxicity/neuroprotection induced by microgila. Media from moderately injured neurons induced the most neuroprotective microglia. **A.** Neuronal survival measured by MAP-2 immunoreactivity (n = 38 from five pooled experiments) and **B.** NSE immunoreactivity (n = 20 from three pooled experiments) yielded comparable results. Significance levels are relative to hypoxia (Hyp).

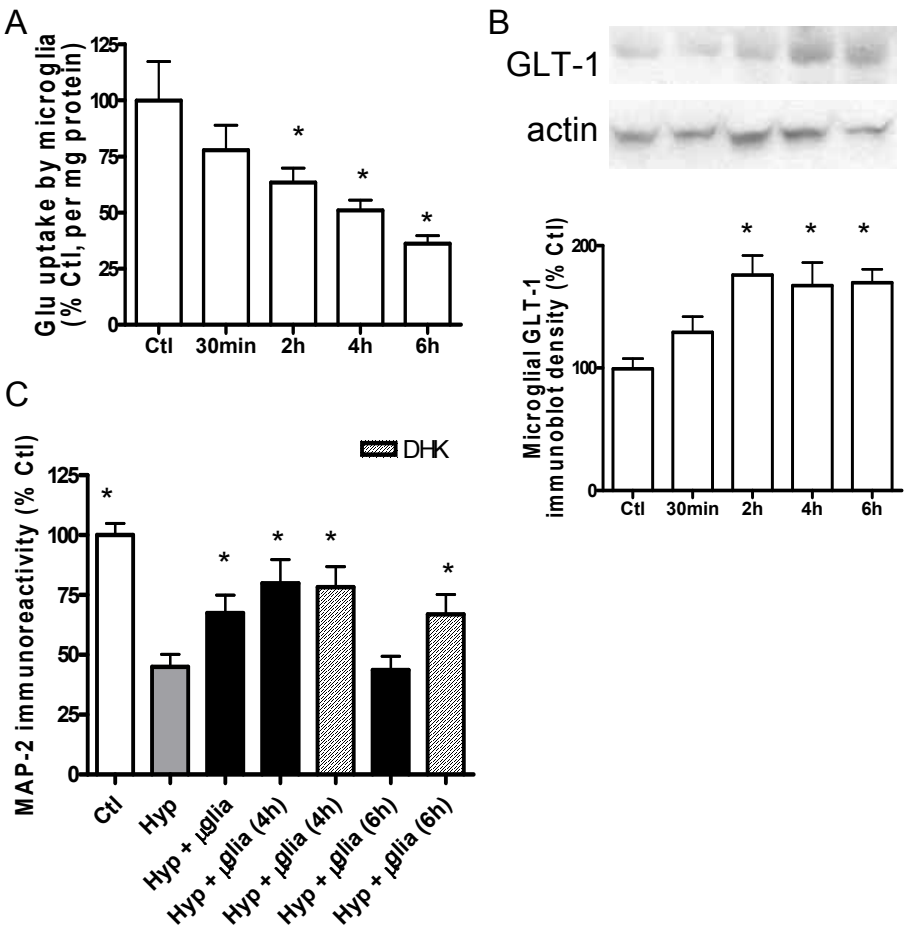

Figure 2-7 – Involvement of GLT-1 in the severity-dependent regulation of microglial activation. A. Microglial GLT-1 activity gradually decreases in response to increased injury severity in neurons (n = 13 from two pooled experiments). Significance levels are relative to Ctl. **B.** Representative blots and densitometric blot quantifications of microglial GLT-1 in response to increased injury severity in neurons (n = 6 from two pooled experiments). Significance levels are relative to Ctl. **C.** Microglia stimulated by severely injured neurons are not neuroprotective in co-cultures, but become neuroprotective when their GLT-1 is blocked by DHK (n = 38 from the same five pooled experiments in **Figure 6A**). Significance levels are relative to Hyp.

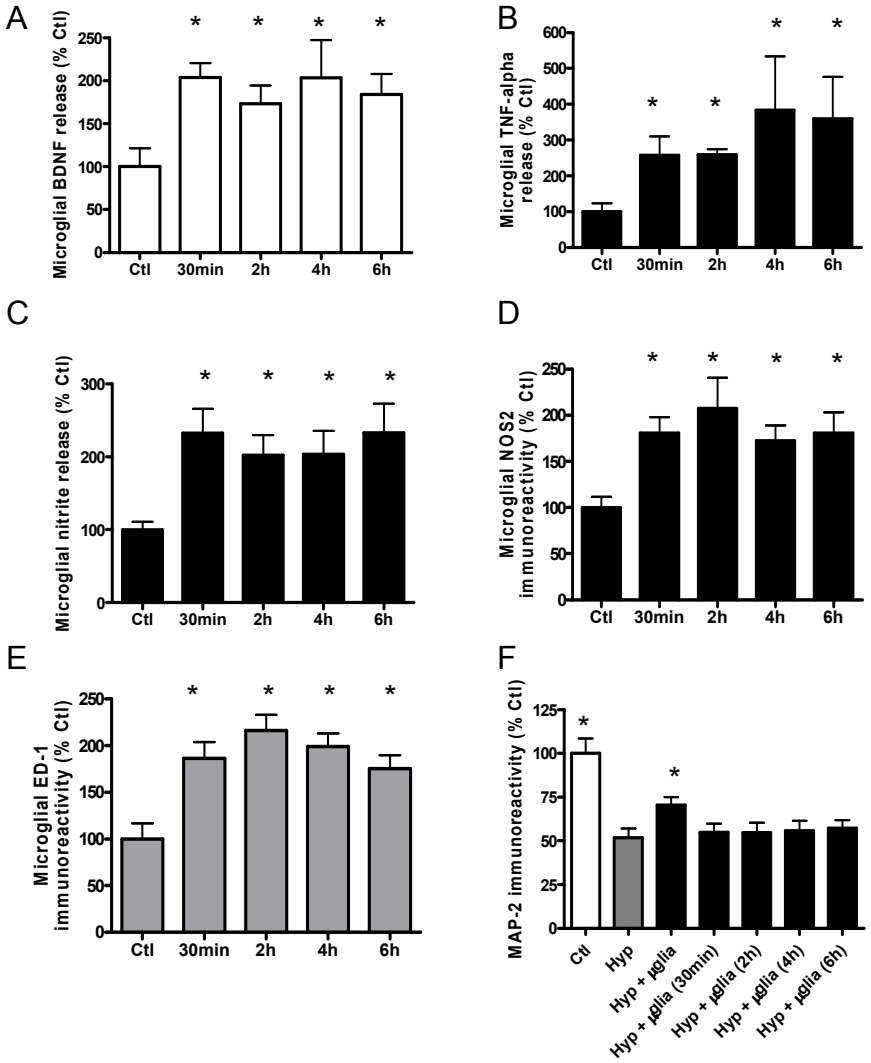

Figure 2-8 – Effects of direct hypoxic insult on microglia. Hypoxia upregulated both trophic and pro-inflammatory effectors in a severity independent, all-or-none manner: ELISA of released **A.** BDNF (n = 15 pooled from two experiments) and **B.** TNF-α (n = 7 pooled from two experiments). **C.** Nitrite release (n = 19 pooled from two experiments). **D.** NOS2 (iNOS) immunoreactivity (n = 20 pooled from two experiments). **E.** ED-1 immunoreactivity (n = 20 pooled from two experiments). **F.** Direct hypoxic insult on microglia negates their basal neuroprotective capabilities in a severity-independent manner (n = 24 from four pooled experiments). Significance levels are relative to Ctl for **A – E** and to Hyp for **F**.

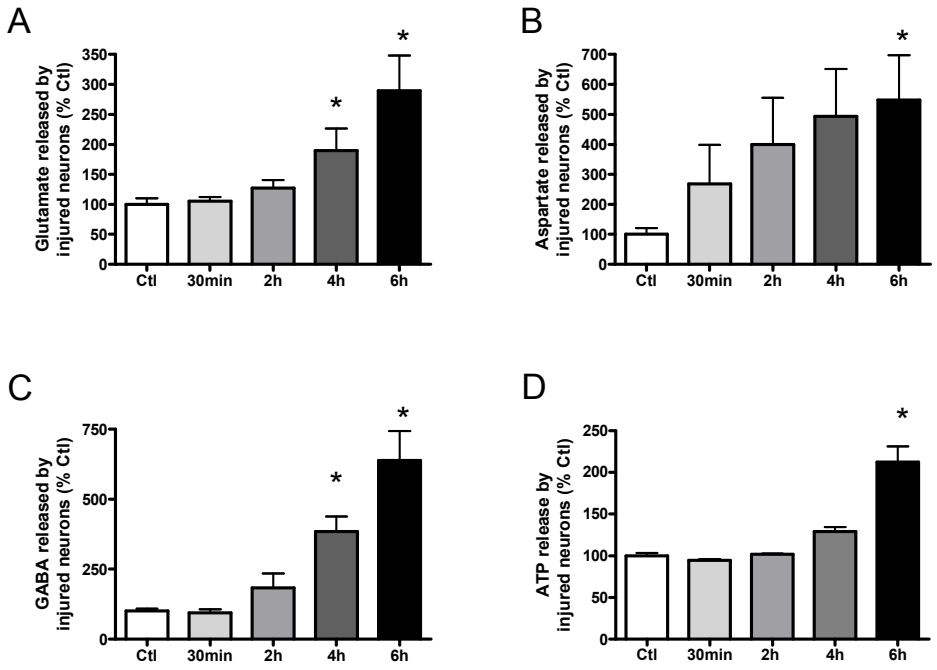

Figure 2-9 – Neurotransmitter release from hypoxia-injured neurons. Hypoxic injury increases the neuronal release of **A.** glutamate, **B.** aspartate, **C.** GABA, and **D.** ATP with increasing injury severity (n = 6 from two pooled experiments; media were collected for assay after 2 hr of hypoxia and 24 hr of re-oxygenation). Significance levels are relative to Ctl.

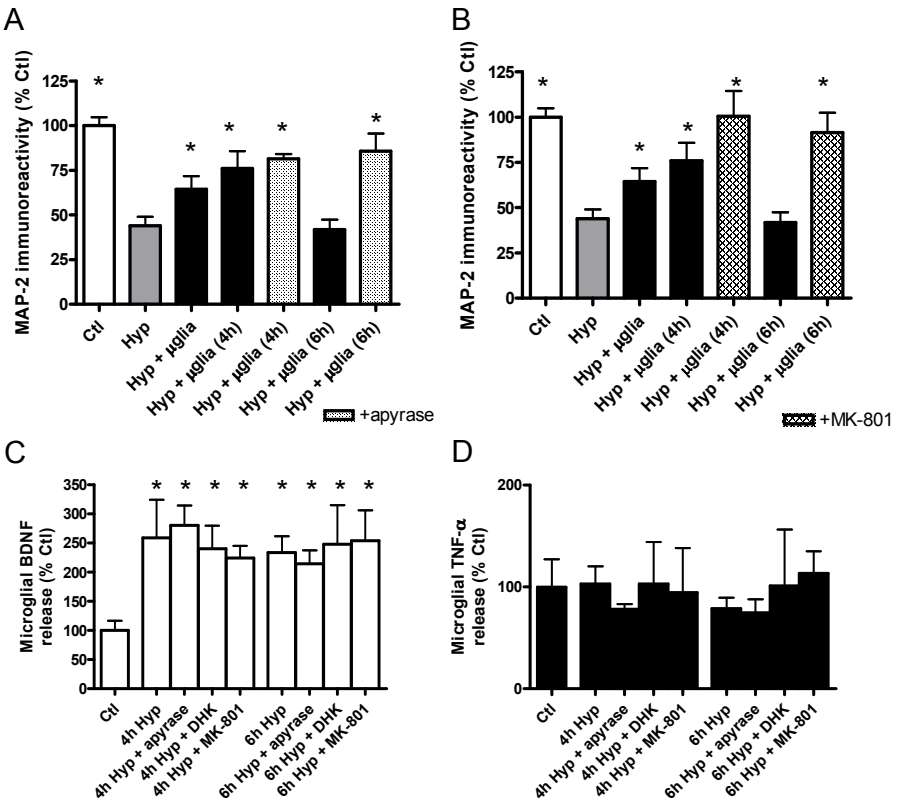

Figure 2-10 – Role of neuronal glutamate and ATP in the severity-dependent regulation of microglial activation. A, B. In co-cultures, inhibition of glutamate or ATP signaling restores the neuroprotective effects that are absent when microglia are stimulated by severely injured neurons (n = 38 from the same five pooled experiments in **Figure 6A**). **C, D.** BDNF and TNF-α release by microglia in response to NCM from hypoxia neurons (4h and 6h) do not change in the presence of either apyrase, DHK, or MK-801 (n = 6 from two pooled experiments). Significance levels are relative to Hyp for **A, B** and to Ctl for **C, D**.

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CHAPTER 3

Varying strength of purinergic stimulation as a determinant of microglial activation phenotype

3.1 Introduction

In the previous chapter, a potential relationship between the concentration of activating signals and the phenotype of activated microglia was shown. However, since the conditioned media from injured neurons (NCM) contains numerous activating agents, it is difficult to distinguish what role is played by each individual agent. Blocking the signaling of a given molecule, as performed in the previous chapter, may shed light on its functions, but other activating agents present in the NCM could have complementary and compensatory activities, rendering the interpretation of the data difficult. To circumvent this caveat, it is necessary to study an individual activator in isolation. The previous chapter showed that glutamate and adenosine 5'-triphosphate (ATP) are two activating agents involved in the neuron-dependent differential activation of microglia. The nucleotide ATP is particularly of interest due to its unique properties as an activator of microglia. ATP is normally at high concentrations intracellularly as an energy substrate, and released in low concentrations as a neuromodulator (Hansson and Ronnback, 2003; Burnstock, 2007a). As shown in the previous chapter, when neurons are injured or stressed, their release of ATP is increased. Released ATP is known to modulate microglial activation both in culture and *in*

vivo, inducing chemotactic behavior in microglia (Davalos et al., 2005) and upregulating several microglial effectors such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, nitric oxide (NO), and brain-derived neurotrophic factor (BDNF) (Hide et al., 2000; Ohtani et al., 2000; Bianco et al., 2005; Coull et al., 2005). As demonstrated in the previous chapter, the amount of ATP released by injured neurons corresponds to the severity of the injury (see **Figure 9-D**). Hence, the amount of ATP can potentially serve as a signal used by microglia to detect injury severity, which was shown to be a determining factor in microglia's activation phenotype.

There are several signaling properties of ATP that are unique compared to other danger signals. The receptor system for ATP is particularly complex. ATP receptors are of the P2 subtype of purinergic receptors, which include the ionotropic P2X subtypes and the metabotropic P2Y subtypes (Burnstock, 2007b). P2 receptors are expressed by all CNS cells, and microglia express the subtypes P2X4, P2X7, P2Y2, P2Y6, and P2Y12 (Inoue, 2002). The complexity of the purinergic receptor subtypes renders ATP a plausible candidate for differential stimulation of microglia. P2X and P2Y receptors trigger different signaling

cascades in microglia, and in addition to this variety, each P2 subtype has a different sensitivity to ATP (Burnstock, 2007b). For example, P2Y6 is almost exclusively a uridine 5'-diphosphate (UDP) receptor and has low sensitivity to ATP (Burnstock, 2007b). The P2X7 receptor is also unique from other ionotropic P2 receptors in that it forms a highly permeable pore in response to high concentrations of ATP (Burnstock, 2007b, Monif et al., 2009). Another interesting aspect of purinergic signaling are the metabolites of ATP. When released into the extracellular space, ATP is rapidly cleaved by nucleotidases into adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), and eventually adenosine (ADO), which can be taken up into the cells by adenosine transporters (Farber and Kettenmann, 2006). While both ADP and AMP have some affinity to P2 receptors, adenosine binds to a different family of purinergic receptors, the P1 receptors (Burnstock, 2007b). Microglia express nucleotidases as well as P1 receptors (Shoen et al., 1992; Burnstock, 2007b; Farber et al., 2008). The possibility exists such that this complex signaling system consisting of P1 and P2 receptors allows microglia to translate different strengths of ATP stimulation into distinct activation phenotypes. In fact, this phenomenon was observed in astrocytes, where LPS-stimulated release of astrocytic TNF-α was attenuated by

high concentrations of ATP, yet potentiated by low concentrations of ATP (Kucher and Neary, 2005). It is likely that microglia have a similar mechanism of bi-directionally regulating their effectors in order to mount the appropriate response to an insult. In the previous chapter, microglial cultures were stimulated by an assortment of danger signals in the NCM. To exclude interfering effects from other danger signals, in the current chapter microglia are stimulated with ATP alone. The aim of this chapter was to determine whether different strengths of ATP stimulation elicit distinct microglial phenotypes. Microglia were treated with both high and low concentrations of ATP. The phenotypes of the ATP-activated microglia were characterized by measuring the various trophic and pro-inflammatory microglial effectors

3.3 Materials and methods

3.2.1 Primary cortical neuronal cultures

Neuronal cultures were prepared from cortices of embryonic day 18 Sprague-Dawley rats based on methods described in previous reports (Brewer et al., 1993; Lai and Todd, 2006). For detailed descriptions of the methods, refer to **Section 2.2.1**.

3.2.2 Primary microglial cultures

Mixed brain cultures were prepared from whole brains of postnatal day 1 Sprague-Dawley rats. For detailed descriptions of the dissociation methods, refer to **Section 2.2.2.** After growing the mixed cultures for 18-24 days *in vitro* (DIV), microglial cells were isolated from the mixed cultures based on the methods of Saura et al. (2003). In brief, mixed cultures were treated with a trypsin-EDTA solution containing calcium (mixing trypsin-EDTA with DMEM 1:1) for 15-30 min. This treatment detaches non-microglial cells, and the remaining adherent cells were verified to be > 95% microglia using the microglia-specific marker ionized calcium-binding adaptor protein 1 (Iba1) and the astrocyte marker glial fibrillary acidic protein (GFAP). Isolated microglial cultures were maintained in serum-free DMEM/F12 and were used for experiments 1 DIV after isolation.

3.2.3 Chemicals

ATP, ADP, AMP, ADO, Brilliant Blue G (BBG, 1 μM), Reactive Blue 2 (RB2, 30 μM), aminoguanidine (AG, 300 μM), 1,9-pyrazoloanthrone (SP600125, 10 μM), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB 202190,

5 μM), and 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-19543, 10 μM) were purchased from Sigma.

2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059, 10 μM) was purchased from Calbiochem. The concentrations used for the antagonists were based on previous *in vitro* studies (Lockhart et al., 1998; Suzuki et al., 2004; Heidemann et al., 2005; Uesugi et al., 2006; Koizumi et al., 2007). Neutralizing anti-BDNF was obtained from Santa Cruz and used at a concentration (1 μg/mL) that neutralized BDNF standards in enzyme-linked immonosorbent assay (ELISA).

3.2.4 Immunocytochemistry

Anti-MAP-2 (1:500), GFAP (1:1000), and Iba1 (1:1000) were purchased from Sigma, Dako, and Wako respectively. Fluorescence-conjugated secondary antibodies (1:500) were obtained from Jackson ImmunoResearch. Immunocytochemistry was performed using methods described in Lai and Todd (2006). For detailed methods refer to **Section 2.2.5**.

Morphological analysis was performed on Iba-1-labeled microglia by dividing

Iba-1-positive cells into three groups: Ramified, amoeboid, and spherical. Microglia with at least one process longer than their respective cell bodies were grouped as ramified, whereas the round microglia that lack processes were grouped as spherical. The rest were grouped as amoeboid. Counting was carried out under the 20X objective. The fields analyzed were selected blindly, and in each field 10-20 cells were counted. For each culture well 4-5 fields were included.

3.2.5 *In vitro* hypoxia and assay for neuronal survival

Hypoxic injury was achieved by incubating neuronal cultures in < 1% oxygen for 2 hours (hr, h) in an oxygen-sensitive chamber (PROOX model 110, BioSpherix). Following hypoxia (Hyp), the neuronal cultures were plated with ATP-treated microglia that were detached from the enriched microglial cultures. The neuron-microglia co-cultures were then re-oxygenated under normoxic conditions for 24 hr before assayed for survival. For the measurement of neurotoxicity in mixed cultures of neurons and microglia, the neuron-specific marker MAP-2 was used to distinguish neurons from microglia. MAP-2 labeling was probed with a fluorescence-conjugated secondary antibody (1:500). The level of fluorescence in

each well was then measured by a plate reader (Gemini EM, Molecular Devices) using the well scan protocol, excitation at 492 nm and emission at 510 nm.

3.2.6 Measurement of oxidative stress mediators

NO levels released by microglia were measured indirectly via its stable metabolite nitrite as outlined in **Section 2.2.7**.

Glutathione (GSH) levels were measured using the methods described by Owens and Belcher (1965), which employs the oxidation of GSH by 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB). Briefly, a reaction mixture containing 1 mM DTNB (Sigma), 1 U/mL GSH reductase (Sigma), and 1 mM reduced β-nicotinamide adenine dinucleotide phosphate (NADPH, Sigma) was added 1:1 to samples and GSH standards (Sigma) in 96-well plates. Once a yellow color developed, the plates were read at 405 nm on a plate reader (Powerwave X, Molecular Devices).

The amount of catalase activity was measured by the decomposition of its substrate hydrogen peroxide, a method adapted from Cohen et al. (1996). In brief,

the samples and catalase standards (Sigma) were incubated with 0.03 % hydrogen peroxide (Fisher) for 10 min. The reaction was stopped by adding a solution containing 30 mN sulphuric acid and 30 mM ferrous sulphate at a 1:1 ratio. Finally, 0.1 M potassium thiocyanate was added to the mixture at 1:2, which develops a brownish color. The plates were then read at 460 nm on a plate reader.

The enzymatic activity of superoxide dismutase (SOD) was measured by its inhibition of the reducing reaction of nitrotetrazolium blue (NBT) by superoxide. Using the methods originally described by Winterbourn et al. (1975), the NBT reaction mixture containing 0.5 mM NBT (Sigma) and 0.05 mM riboflavin (Sigma) was mixed 1:1 with the samples and SOD standards (Sigma) in 96-well plates. Upon addition of the reaction mixture, the plates were exposed to ultraviolet light until a purple dye forms. The plates were then read at 560 nm on a plate reader.

3.2.7 Protein assay

Protein concentrations that were used for normalization of measured effectors were determined by the bicinchoninic acid (BCA) protein assay. Refer to **Section**

2.2.9 for detailed methods.

3.2.8 Enzyme-linked immunosorbent assay

A commercially available ELISA kit for TNF-α (DuoSet) was purchased from R&D Systems. The TNF-α ELISA was carried out according to manufacturer protocols. For detailed procedures refer to **Section 2.2.8**. The BDNF ELISA was carried out using an indirect ELISA method. In brief, media and recombinant BDNF standards (R&D) diluted in phosphate-buffered saline (PBS) were incubated with 96-well plates in the cold room overnight. The plates were then blocked with the ELISA diluent [1% bovine serum albumin (BSA) in PBS] for 1 hr. After blocking, the plates were incubated with rabbit anti-BDNF (1:500, Santa Cruz, in ELISA diluent) for 1 hr followed by horseradish peroxidase (HRP)-conjugated IgG (1:2000, Santa Cruz, in ELISA diluent) for 30 minutes (min). The chromagen tetramethylbenzidine (TMB, Sigma, 0.027 % in 0.82 % sodium acetate, 0.36 % citric acid, and 40% methanol) was added to develop color. Upon development of the chromagen, 1.8 N sulphuric acid was added to stop the reaction, and the plates were read on a plate reader at 450 nm. All procedures were carried out at room temperature unless otherwise specified. All steps prior to

chromagen addition had three PBS washes in between.

3.2.9 Glutamate uptake assay

Glutamate uptake assays were performed based on the method of Persson et al. (2005). Refer to **Section 2.2.9** for detailed methods.

3.2.10 High performance liquid chromatography (HPLC)

The amounts of ATP, ADP, AMP, and ADO were determined by HPLC using the conditions described in Manfredi et al. (2002) with slight modifications. In brief, a C-18 column (Waters Symmetry, 5 μm, 2.1 x 150 mm) with Sentry guard columns was used in a Waters 2695 separation module. The proteins in the media were precipitated out by 0.1 M perchloric acid. The remaining supernatants were then run through the mobile phases. Mobile phase A, adjusted to pH 5 with phosphoric acid, contained 25 mM NaH_2PO_4 and 100 mg/L tetrabutylammonium. Mobile phase B contained 200 mM NaH_2PO_4, 10% acetonitrile, and 100 mg/L tetrabutylammonium adjusted to pH 4 with phosphoric acid. The mobile phase gradient started at 100 % A for 10 min, then shifted linearly to 100 % B for the next 10 min. The total run time was 26 min for each sample. All the compounds

were detected using a Waters 2487 ultraviolet detector at 260 nm.

3.2.11 Western Blot

Refer to **Section 2.2.12** for detailed description of the methods. Goat anti-glutamate transporter (GLT)-1 (1:200) and rabbit anti-inducible nitric oxide synthase (NOS2, 1:200) were obtained from Santa Cruz. Mouse anti-actin (1:5000) was obtained from Sigma. HRP-conjugated secondary antibodies were obtained from Santa Cruz. Densitometric analysis of bands was carried out using Photoshop.

3.2.12 Phagocytosis assay

Phagocytic activity of microglia was measured by their uptake and engulfment of fluorescent latex beads. Carboxylate-modified 1.0 μm beads (Sigma) were incubated at approximately 50 beads per cell (2.5×10^6 beads per well) for 1 hr. After three washes in cold PBS, the cells were lysed and read at a fluorescent plate reader at 470 nm excitation and 505 nm emission.

3.2.13 Statistical analysis

The levels of significance were calculated by analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test, or if unequal variances existed, then the Kruskal-Wallis test was used followed by Dunn's *post hoc* test. Where multiple comparison was not required, the Student's t-test was used. The denotations * and # represent $p < 0.05$. Each n represents a single well of a culture plate.

3.3 Results

3.3.1 Regulation of pro-inflammatory mediators by different ATP concentrations

In the previous chapter, it was found that neurons injured by hypoxia increased the release of ATP more than 2-fold. The actual concentration of ATP, however, was less than 1 μM. To induce the aforementioned pore-like structure in P2X7 receptors, a concentration of 1 mM is required. Therefore, 1 mM ATP was defined as the high concentration of ATP, while 1 μM was the lowest concentration used. Microglial effectors classically defined as pro-inflammatory were examined first. Surprisingly, in response to 24 h treatment with ATP, only 1 mM ATP significantly increased the expression of NOS2 and the release of NO and TNF-α (**Figure 3-1**). Concentrations below 1 mM did not have an effect on these pro-inflammatory

effectors (**Figure 3-1**). Morphological analysis of ATP-treated microglia showed a correlation with this pattern, as 1 mM ATP significantly increased the proportion of microglia having the spherical, process-retracting appearance (**Figure 3-2**), whereas concentrations below 1 mM did not induce changes in overall morphology (**Figure 3-2**).

3.3.2 Regulation of trophic effectors by different ATP concentrations

The next set of experiments investigated whether microglial effectors that have neuroprotective and trophic functions respond to ATP in the same manner as the pro-inflammatory effectors. NO produced by microglial NOS2 is generally considered to promote oxidative stress due to its potential in generating reactive oxygen compounds such as superoxide and peroxynitrite. Anti-oxidative compounds and enzymes that scavenge and degrade reactive oxygen species help protect cells from oxidative stress. Among them, GSH, catalase, and SOD are produced by microglia (Dringen, 2005). GSH is a reducing agent that acts as a scavenger of oxidating compounds, whereas catalase and SOD promote the conversion of hydrogen peroxide and superoxide respectively (Dringen, 2005). Although these antioxidants are normally found intracellularly, stimulating

microglia with ATP did induce their release into the media. Interestingly, the upregulation pattern of these antioxidants did not all correlate with morphological changes: GSH release was upregulated by a relatively low concentration of ATP (10 μM), but not at higher concentrations of ATP (**Figure 3-3A**). Catalase and SOD activities in the media were increased by only 1 mM ATP (**Figure 3-3B – C**), which is similar to the NO pattern. To determine which response pattern is the more common trend among neuroprotective effectors, I also examined the responses of BDNF and GLT-1. BDNF is a neurotrophin that promotes neuronal growth, and GLT-1 prevents neurotoxicity by taking up excess glutamate (Nakajima et al., 2001; Persson et al., 2005). Microglial release of BDNF was increased in response to both high and low concentrations of ATP (**Figure 3-3D**). With regard to GLT-1, while its expression was upregulated only by 100 μM and 1 mM ATP (**Figure 3-3E**), its uptake activity was increased by all tested ATP concentrations, similar to the BDNF pattern (**Figure 3-3F**). The phagocytic activity of microglia, which is thought to clear debris and promote regeneration, was also examined. Only 100 μM and 1 mM ATP were able to significantly increase phagocytic activity (**Figure 3-3G**). In summary, among the neuroprotective effectors examined, several are responsive to weak ATP

stimulation, but there was no common pattern of upregulation shared by all of the examined effectors.

Based on the effector profile, one would expect microglia stimulated with low ATP concentrations to rescue more injured neurons compared to microglia treated with 1 mM ATP. To test this hypothesis, ATP-treated microglial cultures were detached and transferred to neuronal cultures that had been exposed to hypoxia for 2 hr. The co-cultures were then allowed to re-oxygenate for 24 hr, then labeled for MAP-2 to determine the number of neurons remaining. Without the addition of microglia, 2 hr hypoxia induced 50-55% neuronal death (**Figure 3-4**). Adding naive unstimulated microglia induced statistically significant neuroprotection, albeit only to a slight degree (**Figure 3-4A**). This neuroprotection was enhanced if microglia had been treated with 1 μM or 10 μM ATP for 24 hr (**Figure 3-4A**). Because the pro-inflammatory effectors NO and TNF-α were upregulated by higher concentrations of ATP, one would expect microglia treated with 100 μM and 1 mM ATP to be less neuroprotective. Indeed, 100 μM ATP-treated microglia were no more neuroprotective than untreated microglia, and 1 mM ATP-treated microglia were actually more neurotoxic than untreated microglia, inducing more

than 75% neuronal death (**Figure 3-4A**).

3.3.3 Distinct roles of P2X and P2Y receptors

Having established a relationship between ATP signaling strength and microglial phenotype, the next set of experiments focused on elucidating the molecular mechanisms underlying this relationship. As mentioned previously, P2 receptors include the ionotropic P2X receptors and the metabotropic P2Y receptors, each with distinct secondary messenger systems. One would expect that the effects induced by high and low concentrations of ATP are mediated by different subtypes of P2 receptors. Among the P2X and P2Y receptors, P2X7 and P2Y12 subtypes have receptor antagonists that have relatively high specificity, allowing for convenient pharmacological manipulations. The P2Y12 receptor antagonists are, however, not commercially available. As an alternative, the non-specific P2Y antagonist RB2 was used. At 1 mM ATP, the P2X7 receptor antagonist BBG (1 μM), but not RB2, suppressed the ATP-induced release of NO (**Figure 3-5A**). ATP-induced upregulation of SOD (**Figure 3-5B**) and catalase (**Figure 3-5C**) were also blocked by BBG and not RB2. At 10 μM ATP, BBG had no effects at all, while RB2 was able to suppress the ATP-induced release of GSH (**Figure 3-6A**)

and glutamate uptake (**Figure 3-6C**). The data suggest that pro-inflammatory responses from ATP stimulation are mediated by the P2X7 receptor and independent of P2Y receptors. By co-treating the ATP-stimulated microglia with 300 μM AG, a NOS2 inhibitor, the microglial toxicity induced by 1 mM ATP was reduced (**Figure 3-5D**), suggesting that NO is a key molecule in the induction of neurotoxicity. One would then expect that blocking the P2X7 receptor, which prevents the release of NO, would also reduce microglia-induced neuronal death. Indeed, co-treatment of ATP-stimulated microglia with BBG had similar effects to AG treatment in that 1 mM ATP-induced neurotoxicity was blocked (**Figure 3-4B**). Interestingly, RB2 also blocked this neurotoxicity (**Figure 3-4B**). Since RB2 did not alter NO release, its effects are likely mediated through other effectors that have yet to be examined.

With regard to the neuroprotective effects observed at 10 μM ATP, since blocking P2Y receptors with RB2 prevented the ATP-induced upregulation of GSH and glutamate uptake, one would expect RB2 to reduce the neuroprotective effects as well. This was, however, not the case, as neither BBG nor RB2 elicited changes to the microglial neuroprotection induced by 10 μM ATP (**Figure 3-4C**). Since

BDNF release was unaffected by neither antagonist (**Figure 3-6B**), it is possible that the maintenance of BDNF levels accounts for the maintenance of neuroprotection. However, blocking BDNF activity by a neutralizing antibody (1 μg/mL) did not affect the microglial neuroprotection (**Figure 3-6D**), ruling out contributions from BDNF. It is likely that other neuroprotective effectors that have not been investigated here are involved in the process.

3.3.4 Distinct roles of different mitogen-activated protein kinases

Demonstrating the involvement of different P2 receptors in the dose-specific responses to ATP led to speculations that intracellular messengers, which are activated by P2 receptor stimulation, may also be involved. Among the secondary messengers, P2 receptors are known to regulate several MAP kinases (Potucek et al., 2006). To determine whether different MAP kinases are involved in signaling different ATP concentrations, inhibitors of either ERK, JNK, or p38 were co-administered with either 10 μM or 1 mM ATP. At a high concentration of ATP (1 mM), co-treatment with 5 μM SB 202190 (p38 inhibitor) resulted in suppression of the ATP-induced upregulation of NOS2 expression (**Figure 3-7A**). Co-treatment with either 10 μM PD 98059 (ERK inhibitor) or 10 μM SP600125

(JNK inhibitor) had moderate but not significant suppressive effects (**Figure 3-7A**). On the other hand, at a low ATP concentration (10 μM), co-treatment with the p38 inhibitor did not affect the ATP-induced increase of glutamate uptake (**Figure 3-7B**). The JNK inhibitor was able to suppress the glutamate uptake, while the ERK inhibitor actually enhanced the glutamate uptake induced by ATP (**Figure 3-7B**). These results suggest that microglial responses to different strengths of ATP stimulation involve distinct sets of MAP kinases.

3.3.5 Involvement of adenosine

The next set of experiments investigated the role of the metabolites of ATP in the observed microglial responses. As mentioned previously, ATP in the extracellular space is rapidly converted into ADP, AMP, and ADO by nucleotidases expressed by most CNS cells. Since short-term stimulations of ATP (1 hr) did not induce significance changes in NOS2 activity (data not shown), all of the experiments here were thus carried out at 24 hr stimulations. This protocol, however, presents the possibility that the metabolites of ATP contribute to the observed responses. As shown in **Figure 3-8**, 1 mM ATP is degraded by microglia as early as 1 hr, primarily to ADP and AMP. By 24 hr, both ATP and ADP remain in only

negligible amounts, and most of the initial 1 mM ATP has been converted to AMP and ADO. ADO is of particular interest because its signaling of microglia is mediated by P1 receptors which is a separate family of receptors from the P2 receptors. This may explain why in some of the previous experiments, P2 receptor antagonists did not reverse the ATP-induced effects. Treating microglia with 100 μM ADO, which is the amount converted from 1 mM ATP, increased the release of NO to a similar level as that induced by 1 mM ATP (**Figure 3-9A**). Comparing ATP and ADO mole to mole, ADO appears to be a more potent activator of NOS2, as 1 mM ADO induced more than 2-fold increase in NO release (**Figure 3-9A**) compared to the 1.5-fold increase induced by 1 mM ATP (**Figure 3-1A**). To determine whether ADO signaling contributes to the ATP effects, a non-specific P1 receptor antagonist (CGS-19543, 10 μM) was co-treated with 1 mM ATP. As shown in **Figure 3-9B**, P1 receptor antagonism was able to prevent the ATP-induced release of NO, suggesting that ADO signaling is required. However, other effectors of microglia had different responses. In the case of TNF-α (**Figure 3-9C**), 100 μM ADO did not have an effect. Although the same dose of ADO increased the release of BDNF, at a lower dose of 10 μM ADO, BDNF levels were unchanged (**Figure 3-9D**). This suggests that only some ATP-induced responses

have contributions from ADO, and more specifically, in the case of BDNF, although P2 receptor antagonists did not suppress the release induced by ATP, it is likely not due to contributions from ADO and P1 signaling.

3.4 Discussion

The goal of this chapter was to characterize how microglia respond to different strengths of ATP stimulation. The results presented suggest that at least two distinct microglial phenotypes are induced by ATP. While 1 mM ATP induces a pro-inflammatory neurotoxic phenotype, 10 μM or lower concentrations of ATP induce a trophic phenotype. The results further showed that the mechanism underlying this bi-directional regulation involve activation of different P2 receptor subtypes. Specifically, responses induced by 1 mM ATP are mediated by the P2X7 receptor, while the effector responses induced by 10 μM ATP are mediated by P2Y receptors and are independent of the P2X7 receptor. In addition to the differential activation of P2 receptors, MAP kinases are also differentially regulated by 1 mM and 10 μM ATP, with the p38 kinase mediating 1 mM ATP-induced effects and ERK and JNK mediating the responses induced by 10 μM ATP. Finally, the results from the ADO experiments demonstrate the partial

contribution of ADO to the observed effects of ATP.

The finding that the P2X7 receptor does not mediate the responses at 10 μM ATP is not surprising. As previously mentioned, this receptor subtype has both an ion channel conformation and a pore conformation. The channel conformation, when stimulated by ATP, is capable of inducing calcium currents (McLarnon, 2005), but clearly they are not involved in the responses examined in this study. The pore conformation induced by 1 mM ATP is likely responsible for the increased production of NO. However, the specific molecules entering the pore that are directly responsible for the upregulation of NOS2 remain unknown.

The results from the MAP kinases experiments are somewhat surprising. As previous reports have shown that both millimolar and micromolar concentrations of ATP activate all of ERK, JNK, and p38 (Suzuki et al., 2004; Potucek et al., 2006), one would expect that all three are required for the effector responses. Instead, an effector-specific requirement for these MAP kinases was observed (**Figure 3-7**). One possible explanation is that despite the activation of all three kinases by ATP, each kinase leads to a distinct downstream pathway, hence

transcribing a specific set of microglial effectors. It is also possible that at different strengths of ATP stimulation, the MAP kinases are each phosphorylated to different levels, thus becoming either more involved or less involved than the other kinases at a given concentration of ATP. Contributions from other secondary messengers are also a possibility.

One particularly interesting result here is that despite the induction of several neuroprotective effectors by 1 mM ATP (**Figures 3-1, 3-3**), the contributions from these neuroprotective effectors were not enough to counter the neurotoxicity of NO alone (**Figure 3-5**). The lack of contributing effects by neuroprotective effectors at 1 mM ATP was further demonstrated by the P2 receptor antagonists, where the blocking of P2X7 receptor, despite decreasing the production of SOD and catalase, was still able to reduce NO-mediated neurotoxicity (**Figures 3-4, 3-5**). This raises the possibility that increased SOD and catalase were not functioning as effectors, but were rather self-protective mechanisms that responded to the increase in oxidative stress from NO production.

The neuroprotective effects induced by 10 μM ATP are also independent of

contributions from the examined neuroprotective effectors. Blocking P2Y receptors, which suppressed both GSH release and glutamate uptake (**Figure 3-6**), did not affect the neuroprotection mediated by 10 μM ATP (**Figure 3-4**). Based on these results, it is evident that the neuroprotective effectors examined in this study are not the most crucial players. Either they are an epiphenomenon, or their neuroprotective effects are compensated for by other trophic compounds that are not regulated by P2Y receptors. One microglial P2 receptor that is not blocked by either BBG or RB2 is the P2X4 receptor. Due to a lack of a specific antagonist for the P2X4 receptor, its role in the observed responses here remains unclear. Using genetic manipulation, Ulmann et al. (2008) have demonstrated a role for the P2X4 receptor in mediating BDNF release. Although BDNF is not involved in the neuroprotection observed here, it is possible that the P2X4 receptor mediates other important neurotrophic factors such as glia-derived neurotrophic factor (GDNF) and insulin-like growth factor -1 (IGF-1).

An additional point worth noting is that a proportion of ATP-mediated responses, in particular NO release, requires both P2 and P1 signaling. Both P2 and P1 antagonists alone prevented the increased release of NO (**Figures 3-5, 3-9**). This

suggests that at least in the case of NOS2, the initial stimulation of P2 receptors by ATP resembles a priming signal, which allows the subsequent stimulation of P1 receptors by ADO (after ATP has been degraded) to take effect. This priming phenomenon is absent in the cases of TNF-α and BDNF, as both are less responsive to ADO than to ATP. The selectivity of this P2-P1 interaction provides an intriguing mechanism by which ATP differentially activates microglial effectors. The specific molecular substrates involved in the signaling of this interaction are an interesting topic for future investigations.

Despite several issues that remain unanswered, the results from this chapter resolve a major question left from the previous chapter. That is, while the previous chapter demonstrated differential microglial activation dependent on the severity of neuronal injury, it was inconclusive whether the amount and concentrations of danger signals released from injured neurons actually played a role, as opposed to the composition of danger signals. Results presented in the previous chapter showed that more severely injured neurons release higher concentrations of neurotransmitters, which is correlative evidence suggesting that the amount does matter. In this chapter, by studying the neurotransmitter ATP in isolation, the data

showed that varied amounts or concentrations of a danger signal do indeed activate microglia differentially. The results here also elucidated several molecular mechanisms underlying this differential activation, such that the distinct phenotypes induced by different concentrations of ATP are a result of differential receptor activation, differential MAP kinase activation, and also differential interaction between P2 and P1 signaling.

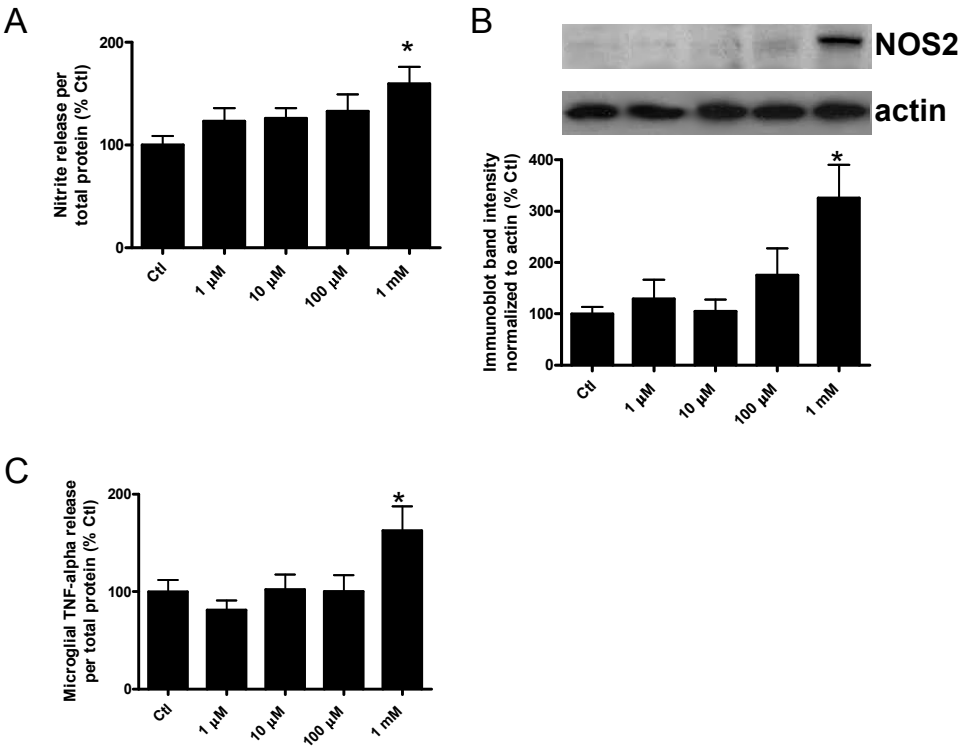

Figure 3-1 – Release of microglial pro-inflammatory mediators in response to different ATP concentrations. Pro-inflammatory mediators in microglia are upregulated by 1 mM, but not lower concentrations of ATP. **A.** NO release (n = 6 pooled from two experiments). **B.** NOS2 expression (n = 15 pooled from four experiments); results represent six pooled experiments. **C.** TNF-α release (n = 18 pooled from three experiments). Significance levels are relative to control (Ctl). Error bars represent standard errors for all figures.

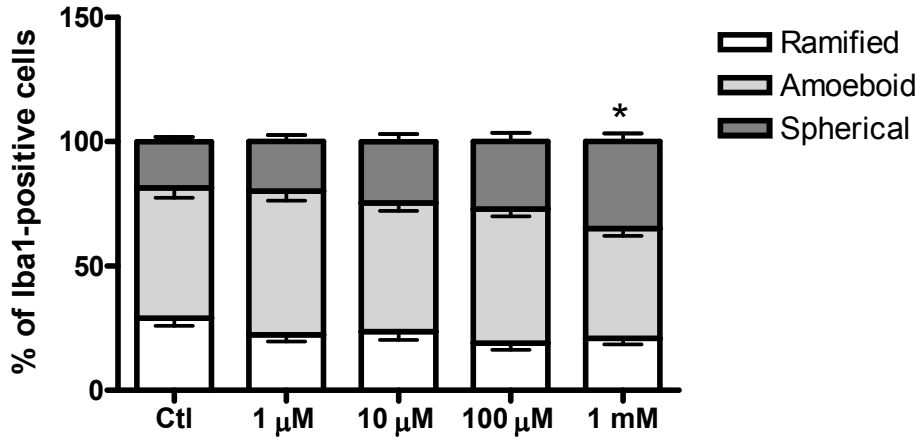

Figure 3-2 – Morphological changes in response to different concentrations of ATP. Morphological analysis of Iba1-positive cells shows that 1 mM ATP induces a higher portion of spherical microglia that have retracted processes. n = 34 pooled from two experiments. Significance levels are relative to Ctl.

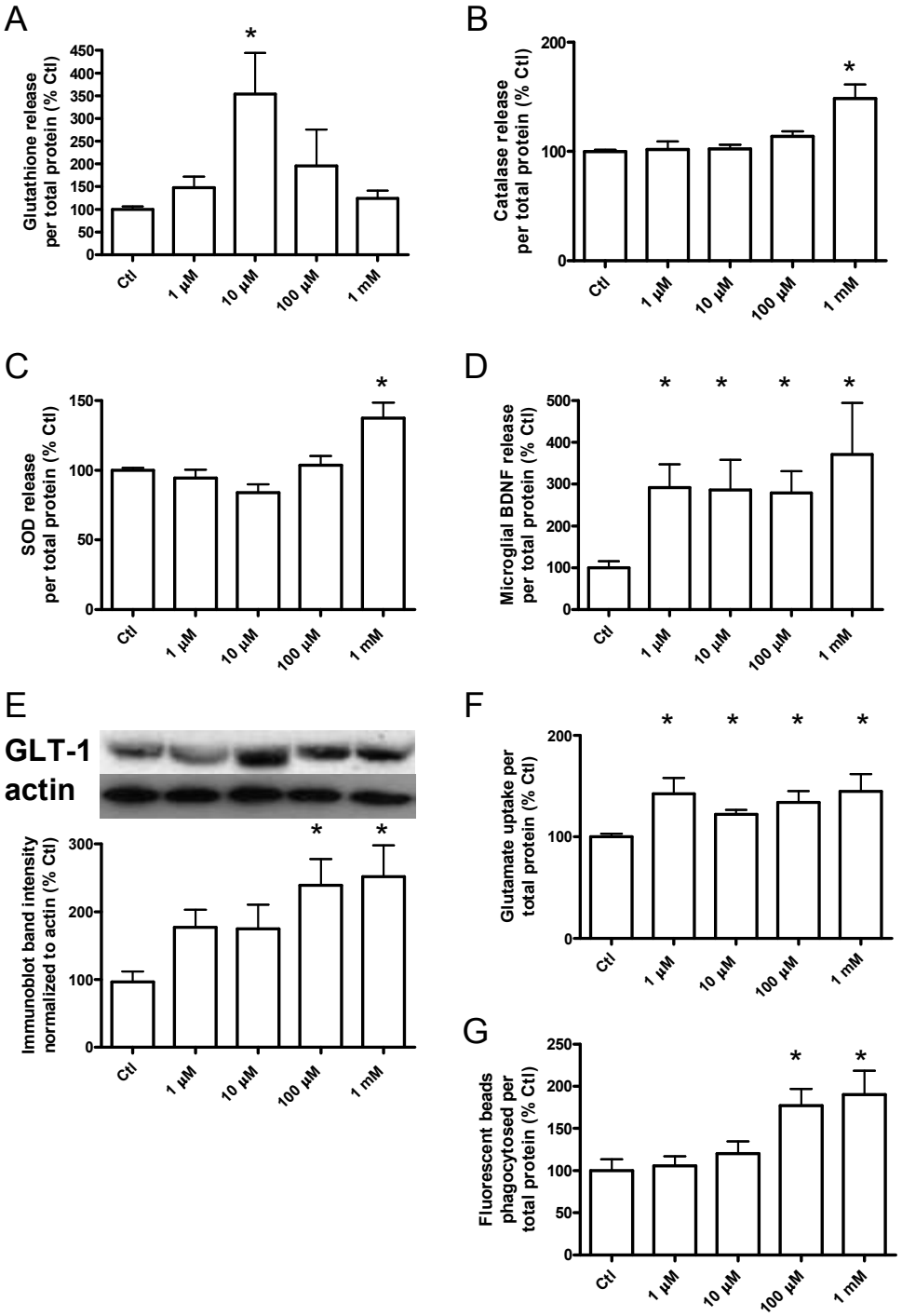

Figure 3-3 – Trophic and neuroprotective effectors in microglia have varied ATP dose response patterns. A. Glutathione release (n = 11 pooled from three experiments). **B.** Catalase release (n = 14 pooled from four experiments). **C.** SOD release (n = 18 pooled from five experiments). **D.** BDNF release (n = 19 pooled from four experiments). **E.** GLT-1 expression (n = 15 pooled from four

experiments). **F.** Glutamate uptake (n = 14 pooled from four experiments). **G.** Phagocytic activity (n = 8 pooled from two experiments). Significance levels are relative to Ctl.

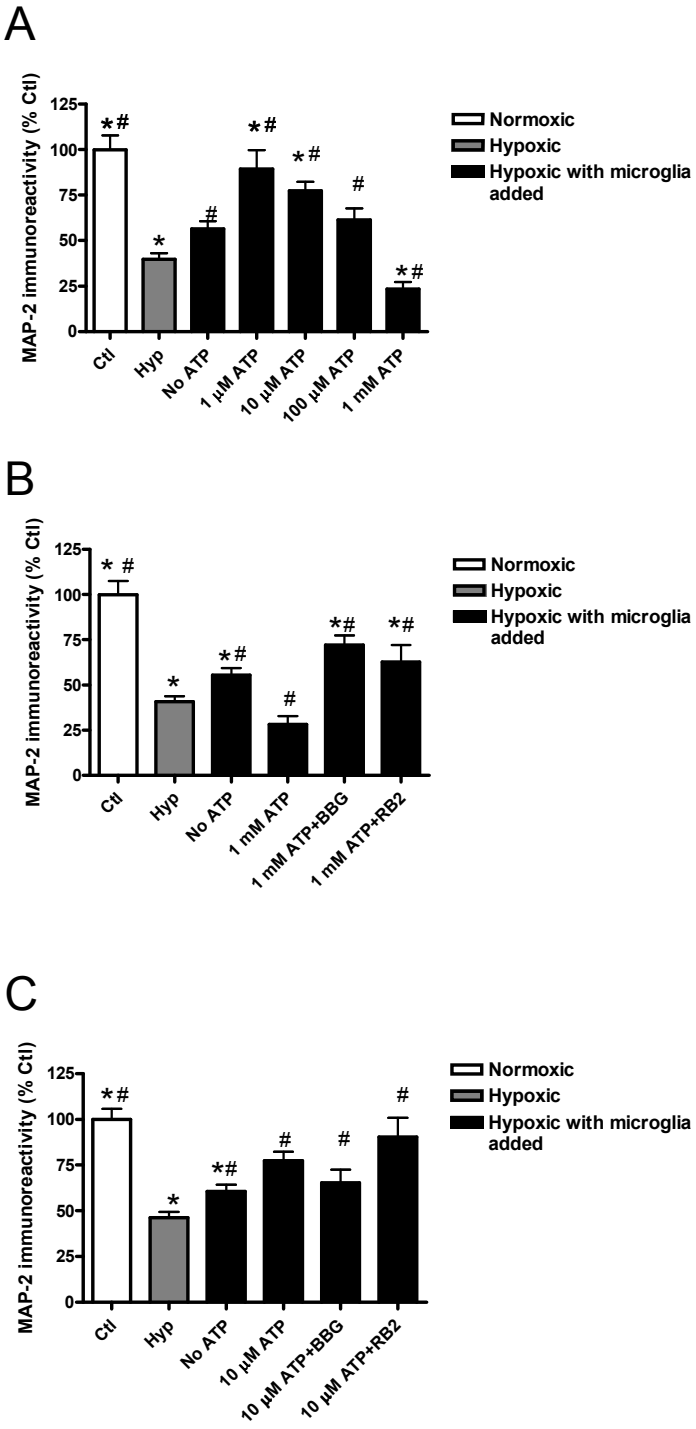

Figure 3-4 – Survival of hypoxia-injured neurons after co-culturing with ATP-treated microglia. A. Microglia treated with 1 and 10 μM ATP protect neurons against hypoxic death while those treated with 1 mM ATP exacerbate hypoxic neuronal death (n = 24 pooled from two experiments). * and # denote

significance relative to No ATP and Hyp respectively. **B.** Blocking either the P2X7 receptor or P2Y receptors prevents microglia-mediated neurotoxicity induced by 1 mM ATP (n = 9 pooled from two experiments). * and # denote significance relative to 1 mM ATP and Hyp respectively. **C.** Neither the blocking of the P2X7 receptor nor P2Y receptors has an effect on microglia-mediated neuroprotection induced by 10 μM ATP (n = 12 pooled from two experiments). * and # denote significance relative to 10 μM ATP and Hyp respectively.

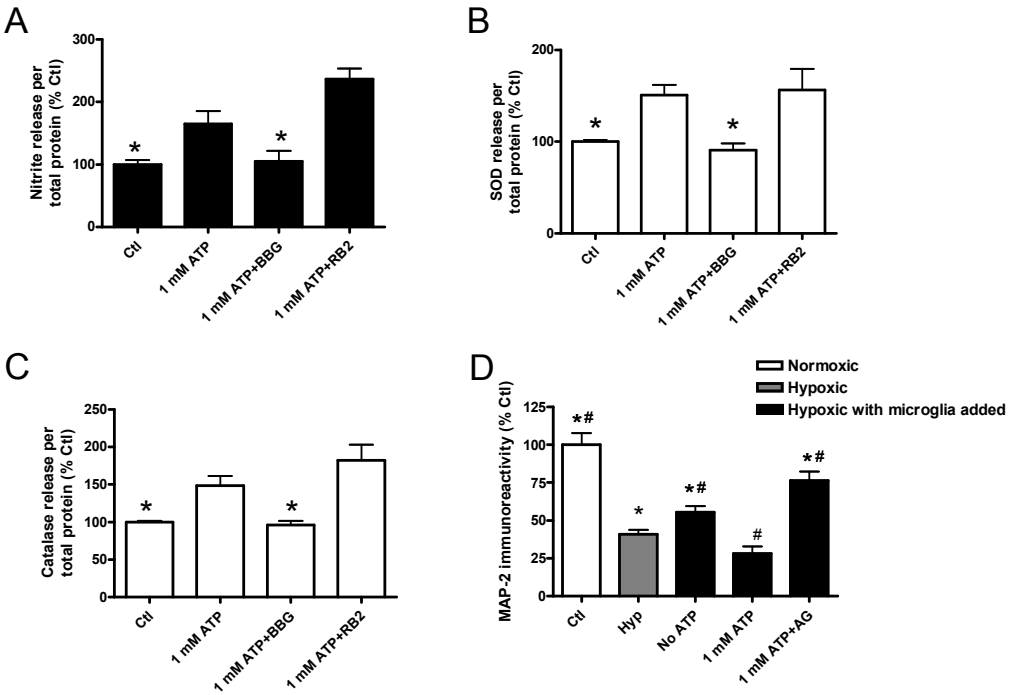

Figure 3-5 – Effects of P2 receptor antagonists on the responses induced by 1 mM ATP. The P2X7 receptor antagonist BBG, but not the non-specific P2Y receptor antagonist RB2, blocks all of **A.** NO release (n = 6 pooled from three experiments), **B.** SOD release (n = 17 pooled from three experiments), and **C.** catalase release (n = 17 pooled from three experiments). **D.** Inhibition of microglial NOS2 prevents neurotoxicity induced by 1 mM ATP (n = 9 pooled from two experiments). * and # denotes significance relative to 1 mM ATP and Hyp respectively.

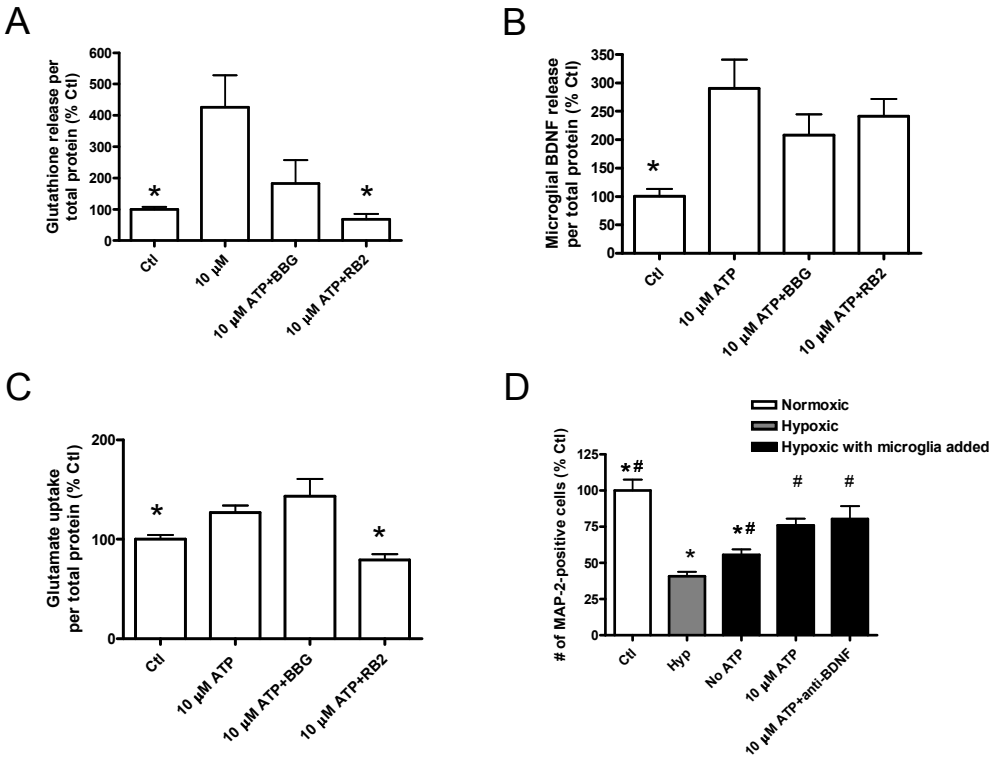

Figure 3-6 – Effects of P2 receptor antagonists on the responses induced by 10 µM ATP. A. Glutathione release induced by 10 µM ATP is blocked by RB2, an antagonist for P2Y receptors, but not by the P2X7 receptor antagonist BBG (n = 14 pooled from three experiments). **B.** BDNF release induced by 10 µM ATP is unaffected by either antagonist (n = 6 pooled from two experiments). **C.** Glutamate uptake induced by 10 µM ATP is blocked by RB2 but not BBG (n = 6 pooled from three experiments). **D.** Microglia-mediated neuroprotection induced by 10 µM is not affected by neutralization of microglial BDNF (n = 9 pooled from two experiments). * and # denote significance relative to 10 µM ATP and Hyp respectively.

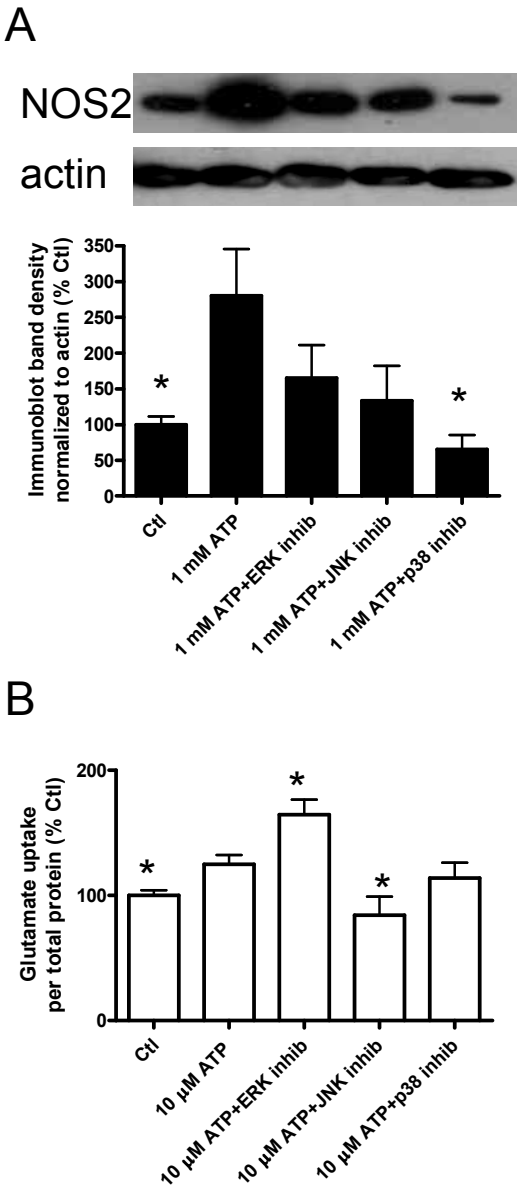

Figure 3-7 – Effects of inhibiting different MAP kinases. Microglial responses induced by 1 mM and 10 µM ATP are mediated by different mitogen-activated protein kinases as demonstrated by inhibitors (inhib) of ERK (10 µM PD 98059), JNK (10 µM SP600125), and p38 (5 µM SB 202190). **A.** NOS2 expression (n = 8 pooled from five experiments). **B.** Glutamate uptake (n = 7 pooled from two experiments). * denote significance relative to either 1 mM ATP or 10 µM ATP.

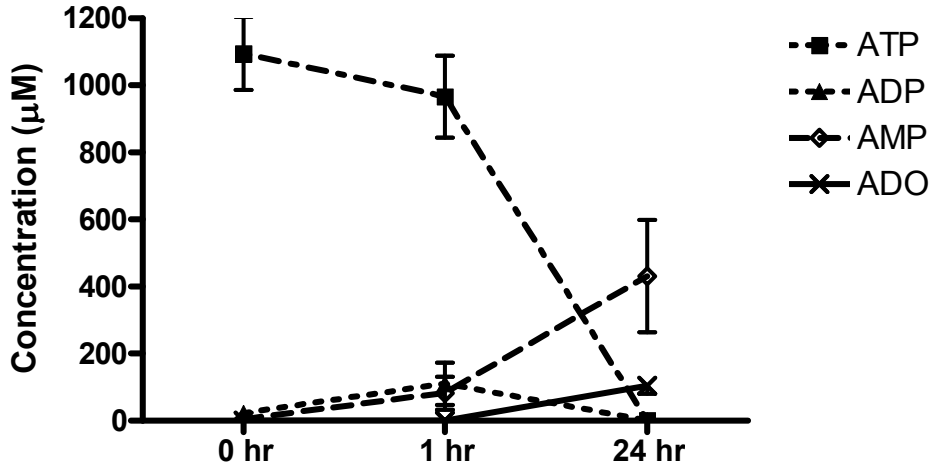

Figure 3-8 – Conversion of ATP to its metabolites by microglia. ATP is completely degraded by 24 h, mostly to AMP and adenosine (n = 6 pooled from two experiments).

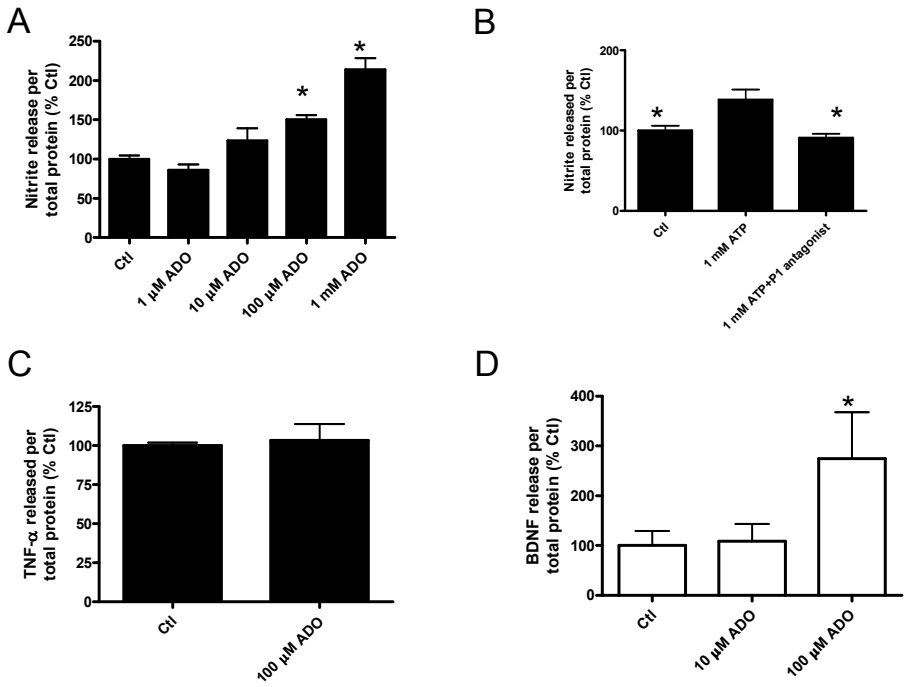

Figure 3-9 – Microglial response to ADO. A. Microglial NO release is upregulated by 100 µM and 1 mM ADO (n = 11 pooled from five experiments). * denotes significance relative to Ctl. **B.** Blocking ADO receptors prevents the upregulation of NO release by ATP (n = 6 pooled from two experiments). * denotes significance relative to 1 mM ATP. **C.** Unlike ATP, ADO at concentrations equivalent to that converted from metabolized ATP does not upregulate the released of **C.** TNF-α (n = 8 pooled from two experiments) or **D.** BDNF (n = 6 pooled from three experiments). * denotes significance relative to Ctl.

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CHAPTER 4

Region of origin as a determinant of microglial activation phenotype

4.1 Introduction

Chapter 2 and **Chapter 3** of this thesis focused on the differential activation of microglia under the regulation of signals from other cells. Since microglia are actively surveying their surroundings and are sensitive even to slight neurochemical alterations, it is not surprising that a superficially insignificant change in signal strength would induce such profound phenotypic differences. While the literature on microglial activation continues to grow, an area in the microglia field that has received much less attention is the study of intrinsic differences among microglial populations. The idea that the same activating signal may yield opposite outcomes due to cell population differences has not been demonstrated in microglia *per se*. Nonetheless, in other brain cell types such as neurons, this is a well-known phenomenon. For example, different neuronal populations in the brain express varying levels of glutamate (GLU) receptors, rendering some populations sensitive to GLU-induced neuronal death and others insensitive in response to the same GLU concentration. In comparison to neurons, there is little evidence supporting the existence of different microglial populations throughout the brain. A large portion of our current knowledge regarding microglia is derived from *in vitro* studies on primary microglial cultures. The

cultures are conventionally prepared from whole brains because it is assumed that microglia are more or less a homogeneous population. However, taking into account the diversity of the neurochemical milieu across the central nervous system (CNS), coupled with the high sensitivity of microglia to changes in surrounding activities, one would expect that throughout the course of development, the microglial phenotype in a given region of the brain would be tailored into a specialized phenotype for that particular region. Indeed, several groups have reported that in the normal CNS, resident microglia in different brain regions do express different levels of immunological markers. (Lawson et al., 1990; Elkabes et al., 1996; Wu et al., 1997; Schmid et al., 2002; Carson et al., 2007; de Haas et al., 2008). Interestingly, the regionality of microglial markers was also observed after microglia had been isolated in culture (Ren et al., 1999), suggesting that the population differences among microglia are robust enough to withstand changes in the surrounding milieu due to culturing. While this evidence supports the existence of heterogeneous microglial populations, in the disturbed CNS, where microglia are activated, it is still unclear whether the phenotypic differences still persist, and if they do, whether they are functionally significant. It has been reported that the density of microglia varies across brain regions, and

that this variation in density accounts in part for the differences in neurotoxicity induced by microglial activators such as lipopolysaccharide (LPS; Kim et al., 2000). However, in addition to variations in density, microglial activation responses may also vary regionally since microglial phenotypes are already regionally distinct at the resting state. The aim of this chapter was to determine whether microglia from different brain regions have diverse responses to activating agents, and if so, whether these diverse responses affect the survival of injured neurons differently. Primary microglial cultures were isolated from five different brain regions: Brainstem (Brs), cortex (Ctx), hippocampus (Hip), striatum (Str), and thalamus (Thl). These microglia were stimulated with activators of microglia, namely adenosine 5'-triphosphate (ATP), glutamate, and LPS. The activation phenotypes of these microglia and the effectors they release were examined. The overall neurotrophic and neurotoxic effects exerted by the activated microglia were also measured by co-culturing them with hypoxia-injured neurons.

4.2 Materials and Methods

4.2.1 Primary cortical neuronal cultures

Neuronal cultures were prepared from cortices of embryonic day 18 Sprague-Dawley rats based on methods described in previous reports (Brewer et al., 1993; Lai and Todd, 2006). For detailed description of the methods, refer to **Section 2.2.1**.

4.2.2 Primary microglial cultures

Microglial cultures were first isolated as mixed brain cultures, which were prepared from brains of postnatal day 1 Sprague-Dawley rats. For each brain, brainstem, cortex, hippocampus, striatum, and thalamus were carefully separated. For detailed description of the dissociation protocol, refer to **Section 2.2.2**. After 18-24 days *in vitro* (DIV), microglial cells were isolated from these mixed cultures using a previously reported trypsin-based method (Saura et al., 2003). For detailed description of the methods refer to **Section 3.2.2**.

4.2.3 Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma. Concentrations used for aminoguanidine (AG; 300 μM), ATP (0.01-1 mM), glutamate (30 μM), and LPS (1 μg/mL) were based on previous *in vitro* studies

(Lockhart et al., 1998; Tanaka et al., 2000; Nakajima et al., 2001; Inoue, 2002) as well as data from the previous chapter. The concentration used for the anti-tumor necrosis factor (TNF)-α antibody (R & D, 0.1 μg/mL) was derived by comparing the measured TNF-α concentrations in the culture media to the neutralization curve provided by the manufacturer. The concentration used for taurine (20 μM) was based on the taurine concentrations measured from the media of the mixed cultures.

4.2.4 Immunocytochemistry

Anti- microtubule-associated protein-2 (MAP-2, 1:500), glial fibrillary acidic protein (GFAP, 1:1000), and ionized calcium-binding adaptor protein 1 (Iba1, 1:1000) were purchased from Sigma, Dako, and Wako respectively. Fluorescence-conjugated secondary antibodies (1:500) were obtained from Jackson ImmunoResearch. Immunocytochemistry was performed using methods described in Lai and Todd (2006). For detailed methods refer to **Section 2.2.5**.

Morphological analysis using the ImageJ (NIH) program was performed on Iba-1-labeled microglia under the 20X objective. For each well, 4-5 fields were

analyzed. The fields analyzed were selected blindly, and in each field 8 cells that were closest to the center of the field were included in the analysis.

4.2.5 *In vitro* hypoxia and measurement of neuronal survival

Hypoxic injury was induced by exposing neuronal cultures to hypoxia (< 1% oxygen) for two hours (h, hr), incubating the cultures in an oxygen-sensitive chamber (PROOX model 110, BioSpherix). Following hypoxia, the cultures were re-oxygenated under normoxic conditions for 24 hr before they were assayed. To measure the neurotoxicity in mixed cultures of neurons and microglia, the microtubule-associated protein-2 (MAP-2) antigen, a neuron-specific marker, was used to distinguish neurons from microglia. Refer to **Section 3.2.5** for detailed description of the methods.

4.2.6 Nitric oxide assay

Nitric oxide (NO) in the media was assessed indirectly via its stable metabolite nitrite. Refer to **Section 2.2.7** for detailed description of the methods.

4.2.7 Protein assay

Protein concentrations that were used for normalization of measured effectors were determined by the bicinchoninic acid (BCA) protein assay. Refer to **Section 2.2.9** for detailed methods.

4.2.8 Enzyme-linked immunosorbent assay (ELISA)

A commercially available DuoSet ELISA kit for tumor necrosis factor (TNF)-α was purchased from R&D Systems. The TNF-α ELISA was carried out according to manufacturer protocols. For detailed methods refer to **Section 2.2.8**. The ELISA for brain-derived neurotrophic factor (BDNF) was carried out using an indirect ELISA method as described in **Section 3.2.8**.

4.2.9 Glutamate uptake assay

Glutamate uptake assays were performed based on the method of Persson et al. (2005). Refer to **Section 2.2.9** for detailed methods.

4.2.10 High performance liquid chromatography (HPLC)

HPLC was used to measure the levels of various amino acids in the culture media. A C-18 column (Waters Symmetry, 5 μm, 4.6 x 150 mm) with a Sentry guard

column was used in a Waters 2695 separation module. The proteins in the media were precipitated out with methanol (2:1 volume methanol:sample); the remaining supernatants were then subjected to fluorescence derivatization by o-phthaldialdehyde/isobutyryl-L-cysteine (OPA/IBLC; 1:1 mixture; the derivatization reagent contains 1 mg OPA and 2 mg IBLC dissolved in 0.1 mL methanol, then mixed with 0.9 mL 0.2 M pH 10 sodium borate) prior to running through mobile phases. Mobile phase A, adjusted to pH 6.2 with NaOH, per 2 L contained 300 mL methanol, 8.4 g NaH_2PO_4, and 1.4 g Na_2HPO_4. Mobile phase B, adjusted to pH 6.2 with NaOH, per 2.5 L contained 1110 mL methanol, 60 mL tetrahydrofuran, and 6.4 g NaH_2PO_4. The mobile phase gradient started at 85% A:15% B and shifted to 100% B over 35 min. The total runtime for each sample was 45 min with all compounds eluting by 30 min. The fluorescent-derivatives were detected using a Shidmazu RF10A detector, excitation at 344 nm and emission at 433 nm.

4.2.11 Immunoblotting

The following antibodies were used: Rabbit anti-P2XR7 (1:200, Chemicon), goat anti-P2YR12 (1:200, Santa Cruz), and mouse anti-actin (1:5,000, Sigma).

Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) were obtained from Santa Cruz. For detailed description of the blotting methods, refer to **Section 2.2.12**. Densitometric analysis of the protein bands were carried out using ImageJ.

4.2.12 Statistical analysis

Significance levels were calculated using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. For the HPLC data sets where two-way ANOVA is not necessary, one-way ANOVA followed by the Newman-Keuls *post hoc* test was used. For the MAP-2 immunoreactivity data, where there is unbalanced grouping, one-way ANOVA and the Newman-Keuls test were also used instead of the two-way. The denotations *, #, +, and & represent $p < 0.05$. Each n represents a single well of a culture plate.

4.3 Results

4.3.1 Regional differences in microglial morphology

Although the morphological features of microglia do not necessarily correlate with their functions, they can nonetheless serve as a marker for a particular

microglial phenotype and perhaps give an indication of potential regional differences. Using the myeloid marker Iba1, the morphological features of microglia can be visualized in detail. As mixed cultures, there were no apparent regional differences in the gross appearance of the cultures. However, when isolated as enriched microglial cultures, there was noticeable regionality. As shown in **Figure 4-1**, microglial cultures derived from Ctx contain mostly round and amoeboid cells (arrow), as opposed to most Str microglia which have extensive processes (chevron). The right column of **Figure 4-1** shows the 1 mM ATP-treated cultures, which have regionally distinct morphologies as well. In particular, ATP-stimulated microglia from Str appear to be smaller overall than those from other regions. To quantitatively describe these morphological differences, the number of branches (stemming from the cell body, **Figure 4-2A**), the size of the cell body (**Figure 4-2B**), and the length of the longest branch (**Figure 4-2C**), were measured. Quantitative analysis showed that without ATP stimulation, Ctx microglia had the least number of branches as well as the shortest branch length (**Figure 4-2**). In contrast, untreated Str microglia were characterized by high branch number, short branch length, yet also smaller cell bodies than the other regions (**Figure 4-2**). ATP stimulation induced significant morphological

changes in Brs, Hip, and Thl, but not Ctx and Str microglia (**Figure 4-2**). There were no regional differences among ATP-treated microglia in branch number and branch length; however, the cell bodies of ATP-treated Str and Thl microglia were significantly smaller than those from other regions (**Figure 4-2**).

4.3.2 Regional differences in released effectors of microglia

The overall observation from morphological analyses suggested the presence of regionality among microglial populations. Ctx and Str microglia were particularly distinct morphologically from microglia of other regions. To determine whether these morphological differences have functional significance, several effectors produced by activated microglia were measured. The pro-inflammatory mediator NO was examined first. As shown in **Figure 4-3A**, stimulating microglial cultures with 1 mM ATP for 24 hr resulted in upregulation of NO release in microglia from all of Brs, Ctx, Hip, and Thl, but not in those derived from Str. This pattern of NO upregulation, however, was dependent on the specific activating agent: Stimulating microglia with either 30 μM glutamate or 1 μg/mL LPS for the same duration did not yield a similar pattern. In the case of glutamate stimulation, NO release was not altered in any of the examined regions (**Figure 4-3B**). Stimulation

with LPS yielded a regional profile distinct from that of ATP, where microglia from Brs and Str demonstrated upregulated NO release (**Figure 4-3C**). This activator-dependent pattern was not restricted to pro-inflammatory effectors, as microglial release of the neurotrophin BDNF also showed regionality specific to the activating agent. At 1 mM ATP, BDNF release was upregulated in microglia derived from Brs and Ctx (**Figure 4-4A**). Interestingly, although the same ATP concentration had no effects on Hip microglia, a lower ATP dose at 10 μM did induce the release of BDNF (**Figure 4-4A**). This was not surprising as the previous chapter had demonstrated that BDNF release can be upregulated by micromolar concentrations of ATP. In the case of glutamate stimulation, only Str microglia upregulated BDNF release (**Figure 4-4B**). The LPS response was more ubiquitous and less region-specific, as all of the regions examined except Thl increased BDNF release after LPS treatment (**Figure 4-4C**).

Having demonstrated that regionality of microglial activation is activator specific, the next set of experiments focused on only the ATP-induced responses, considering that it is an endogenous activator (as opposed to LPS), and as shown in **Figure 4-3**, is a more potent activator than glutamate. In addition to NO and

BDNF, two other microglial effectors, TNF-α and glutamate transporter, were also examined. TNF-α release normally coincides with NO production due to their common effects on promoting neuroinflammation. Although one would expect a similar pattern of regionality between TNF-α and NO, it was not the case here. At 1 mM ATP, TNF-α release was significantly increased only in Ctx and Hip microglia and not other regions (**Figure 4-5A**). The patterns and TNF-α and NO were nonetheless similar in that neither effector was upregulated in Str microglia. With regard to glutamate transport, microglia from Str were again distinct from other regions. Without ATP stimulation, the basal glutamate uptake activity of Str microglia was higher than other regions. Interestingly, although the previous chapter showed increased glutamate uptake in response to ATP, no significant increases in glutamate uptake were observed in any of the regions examined (**Figure 4-5B**). In fact, Thl microglia at 1 mM ATP actually showed a decrease in uptake (**Figure 4-5B**).

4.3.3 Region-specific effects on neuronal survival

Taken together, the regional profiles of NO, TNF-α, and BDNF release, as well as glutamate uptake each have a unique pattern. A general trend though was

observed, such that microglia from Str had the most distinct phenotype with respect to other regions, particularly in comparison to microglia from Ctx (in terms of morphology) and Hip (in terms of effector profile). The results thus far are indirect measures of the physiological activities of microglia. How the regional differences in effector profiles translate towards functional significance has yet to be determined. Therefore, the next set of experiments investigated how ATP-stimulated microglia from various brain regions affected the survival of neurons after insult/injury. To induce an insult to neuronal cultures, they were exposed to hypoxia for 2 hr followed by 24 hr of re-oxygenation. This procedure induced 50-55% neuronal death. To assess the effects of microglia on these injured neuronal cultures, ATP-stimulated microglia were manually detached and transferred to the neuronal cultures immediately following hypoxia. Under this experimental setup, ATP-stimulated microglia from Ctx and Hip induced significantly more toxicity to neurons compared to their respective non-stimulated controls (**Figure 4-6A**). In contrast, ATP-stimulated microglia from the other regions did not induce any significant toxicity (**Figure 4-6A**). This pattern correlates with that of the pro-inflammatory mediators, especially TNF-α, which was upregulated in Ctx and Hip microglia only. Thus, the possibility exists that

the neurotoxicity induced by Ctx and Hip microglia was due, in part, to the increased release of pro-inflammatory mediators such as NO and TNF-α. To test this hypothesis, an anti-TNF-α antibody (0.1 μg/mL) and the iNOS inhibitor aminoguanidine (AG, 300 μM) were added to the culture media immediately after the addition of microglia to the neuronal cultures. Treatment with either anti-TNF-α or AG reversed the toxicity induced by ATP-stimulated microglia from Ctx (**Figure 4-6B**). Unexpectedly, neither treatment was able to reverse the toxicity induced by ATP-treated Hip microglia; the anti-TNF-α antibody in fact exacerbated the neurotoxic effects (**Figure 4-6B**). A similarly surprising observation was that ATP-stimulated microglia from Str and Thl, neither of which induced toxicity, when treated with either anti-TNF-α or AG, actually induced a significant amount of toxicity (**Figure 4-6B**). To rule out the possibility that the drugs themselves were neurotoxic, anti-TNF-α and AG were added to neurons alone. Neither treatment had neurotoxic effects on neuronal cultures (data not shown). This in turn suggests that NO and TNF-α released by microglia from Str and Thl were actually beneficial to the survival of injured neurons, unlike their generally perceived detrimental nature.

4.3.4 Regionality of ATP receptors

The next set of experiments investigated whether or not the expression of ATP receptors on microglia was also regionally unique. The possibility exists such that regional differences in the expression of these receptors on microglia play a role in their region-specific responses to ATP. As described in the previous chapter, two major receptors for ATP expressed by microglia are the purinergic receptors P2XR7 and P2YR12 (Inoue, 2002). By immunoblotting, the regional expressions of these receptors were determined. Without ATP stimulation, the basal expression of both microglial P2XR7 and P2YR12 were found to be regionally distinct, such that Str microglia expressed higher levels of both receptors compared to other regions (**Figure 4-7**). Although stimulation with ATP did not induce significant changes in expression (**Figure 4-7**), the fact that basal P2XR7 and P2YR12 expressions were higher in Str microglia correlates well with the previously shown patterns of regionality. The possibility exists such that higher expression of ATP receptors in Str microglia sets a higher activation threshold, which may explain why ATP did not upregulate any of the examined effectors in Str microglia

4.3.5 Origin of regional heterogeneity

Having established that regional heterogeneity does exist among activated microglia, the question then arises as to how this heterogeneity came about. One possibility is that microglia from different brain regions were influenced by their surroundings during development, and by the time they matured had already acquired a fixed pool of transcripts that provide each microglial population with a hardwired identity. The other possibility was that rather than having a fixed phenotype, mature microglia remain dynamic such that changes in the neurochemical environment can readily alter their inflammatory machinery, and in turn, their responses to activators. To test which of these speculations was true, a conditioning experiment was performed. Under this conditioning procedure, conditioned media (CM) from mixed cultures of Hip and Str were collected just prior to microglial isolation. These CM were then incubated 24 hr with microglia isolated from an ectopic region, then stimulated with ATP for an additional 24 hr. Measuring NO release from these conditioned microglia showed that when Hip and Thl microglia were ectopically incubated with Str CM, they obtained Str microglia-like behavior such that they no longer upregulated NO release in response to ATP (**Figure 4-8**). Conversely, when Str microglia were incubated with Hip CM, they also obtained a Hip microglia-like phenotype, upregulating

NO release in response to ATP (**Figure 4-8**). This result suggests that regional heterogeneity of microglial activation is indeed influenced by the surrounding environment, and can be 're-influenced' when that environment changes. To further verify this hypothesis, the microglia that were incubated with ectopic CM for 24 hr were re-conditioned with CM from their region of origin for an additional 24 hr. In theory, if the regionality of microglial activation is more dynamic than hardwired, re-incubation with CM from the region of origin should restore the original phenotype. This was the case for Str microglia; when Str microglia were incubated ectopically with Hip CM then followed by Str CM, they did not increase NO release in response to ATP, resembling a Str phenotype (**Figure 4-8**). However, the same restoration of phenotype did not occur in Hip and Thl microglia. Neither had ATP-induced increase in NO after incubation with CM from their regions of origin and still possessed a Str microglia-like phenotype (**Figure 4-8**). This suggests that the regionality of microglial activation may only be dynamic and plastic to a certain degree.

The mechanism that underlies this conditioning phenomenon then comes into question. What are the specific signaling molecules involved in conditioning

microglia from one regional phenotype to another? In addition to purinergic receptors, microglia express receptors for various neurotransmitters, some of which are known to possess modulating functions on microglial activation (Pocock and Kettenmann, 2007). These neurotransmitters may act as environmental cues that condition microglia into distinct phenotypes. If this is indeed the case, the CM from mixed cultures of distinct regions should have region-specific profiles of neurotransmitters. Using HPLC, CM media from mixed cultures of different regions were measured for levels of various brain amino acids. Surprisingly, levels of glutamate (GLU) and γ-aminobutyric acid (GABA), both of which are known to modulate microglial activity (Pocock and Kettenmann, 2007), were not regionally different (**Figure 4-9**). There were also no differences across regions in levels of glutamine (GLN) and aspartate (ASP) (**Figure 4-9**). The levels of asparagine (ASN), L-serine (L-SER), D-serine (D-SER), glycine (GLY), taurine (TAU), and alanine (ALA) showed significant differences between regions (**Figure 4-9**), suggesting their potential involvement in conditioning microglial phenotypes. The amino acid TAU is of particular interest because it is found in lower concentrations in the Str CM compared to CM of other regions, which correlates with the previously described patterns of regionality. To determine the

precise role of TAU, microglia isolated from different regions were conditioned with 20 μM TAU (the highest level of TAU measured in the CM) for 24 hr prior to ATP stimulation. Based on the HPLC data, the low level of TAU in the Str CM may be responsible for the lack of increased NO release in ATP-stimulated microglia from Str. Indeed, when Str microglia were pre-conditioned with TAU, an increased NO release after ATP stimulation was observed (**Figure 4-10A**). TAU pre-conditioning of Hip and Thl microglia had no effects on their release of NO, whereas in Brs and Ctx microglia, TAU pre-conditioning actually attenuated ATP-induced NO release to a noticeable but not statistically significant level (**Figure 4-10A**). The effects of TAU pre-conditioning were also evident with respect to the overall toxicity induced by microglia: Using a similar setup as previously described, ATP-stimulated microglia were transferred to neurons that were exposed to hypoxia. As expected, when pre-conditioned with TAU, ATP-treated microglia from Str, which were not neurotoxic without TAU treatment, induced significantly more neuronal death (**Figure 4-10B**). Notably, TAU pre-conditioning had the same effect for Brs and Hip microglia despite the fact that TAU did not potentiate their release of NO (**Figure 4-10B**), suggesting that TAU may modulate other microglial effectors involved in neurotoxicity.

In addition to amino acids, several biogenic amines that function as neurotransmitters and neuromodulators also have effects on microglial activity. Microglia express receptors for amines such as dopamine and noradrenaline (Pocock and Kettenmann, 2007). However, HPLC measurement of CM from mixed cultures did not detect regional differences for these compounds (data not shown). Other biogenic amines and their metabolites such as 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic (HVA), and serotonin (5-HT), were measured as well, but also did not show significant regional differences (data not shown).

4.4 Discussion

Taken together, the data in this chapter demonstrate that microglia derived from different regions of the brain mount regionally distinct responses upon activation *in vitro*. Different populations of activated microglia were shown to induce different levels of neurotoxicity when co-cultured with injured neurons. The results also showed that these region-specific responses are not hardwired, but are plastic and can be altered by changing the surrounding neurochemical milieu.

It is well known that similar degrees of CNS insult to different regions of the brain often yield variable levels of damage. Similarly, classic CNS diseases such as Parkinson's Disease display regional vulnerability (Lee et al., 2009). Intrinsic differences between neurons of different regions play a large role in this selectivity. In addition to heterogeneity between neurons, regional differences between glial cells play as important a role, considering that glial cells often dictate the outcome of neuronal survival and disease progression. There have been in-depth studies on the regional heterogeneity of astrocytes, in particular the astrocytic glutamate transport (Schluter et al, 2002; Han et al., 2004). Similar studies on microglia have focused on regional differences under their resting/ramified state (Lawson et al., 1990; Elkabes et al., 1996; Wu et al., 1997; Ren et al., 1999; Schmid et al., 2002; Carson et al., 2007; de Haas et al., 2008), but not their responses after activation, nor how these region-specific microglial responses affect the survival of neurons. Using an *in vitro* system, the results from this chapter were able to address these questions. An apparent disadvantage of culture systems is the removal from their native environment in the brain. Growing in a serum-supplemented medium simulates a brain environment that

more closely resembles one with a disrupted blood-brain barrier (BBB) rather than an undisturbed state. Nonetheless, the mixed cultures by the time of microglial isolation still contain most of the CNS components, including neurons, astrocytes, oligodendrocytes, and endothelial cells (data not shown). It is safe to assume then that whatever signals microglia have received from other cell types while in their native tissue are still present even after culture. This would explain why in a similar study by Ren et al. (1999), despite being in culture for more than two weeks, microglia still maintained their regionality. Another limitation of this culture model is the fact that the cultures are derived from neonatal brains. A major difference between neonatal and adult brains is the incompleteness of myelination and lack of white matter in the neonatal CNS. This would then exclude the microglial population that resides along white matter tracts, which some have suggested to have a specialized phenotype (Hanisch and Kettenmann, 2007). Even when isolated in culture, there are results demonstrating that microglia derived from adult and neonatal brains respond differently to activating agents (Floden and Combs, 2006). One should therefore use caution in extending the results here toward different age groups. Regardless, the regionality of microglial activation is likely a universal phenomenon, and one would only expect

microglial populations to grow more distinct from one another with increasing age, as they receive increasing exposure from their respective milieus. One would expect, however, that regionality among adult microglia is less dynamic and more hardwired than that observed in this study.

Aside from the limitations, the culture model is advantageous in that microglia can be activated alone, and their influence on neuronal survival can be readily attributed as microglia-specific effects. This study showed that ATP-activated microglia from Ctx and Hip were more neurotoxic, and that this toxicity, at least in the case of Ctx microglia, likely involves the overproduction of NO and TNF-α. The finding that NO and TNF-α inhibition in Hip, Str, and Thl microglia exacerbated neuronal death (**Figure 4-6B**) is unexpected. This result suggests that NO and TNF-α may be either neurotoxic or neuroprotective depending on microglias' region of origin. An explanation for this seemingly irreconcilable finding is that the NO and TNF-α released by microglia may function in concert with other effectors, which are likely produced in a region-specific manner themselves. The possibility exists that NO and TNF-α yield an overall protective effect in combination with certain effectors, yet produce toxic effects in the

presence of another set of effectors.

A key question raised in this study concerns the origin of microglial regionality, which was addressed by the pre-conditioning experiments. The results showed that 1) the surrounding neurochemical environment plays an important role, that 2) the establishment of microglial heterogeneity is dynamic and reversible, where altering the neurochemical environment for 24 hr was sufficient to make microglia from one brain region 'behave' like those from a different region, and that 3) the neurochemical milieu in the Str contain signals more potent in conditioning microglia compared to signals from other regions. This may explain why after incubation with Str CM, Hip and Thl microglia were not able to be conditioned back to their original phenotype. The regional profile of the amino acids measured by HPLC implicates the involvement of several molecules in the establishment of microgilal regionality. TAU was shown to play a role, but it is likely that GLY and D-SER are involved as well considering their known modulatory activity on glutamate receptors (which are expressed by microglia) (Wolosker, 2007). Although no regional differences were found in glutamate, GABA, or other classical neurotransmitters such as dopamine and noredrenaline,

all of which are known to modulate microglial activity (Pocock and Kettenmann, 2007), it still remains a possibility that they contribute to the regionality of microglia since these neurotransmitters could have been taken up or degraded by the time they were measured. Future investigations are needed to better understand the role(s) each neurochemical has in influencing the microglial phenotype.

In conclusion, data from this chapter provide novel evidence that microglia respond to activators in a region-specific manner, and that this regional heterogeneity originates from differences in the surrounding chemical environment. This is also the first study to show a relationship between taurine and microglia, which, as the results suggest, is involved in the establishment of microglial regionality. The regionality of activated microglia provides yet another explanation for the regional selectivity/vulnerability of CNS disorders. Whereas most studies on microglia, as well the previous two results chapters, have focused on neuronal input, this chapter has demonstrated the importance of intrinsic differences among microglia themselves. Most importantly, the findings here demonstrate the dynamic nature of microglia, which is an important factor to

consider when conducting experiments on these cells that have the potential to readily alter their phenotypes upon changes in the surroundings.

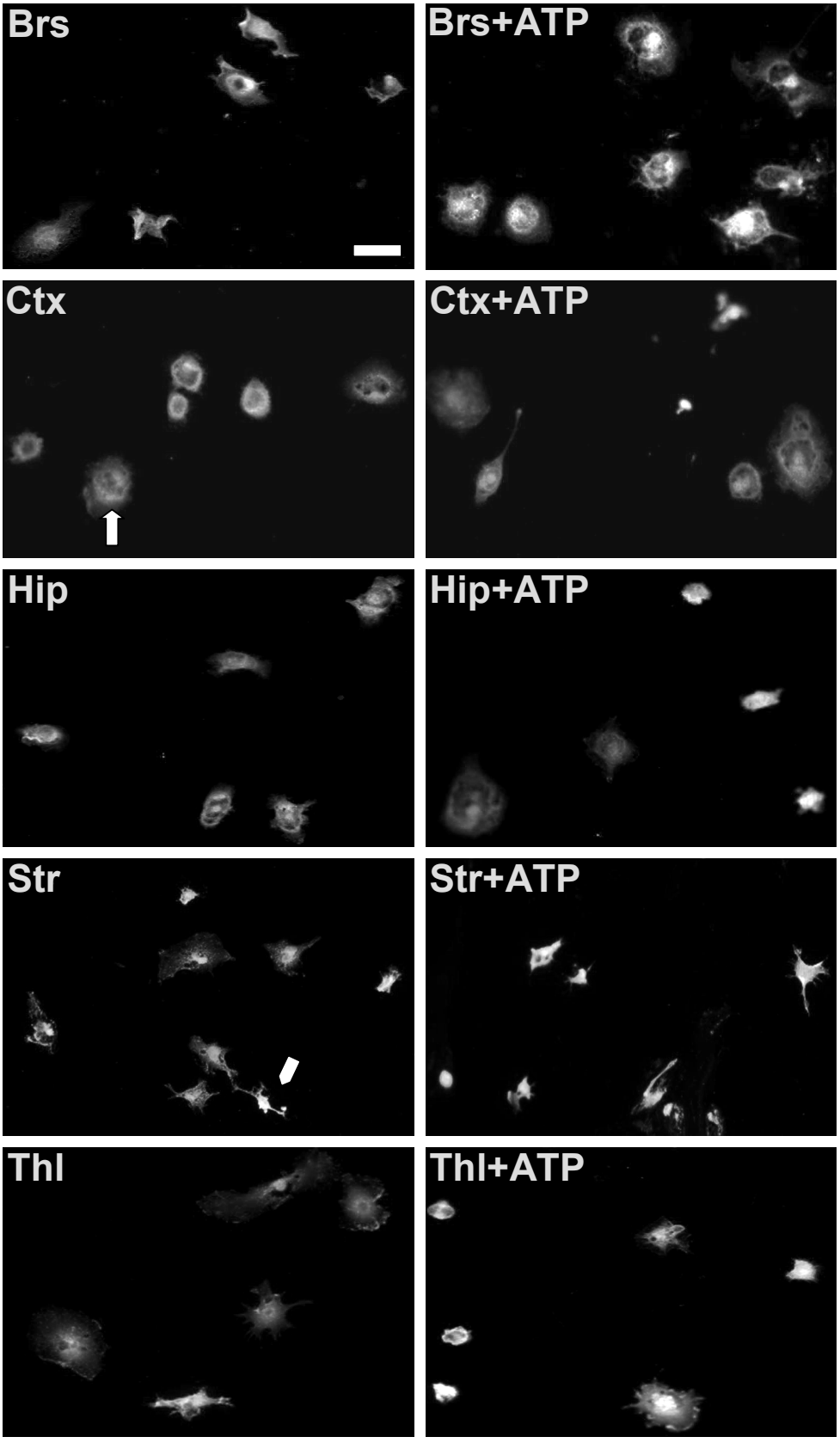
Brs
Brs+ATP
Ctx
Ctx+ATP
Hip
Hip+ATP
Str
Str+ATP
Thl
Thl+ATP

Figure 4-1 – Representative micrographs of untreated and ATP-stimulated primary microglia derived from Brs, Ctx, Hip, Str, and Thl. Microglia from different regions show distinct morphological features. Total magnification = 200X. Scale bar = 20 μm. Arrow points to an example of amoeboid microglia. Chevron points to an example of ramified microglia with extensive processes.

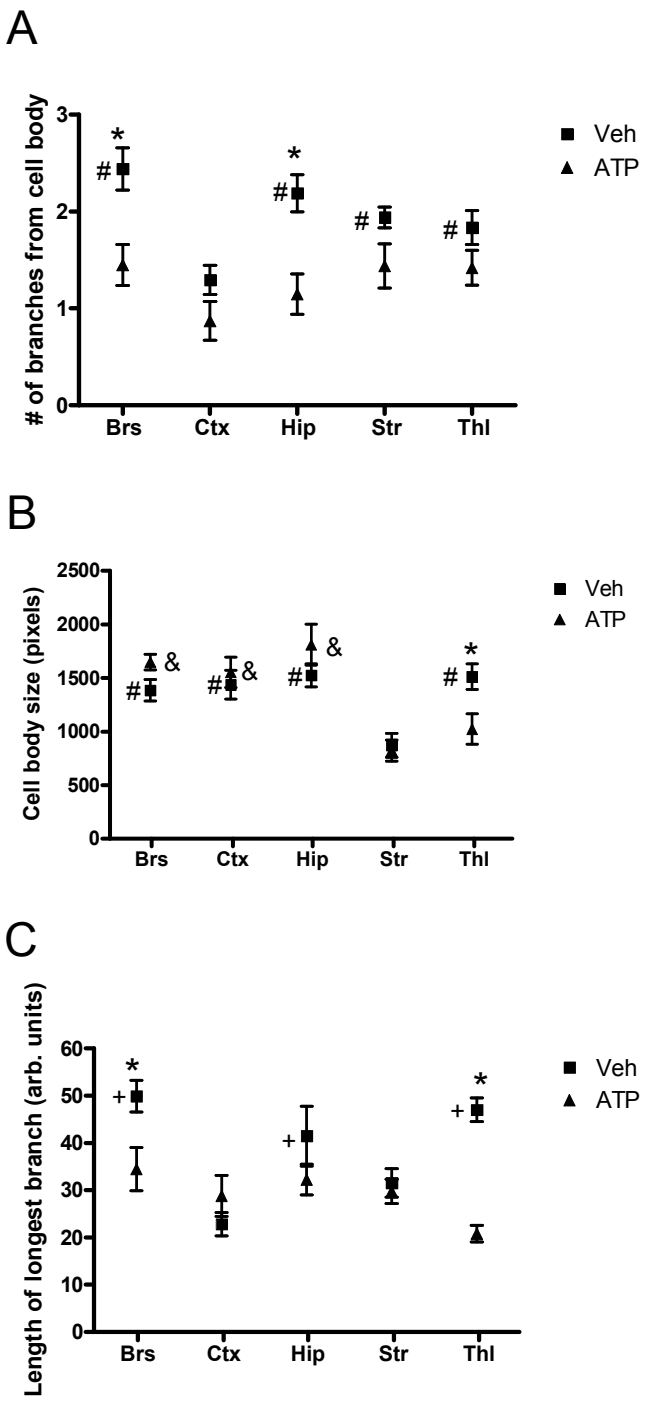

Figure 4-2 – Quantitative morphological analysis of Iba1-labeled microglia from various regions. Microglia have region-specific differences in various morphological features such as **A.** Total number of branches from the cell body, **B.** size of the cell body, and **C.** length of the longest branch of a cell. * denotes significance between the non-treated and ATP-treated groups in each region. #

denotes significance within the non-treated groups (relative to Ctx for **A**, to Str for **B**, to Ctx for **C**). + denotes significance within the non-treated groups relative to both Ctx and Str (for **C**). & denotes significance within the ATP-treated groups relative to both Str and Thl (for **B**). n = 8 from two pooled experiments. Error bars represent standard error for all figures.

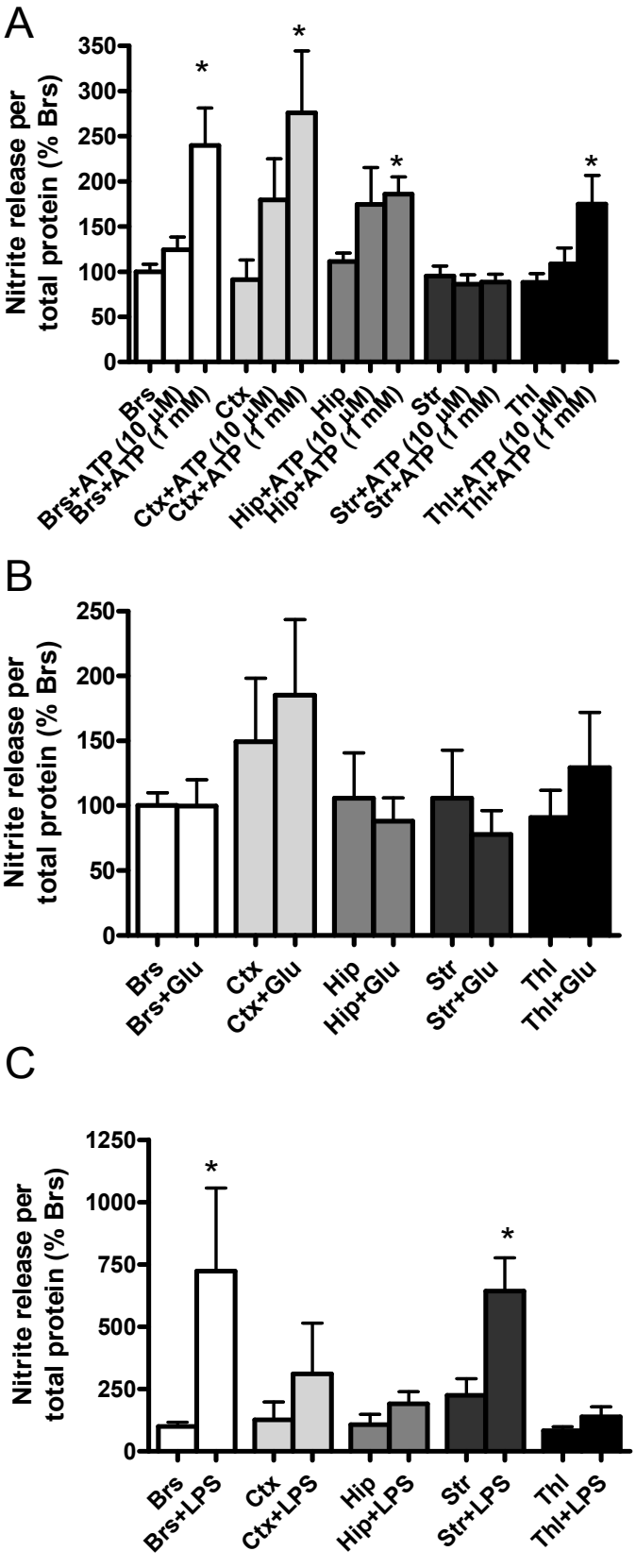
A
Nitrite release per total protein (% Brs)
350
300
250
200
150
100
50
0
*
*
*
*
Brs
Brs+ATP (10 µM)
Brs+ATP (1 mM)
Ctx
Ctx+ATP (10 µM)
Ctx+ATP (1 mM)
Hip
Hip+ATP (10 µM)
Hip+ATP (1 mM)
Str
Str+ATP (10 µM)
Str+ATP (1 mM)
Thl
Thl+ATP (10 µM)
Thl+ATP (1 mM)
B
Nitrite release per total protein (% Brs)
250
200
150
100
50
0
Brs
Brs+Glu
Ctx
Ctx+Glu
Hip
Hip+Glu
Str
Str+Glu
Thl
Thl+Glu
C
Nitrite release per total protein (% Brs)
1250
1000
750
500
250
0
*
*
Brs
Brs+LPS
Ctx
Ctx+LPS
Hip
Hip+LPS
Str
Str+LPS
Thl
Thl+LPS

Figure 4-3 – Activator-induced release of NO from microglia is region-dependent and activator dependent. A. ATP-stimulated NO release (n = 12 from five pooled experiments). **B.** Glutamate-stimulated NO release (n = 8 from three pooled experiments). **C.** LPS-stimulated NO release (n = 5 from two pooled experiments). * denotes significance relative to the untreated condition in each region.

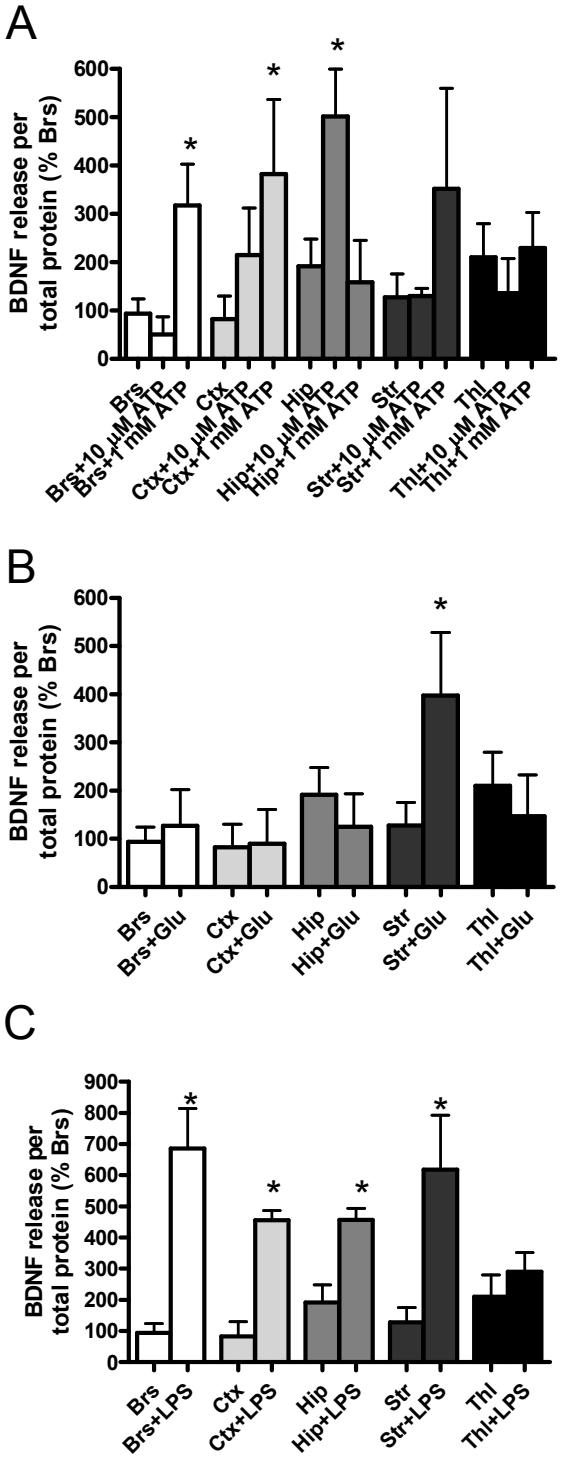

Figure 4-4 – Region-dependent and activator-dependent induction of BDNF release from microglia. A. ATP-stimulated release of BDNF (n = 5 from two pooled experiments). **B.** Glutamate-stimulated release of BDNF (n = 5 from two pooled experiments). **C.** LPS-stimulated release of BDNF (n = 4 from two pooled

experiments). * denotes significance relative to the untreated condition in each region.

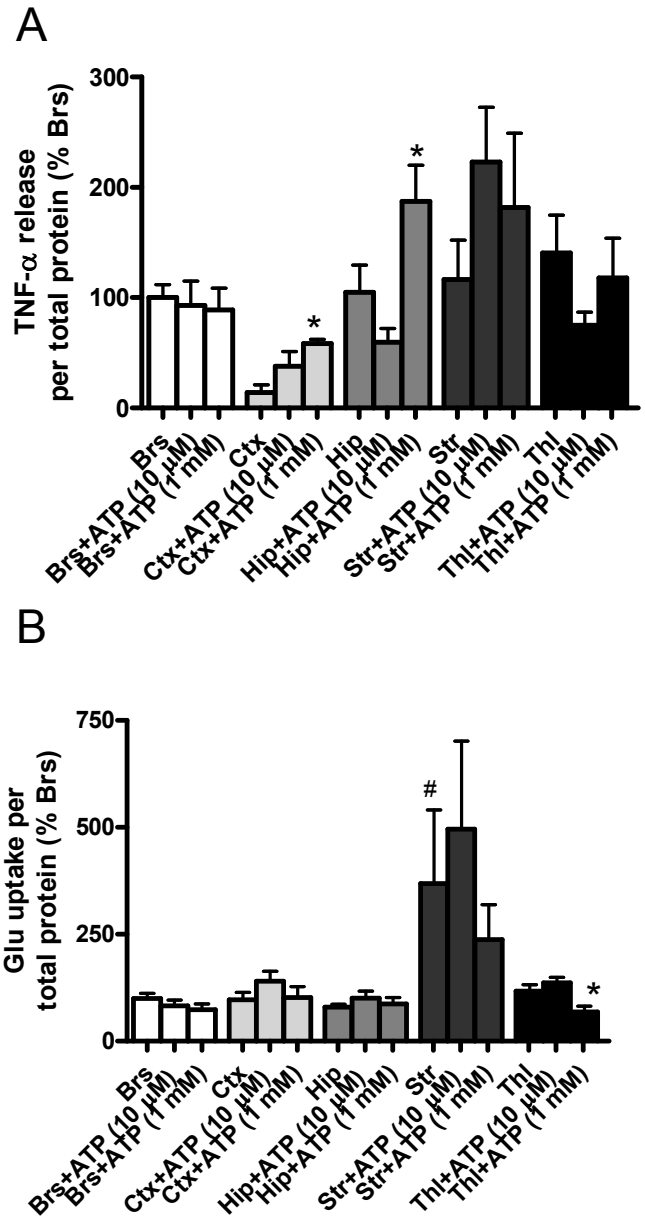

Figure 4-5 – Region-specific regulation of microglial TNF-α and glutamate uptake. A. TNF-α release (n = 12 from six pooled experiments) and **B.** glutamate uptake (n = 6 from two pooled experiments) by ATP. * denotes significance relative to the untreated condition in each region. # denotes significance relative to Hip.

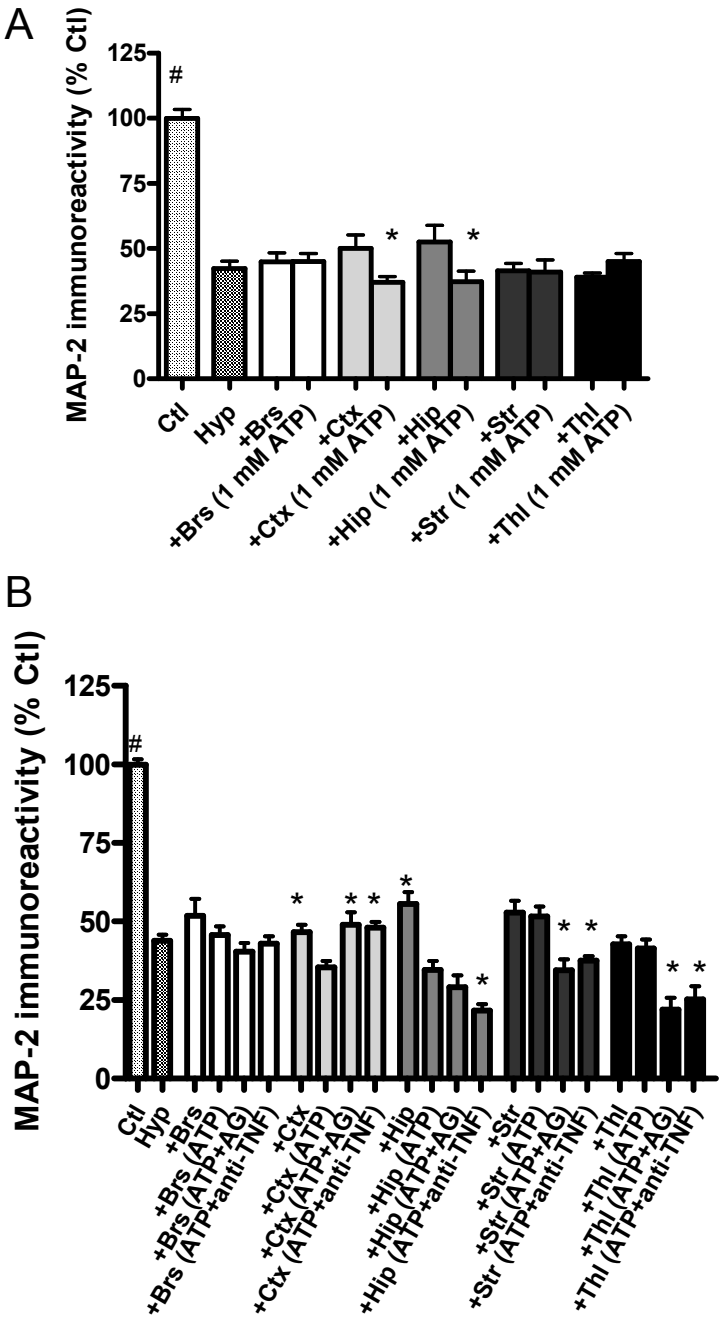

Figure 4-6 – Neurotoxicity induced by microglia derived from different regions. A. ATP-activated microglia induce region-dependent neurotoxicity when co-cultured with hypoxia-injured neurons (n = 18 from three pooled experiments). **B.** Region specific effects of aminoguanidine and anti-TNF-α (n = 12 from three pooled experiments). * denotes significance relative to the untreated condition in each region for **A**, and relative to the ATP condition for **B**.

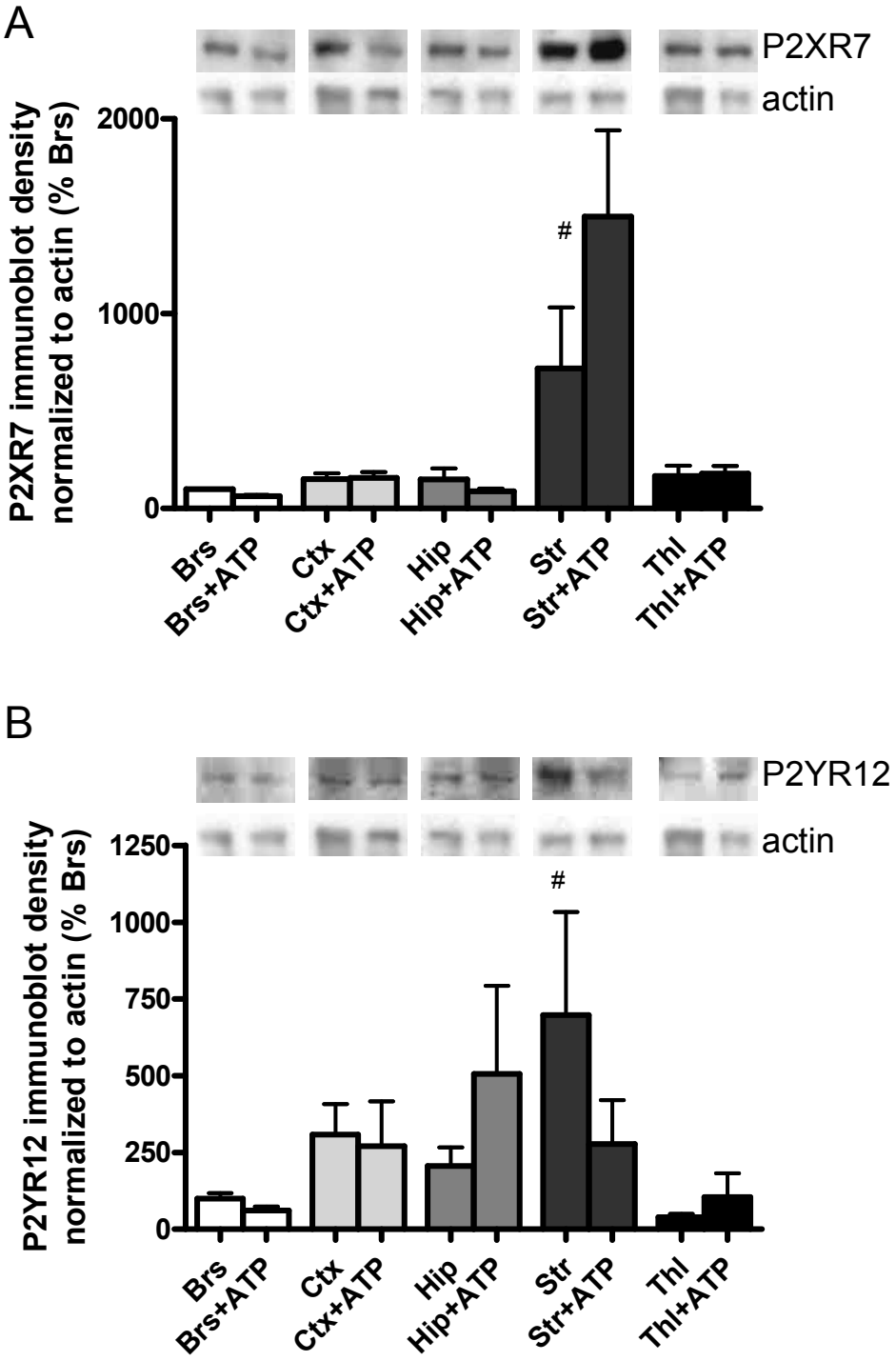

Figure 4-7 – Striatal microglia express higher levels of P2X7 and P2Y12 receptors compared to microglia from other regions. A. P2X7 receptor immunoblot (n = 5 from five pooled experiments). **B.** P2Y12 receptor immunoblot (n = 4 from four pooled experiments). # denotes significance relative

to Brs for **A** and to Thl for **B.**

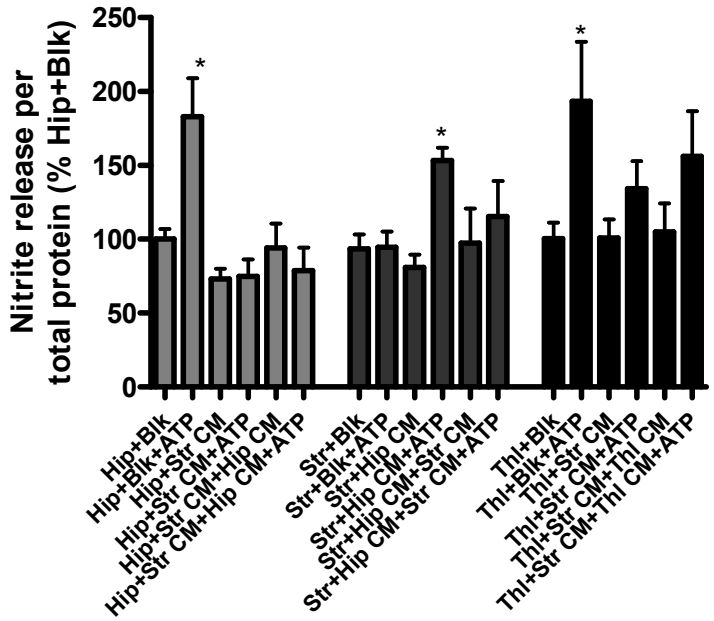

Figure 4-8 – Ectopic regional conditioning of microglia. Release of NO by hippocampal, striatal, and thalamic microglia after treatment of conditioned media from an ectopic region, and also after re-conditioning with media from their original regions. n = 5 from two pooled experiments. * denotes significance relative to the untreated condition of each conditioning paradigm.

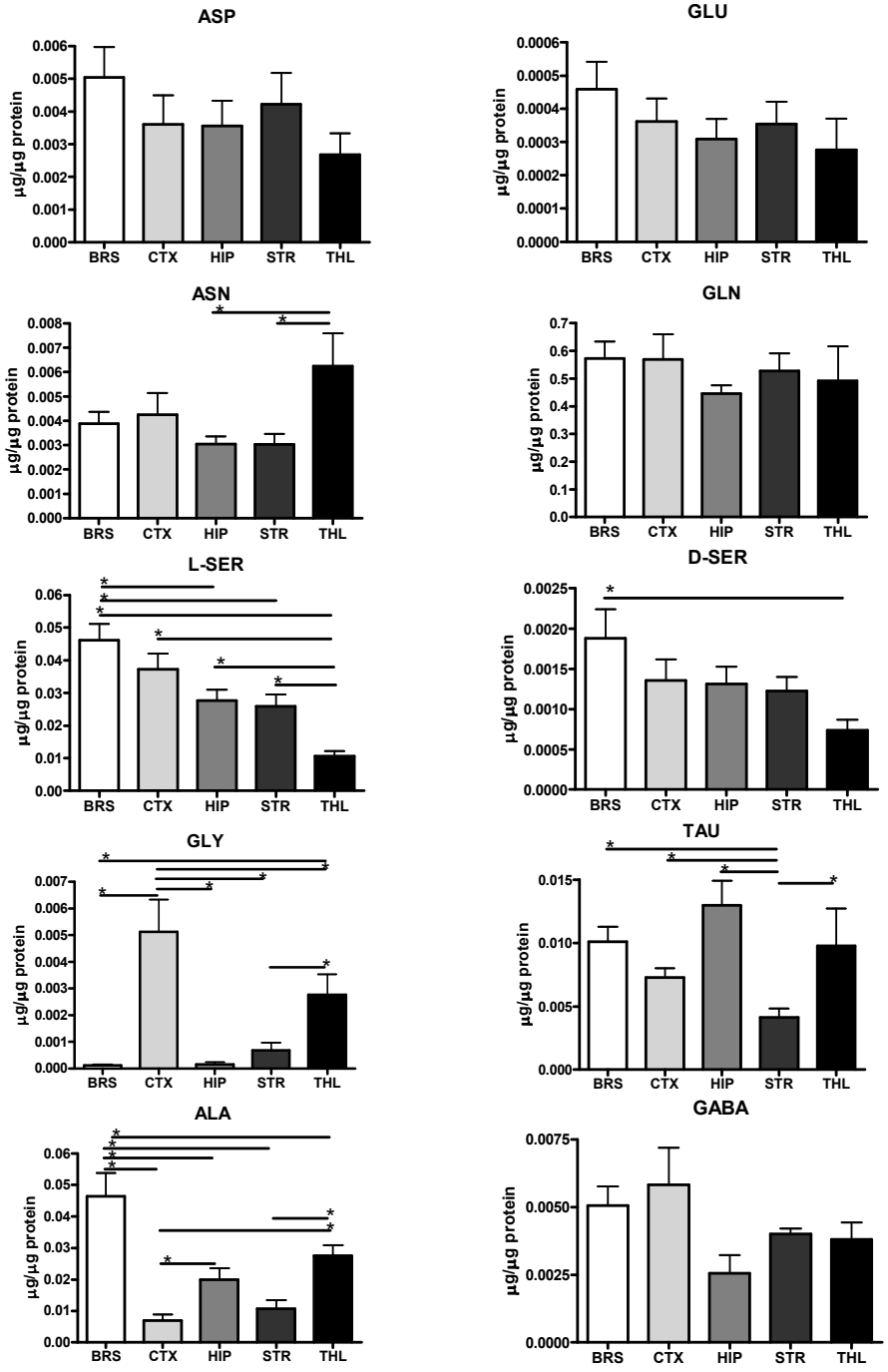

Figure 4-9 – Levels of amino acids present in the media from the mixed cultures that microglia are derived from. Each region shows a distinct profile of amino acids. n = 6 from two pooled experiments.

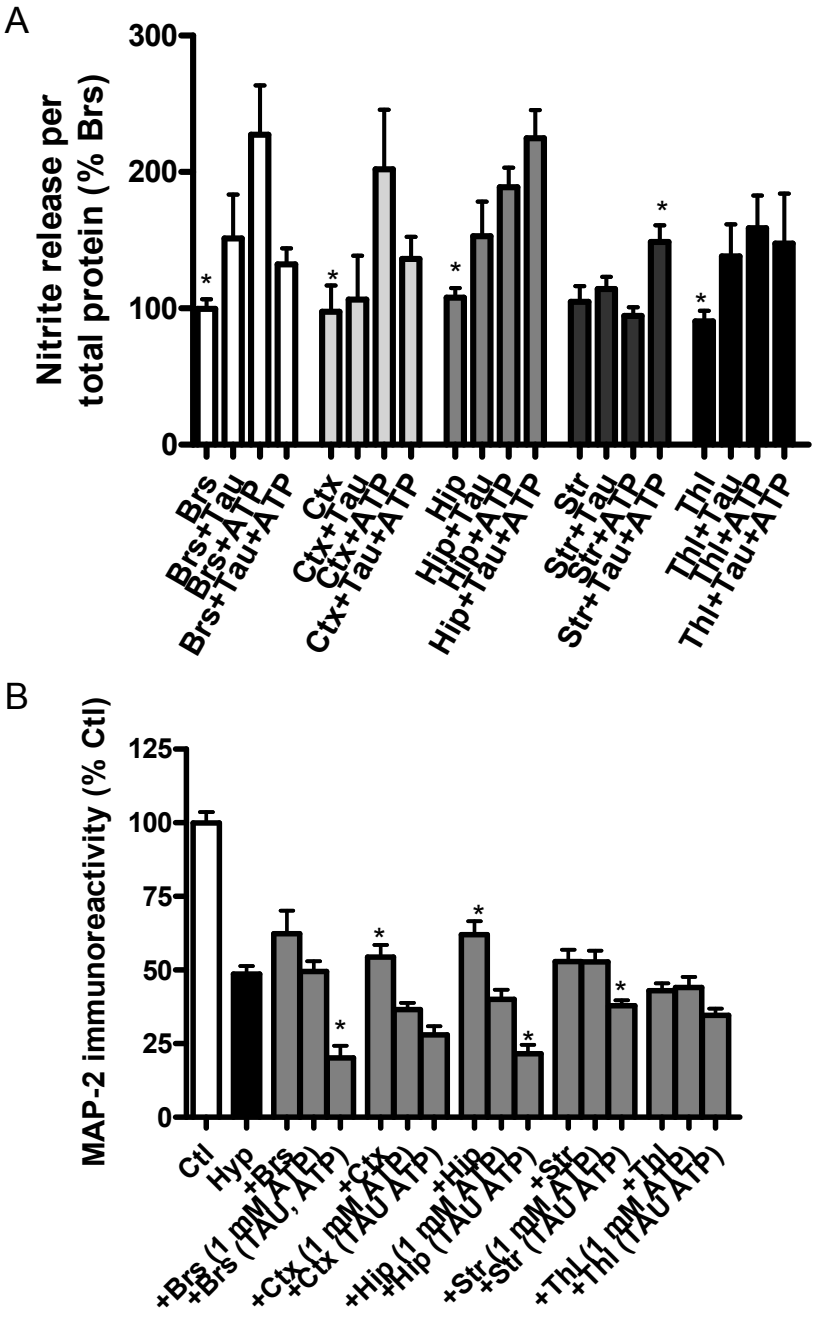

Figure 4-10 – Effects of taurine conditioning. A. Effect of taurine pre-conditioning on the ATP-induced release of NO by microglia of various regions (n = 6 from three pooled experiments). **B.** Effect of taurine pre-conditioning on the ATP-induced neurotoxicity of microglia (n = 8 from three pooled experiments). * denotes significance relative to the ATP-treated group in each region.

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CHAPTER 5

Age as a determinant of microglial activation phenotype

5.1 Introduction

The previous chapter focused on the rarely discussed issue of intrinsic differences among microglial populations. Specifically, regional variations were found to be a critical factor in determining the phenotype of activated microglia. One of the limitations mentioned in the chapter was the issue of using neonatal microglia as the experimental model. Throughout this thesis and in most *in vitro* studies on microglia, primary neonatal microglial cultures were used due to difficulties in obtaining a high yield of cells from adult animals. As using whole brain cultures overlooks regional variations, studying cultures derived from one developmental age in turn overlooks age-related changes. It has long been known that microglial morphology changes with age (Perry et al., 1993). Immunological markers such as ED-1 and major histocompatibility complex (MHC) II, which are not normally expressed in young ramified microglia, also show increased expression with age (Perry et al., 1993). There has been a wealth of literature and theories surrounding the issue of aging in microglia. Streit and colleagues have long advocated that microglia become senescent with age and lose some of their functions (Miller and Streit, 2007). They have also shown that this senescence may be caused by telomere shortening from continuous proliferation to replenish the parenchymal

microglial population (Flanary and Streit, 2003; Flanary and Streit, 2004). Another hypothesis proposes a similar mechanism, where microglial organelles that have important effector functions, such as lysosomes and mitochondria, undergo dysfunctional changes in the aging process (Nakanishi and Wu, 2009). Decline of lysosomal functions not only impedes phagocytic activity but also prevents autophagy-dependent turnover of organelles including mitochondria, leading to mitochondrial dysfunction, generation of reactive oxygen species, and modification of the overall microglial phenotype (Nakanishi and Wu, 2009).

In addition to aging of microglia themselves, the aging of the brain as a whole changes the neurochemical milieu of the brain, which, as shown in the previous chapter, may affect microglial phenotype. Major structural changes such as loss of brain volume and synapses take place even after middle age (Peters, 2006). Several neurotransmitters including dopamine, serotonin, and brain-derived neurotrophic factor (BDNF) show decreased levels with aging, while monoamine oxidase, an enzyme involved in neurotransmitter metabolism, increases with age (Peters, 2006). Reports have also have shown that nutritional supplements of the amino acid taurine (TAU) alleviate age-related cognitive loss, suggesting that

TAU levels also drop with age (El Idrissi et al., 2009). Although there is no evidence that these neurochemical changes directly contribute to phenotypic changes in microglia, age-related changes in microglial phenotype even long before a senescent age have been observed. In systemic inflammation, where microglia receive activating and priming signals from the circulation, two distinct immunological profiles are expressed by microglia in young versus middle-aged rats (Wu et al., 2008). *In vitro*, cultures of neonatal and adult microglia also show differential ability to phagocytose amyloid aggregates (Floden and Combs, 2006). This suggests that age differences in microglia, similar to regional differences, are resistant to culturing. Studying age-related changes in culture has several advantages. As mentioned in the previous chapter, an *in vitro* system allows one to assess the functional significance of the effectors produced by microglia. It also allows for manipulations of the neurochemical milieu that the microglia are exposed to, which may elucidate how their age-specific phenotypes have evolved. Based on the current literature on microglial aging, an apparent missing link is a study that investigates differences across developmental ages, as opposed to differences between a senescent and non-senescent age. Considering the widespread use of neonatal microglial cultures, gaining a better understanding of

phenotypic variations between activated microglia of neonatal, young, and old ages are of particular significance. The aim of this chapter, then, is to determine the phenotypic and functional variations in cultured microglia derived from different age groups. Six different age groups of rats were investigated: Embryonic (Em) day 18, neonatal (Neo) one day old, 2-3 months old, 6-8 months old, 9-11 months old, and 13-15 months old. Microglia from all age groups were stimulated with the activating agents adenosine 5'-triphosphate (ATP) and lipopolysaccharide (LPS). The microglial effector profiles after activation, as well as how the effectors influence neuronal survival, were assessed. In addition, the mechanisms that underlie the development of age-specific differences were also investigated. Finally, whether or not changes in brain chemistry and neurotransmitters play a role was determined.

5.2 Materials and Methods

5.2.1 Primary cortical neuronal cultures

Neuronal cultures were prepared from cortices of embryonic day 18 Sprague-Dawley rats based on methods described in previous reports (Brewer et al., 1993; Lai and Todd, 2006). For detailed descriptions of the methods, refer to

Section 2.2.1.

5.2.2 Primary microglial cultures

Embryonic and neonatal microglia were first isolated as mixed brain cultures, which were prepared from brains of embryonic day 18 and postnatal day 1 Sprague-Dawley rats respectively. For detailed descriptions of culture dissociation methods, refer to **Section 2.2.2**. After 18-24 days *in vitro* (DIV), microglial cells were isolated from these mixed cultures using a previously reported trypsin-based method (Saura et al., 2003). For detailed description of the methods refer to **Section 3.2.2**.

For obtaining adult microglia, a Percoll (Sigma)-based method adapted from Cardona et al. (2006) was used. In brief, brains of adult Sprague-Dawley rats of various ages were isolated and mechanically chopped to small pieces of approximately 1 mm length while on ice. The chopped pieces were then enzymatically digested by 0.25 % trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco) at 37 ℃ for 15 minutes (min). After gentle mechanical titurations in Dulbecco's modified Eagle's media with Ham's F12 (DMEM/F12) and 10% fetal

bovine serum (FBS), the cell suspension was centrifuged to a pellet and resuspended in 37% Percoll (diluted in DMEM/F12). This Percoll fraction with cells was then carefully laid on top of a 70% Percoll layer. Afterwards, a 30% layer followed by a 0% (DMEM/F12 only) layer was sequentially laid on top of the 37% layer. The tube with a 70%-37%-30%-0% gradient was centrifuged at 500 x g for 45 min at 18 ℃. After centrifugation, the interface between the 70% and the 37% layer, which contained microglia, was collected using a Pasteur pipet. The collected portion was diluted five times in DMEM/F12, mixed well, then centrifuged at 3000 x g to remove leftover Percoll. The remaining pellet was resuspended in DMEM/F12 with 10% FBS and plated at a density of 1/4 plates per brain. This yields a microglial density comparative to that in embryonic and neonatal microglial cultures. After one DIV, the cultures were switched to a serum-free DMEM/F12, and were used for experiments after another DIV. The cultures were verified to be >95% microglia using the microglia-specific marker ionized calcium-binding adaptor protein 1 (Iba1) and the astrocyte marker glial fibrillary acidic protein (GFAP).

5.2.3 Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma. Concentrations used for aminoguanidine (AG; 300 μM), ATP (1 mM), Brilliant Blue G (BBG, 1 μM), Reactive Blue 2 (RB2, 30 μM), TAU (20 μM), and LPS (1 μg/mL) were based on previous *in vitro* studies as well as data from previous chapters (Lockhart et al., 1998; Nakajima et al., 2001; Inoue, 2002; Suzuki et al., 2004; Koizumi et al., 2007).

5.2.4 Immunocytochemistry

Anti- microtubule-associated protein-2 (MAP-2, 1:500), GFAP (1:1000), and Iba1 (1:1000) were purchased from Sigma, Dako, and Wako respectively. Fluorescence-conjugated secondary antibodies (1:500) were obtained from Jackson ImmunoResearch. Immunocytochemistry was performed using methods described in Lai and Todd (2006). For detailed methods refer to **Section 2.2.5**. Detailed methods for morphological analysis were described in **Section 4.2.4**.

5.2.5 *In vitro* hypoxia and measurement of neuronal survival

Neuronal injury was induced by exposing neuronal cultures to hypoxia (< 1%

oxygen) for two hours (h, hr), incubating the cultures in an oxygen-sensitive chamber (PROOX model 110, BioSpherix). Following hypoxia, the cultures were re-oxygenated at atmospheric oxygen level for 24 hr before they were assayed. To measure the neurotoxicity in mixed cultures of neurons and microglia, MAP-2, a neuron-specific marker, was used to distinguish neurons from microglia. Refer to **Section 3.2.5** for detailed description of the methods.

5.2.6 Nitric oxide assay

Nitric oxide (NO) in the media was assessed indirectly via its stable metabolite nitrite. Refer to **Section 2.2.7** for detailed description of the methods.

5.2.7 Protein assay

Total protein concentrations were used for normalization of measured effectors. The bicinchoninic acid (BCA) protein assay was used to determine protein concentrations. Refer to **Section 2.2.9** for detailed methods.

5.2.8 Enzyme-linked immunosorbent assay (ELISA)

A DuoSet ELISA kit for tumor necrosis factor (TNF)-α was purchased from R&D

Systems. The TNF-α ELISA was carried out according to manufacturer protocols. For detailed methods refer to **Section 2.2.8**. The ELISA for BDNF was carried out using an indirect ELISA method as described in **Section 3.2.8**.

5.2.9 Glutamate uptake assay

Glutamate uptake assays were adapted from a previous report (Persson et al., 2005). Refer to **Section 2.2.9** for detailed methods.

5.2.10 Immunoblotting

The following antibodies were used: Rabbit anti-P2XR7 (1:200, Chemicon), goat anti-P2YR12 (1:200, Santa Cruz), rabbit anti-P2XR4 (1:200, Chemicon), rabbit anti-P2YR2 (1:200, Chemicon), and mouse anti-actin (1:5,000, Sigma). Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) were obtained from Santa Cruz. For detailed description of the blotting methods, refer to **Section 2.2.12**. Densitometric analysis of the protein bands were carried out using ImageJ (NIH).

5.2.11 Statistical analysis

Significance levels were calculated using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. In cases where the groupings were unbalanced, or in cases where only one independent variable was present, one-way ANOVA followed by a Newman-Keuls test *post hoc* was used instead of the two-way. One-way ANOVA was used for the following data sets: The MAP-2 immunoreactivity data, the P2 receptor antagonists data, and data for the conditioning experiments. For all of the figures, the denotations *, #, +, and & represent $p < 0.05$. Each n represents a single well of a culture plate.

5.3 Results

5.3.1 Age-specific variations in morphology

Morphological features of microglia are often useful markers for their phenotypic differences. As previously mentioned, age-specific differences in microglial morphology have been observed both *in vivo* and *in vitro* (Perry et al., 1993; Floden and Combs, 2006; Sierra et al., 2007). Here, six age groups of microglia ranging from embryonic to old were examined. Microglia were labeled with the cell-specific marker Iba1, which outlined their morphological features in detail. As shown in **Figure 5-1**, even in the absence of an activating agent, microglia of

different age groups showed noticeable differences in appearance. Specifically, adult microglia had a more ramified morphology with thicker and more extensive processes (chevron) compared to Em and Neo microglia. Em microglia were the most amoeboid (triangle), which is not surprising considering that microglia are known to exhibit this morphology during brain development (del Rio-Hortega, 1932; Hanisch and Kettenmann, 2007). Interestingly, microglia cultured from old rats of 13-15 months (mo) did not exhibit extensive processes and showed a semi-amoeboid morphology similar to that of Neo microglia. In addition, 13-15 mo microglial cultures contained several cells that had broken processes (**Figure 5-1** arrow), which may be the senescent microglia similar to those mentioned by Streit and colleagues (Miller and Streit, 2007). If treated with 1 mM ATP for 24 h, 13-15 mo microglia were also distinct morphologically from other age groups. ATP induced retraction of processes for microglia of most ages, but in 13-15 mo cultures, ATP actually stimulated a modest amount of process extension.

To assess the morphological differences in a more objective manner, three morphological features including the number of branches, cell body size, and length of longest branch, were analyzed quantitatively. Overall, the quantitative

data corroborate previously described trends. With regard to branch number, with the exception of 13-15 mo microglia, the adult ages had more branches compared to Em and Neo microglia in the absence of ATP (**Figure 5-2A**). Even with ATP treatment, adult microglia still had more branches in general (**Figure 5-2A**). The cell body size showed a similar trend in that the size increased progressively with age, reaching peak size at 9-11 mo (**Figure 5-2B**). However, with ATP treatment, 13-15 mo microglia had the largest cell body size of all ages (**Figure 5-2B**). With regard to branch length, adult microglia had longer branches than Neo microglia, with 13-15 mo again being the exception (**Figure 5-2C**). In the presence of ATP stimulation, morphological changes from ATP treatment were the most evident in 9-11 mo microglia, whereas in Em, Neo, and 6-8 mo cultures, ATP did not induce changes in the examined morphological features (**Figure 5-2**).

5.3.2 Age differences in pro-inflammatory effectors

Although microglia of different ages exhibited clear differences in morphology, the functional significance of these observed differences was unclear. The next set of experiments thus investigated whether age differences also exist in the production of microglial effectors. In response to 24 h treatment of 1 mM ATP,

nitric oxide (NO) release was increased in Neo microglia (**Figure 5-3A**). The same increase was, however, not observed in microglia of other ages (**Figure 5-3A**). To determine whether a different microglial activator would yield the same trend, microglia were also stimulated with 1 μg/mL LPS for the same duration. In response to LPS, NO release was again upregulated in Neo microglia, but unlike ATP stimulation, LPS increased NO release in adult microglia as well (**Figure 5-3B**), suggesting that age-specific differences are activator-dependent. Em microglia did not increase NO production in response to either ATP or LPS, although, interestingly, their basal production of NO in culture was higher than other age groups (**Figures 5-3A**, **5-3B**).

Another pro-inflammatory effector, tumor necrosis factor-α (TNF-α), was also examined as a comparator to NO. Unlike NO, the basal level of released TNF-α increased with age, peaking at 9-11 mo (**Figure 5-3C, 5-3D**). The response to ATP was also different from that of NO, such that ATP increased TNF-α release in EM, Neo, and 13-15 mo microglia, with 13-15 mo microglia having the largest upregulation (**Figure 5-3C**). This correlates with the trend observed in the morphological analysis, where Em, Neo, and 13-15 mo microglia showed the

most amoeboid morphology. In response to LPS, TNF-α release did not show age specificity as LPS was able to upregulate TNF-α in all age groups (**Figure 5-3D**), corroborating the NO results that age differences in microglial activation is activator-specific.

5.3.3 Age differences in neuroprotective effectors

Since LPS appeared to have little selectivity for age, the remaining experiments focused on the ATP-induced responses. To determine whether the trophic effectors of microglia also have age-specific regulatory patterns, glutamate uptake and BDNF release in response to ATP were measured. Unlike pro-inflammatory effectors, ATP increased glutamate uptake in young adult microglia that were 2-3 mo old (**Figure 5-4A**). Similar increases were observed also in Neo and 13-15 mo microglia; however, in EM microglia, ATP actually downregulated glutamate uptake (**Figure 5-4A**). The basal level of glutamate uptake showed a similar trend to that of TNF-α such that it increased with age, peaking at 6-8 mo (**Figure 5-4A**). In the case of BDNF, basal levels were also different between the age groups, and were increased with increasing age, peaking also at 6-8 mo (**Figure 5-4B**). However, in the ATP-treated conditions, only Neo microglia and no other age

group upregulated BDNF release, showing a trend similar to that of NO. Overall, the neuroprotective effectors of microglia exhibited age-specificity in a pattern similar to that observed with the pro-inflammatory effectors, albeit with slight variations.

5.3.4 Age differences in the expression of purinergic receptors

The observation that age-specific responses of microglia depend on the activating agent is quite intruiging. There are several possible explanations that might justify why LPS was able to upregulate NO and TNF-α in most ages of microglia, while ATP could not. One possibility is that secondary messengers of LPS signaling are expressed ubiquitously while those of ATP signaling are expressed differentially. The more likely explanation though is the differential expression of ATP receptors themselves in the different age groups. As mentioned in previous chapters, microglia express several subtypes of type 2 purinergic (P2) receptors for ATP, and data from previous chapters have demonstrated that each subtype is responsible for different components of microglial activation. The age-specific responses to ATP could be related to differential expression of P2 receptors. To determine if this was the case, expressions of the P2X7, P2Y12, P2X4, and P2Y2

receptors in untreated and ATP-treated microglia from different ages were determined by immunoblotting. Basal expressions of P2XR4 were similar between the age groups (**Figures 5-5A, 5-5D**). In contrast, both P2XR7 and P2YR12 expressions increased with age, both peaking at 6-8 mo, with P2YR12 decreasing its expression significantly at 9-11 mo (**Figures 5-5A, 5-5B, 5-5C**). P2YR2 had its own expression profile, having the highest level in Em microglia (**Figure 5-5A, 5-5E**). Notably, ATP treatment did not affect the expression of the receptors in most age groups except in Neo microglia, where both P2YR12 and P2YR2 expression were increased in response to ATP (**Figure 5-5**). Collectively, the expression profile of the P2 receptors correlated closely to the effector profiles, such that adult and Neo microglia express different subtypes of P2 receptors and the expression profile of 13-15 mo old microglia is more similar to that of Em and Neo microglia than to that of adult microglia of other ages.

5.3.5 Age differences in regulation of neuronal survival

Based on the effector profiles alone, it is clear that phenotypic differences exist between activated microglia of different ages. However, it remains inconclusive whether these activated microglia of different ages are also functionally distinct.

To address this question, the effects of microglial activity on neuronal survival were measured. Neuronal cultures were exposed to 2 hr hypoxic injury followed by 24 hr re-oxygenation. This procedure induced 50-55% neuronal death. Immediately after hypoxia, ATP-treated microglial cultures of various age groups were manually detached and plated with the neurons to assess their regulation on neuronal survival. Addition of untreated Neo microglia, but not microglia of other age groups, significantly improved the survival of injured neurons (**Figure 5-6A**). Pre-treatment of Neo microglia with ATP yielded the opposite effect, inducing significant neurotoxicity (**Figure 5-6A**). ATP had no effects on microglia of other age groups except on 2-3 mo old microglia, which, when pre-treated with ATP, promoted neuroprotection (**Figure 5-6A**). This is not surprising since 2-3 mo old microglia demonstrated increased glutamate uptake in response to ATP without increasing the production of pro-inflammatory mediators. Unlike 2-3 mo old microglia, Neo microglia increased both NO and TNF-α after ATP stimulation, which may account for the observed neurotoxicity. To verify this hypothesis, 300 μM aminoguanidine (AG), an inhibitor of inducible nitric oxide synthase (iNOS), was added to the co-cultures immediately after hypoxia. While AG had no effects on neuronal survival in other age groups, it significantly reduced the neurotoxicity

induced by ATP-treated Neo microglia (**Figure 5-6A**), suggesting the involvement of NO in the observed neurotoxicity.

Taking into consideration the aforementioned complexity of the P2 receptor system, it was also of interest to determine if different subtypes of P2 receptors mediate the neurotoxicity/neuroprotection induced by microglia of different age groups. 1 μM Brilliant Blue G (BBG), a P2XR7-specific antagonist, and 30 μM Reactive Blue 2 (RB2), a non-specific P2YR antagonist, were co-treated with ATP on microglia 24 hr prior to co-culturing with neurons. The neurotoxicity induced by ATP-treated Neo microglia was negated by both BBG and RB2 (**Figure 5-6B**), suggesting the involvement of both P2X and P2Y receptors. On the other hand, neuroprotective effects mediated by ATP-treated 2-3 mo old microglia were not affected by either antagonist (**Figure 5-6B**). Interestingly, at 9-11 mo and 13-15 mo, where ATP-treated microglia had no effect on neuronal survival, co-treatment with either antagonist induced significant neuroprotective effects; for 9-11 mo microglia, only RB2 induced neuroprotection, while both BBG and RB2 induced neuroprotection from 13-15 mo microglia (**Figure 5-6B**). This suggests two possibilities: One explanation is that the antagonists not only blocked signaling

from added ATP, but the basal autocrine purinergic signals as well, leading to reduced basal production of neurotoxic effectors such as NO; the other possibility is that blocking a subset of P2 receptors triggered compensatory mechanisms from other P2 subtypes that were not blocked. The compensatory hyper-activation of those P2 receptors then induced upregulation of neuroprotective effectors. To determine which of these speculations was true, NO, TNF-α, glutamate uptake, and BDNF were measured in 9-11 mo old and 13-15 mo old microglia that had been co-treated with ATP and one of BBG and RB2. Neither antagonist had effects on levels of NO, TNF-α, and BDNF (data not shown). However, co-treatment of RB2 and ATP significantly amplified glutamate uptake in both 9-11 mo old and 13-15 mo old microglia (**Figure 5-7**), suggesting that increased glutamate uptake could explain the observed neuroprotective effects. Co-treatment with BBG, on the other hand, did not have effects on glutamate uptake (**Figure 5-7**), so its neuroprotective mechanisms are dependent upon other factors that have yet to be examined.

5.3.6 Plasticity of age differences in activated microglia

The data thus far suggest that Neo microglia have the lowest threshold for

activation by ATP, and as microglial age increases, they also become increasingly difficult to stimulate until they reach the age of 13-15 mo. A question that has not been addressed is how this age-specific activation threshold originated. One possibility is that the biochemistry of microglia themselves alter as they age. The other possibility involves the conditioning of microglia by different neurochemical milieu that results from aging of the brain as a whole. If the latter is true, then exposing microglia of a given age to a neurochemical environment of another age would alter their age-specific phenotype. To test this possibility, conditioned media (CM) from Neo mixed cultures were isolated. This CM theoretically contained the elements that make up the neurochemical milieu to which Neo microglia were exposed. Neo CM were then incubated with 9-11 mo old microglia for 24 hr just after they were isolated. If age-specific phenotypes were sensitive to changes in the surrounding environment, then Neo CM should condition the 9-11 mo old microglia into a Neo phenotype. This was, however, not the case. While Neo microglia conditioned with Neo CM increased NO release in response to ATP, 9-11 mo microglia conditioned with Neo CM did not (**Figure 5-8A**). It is possible that age-specific phenotypes are relatively hardwired and require stronger conditioning signals for them to alter. The amino acid TAU is a

potential candidate signal. The concentration of TAU in the brain alters as a function of age (El Idrissi et al., 2009). TAU was also shown in the previous chapter to condition the region-specific phenotypes, rendering microglial populations that were previously unresponsive to ATP to become ATP-sensitive. Microglia derived from 2-3 mo old and 9-11 mo old rats were conditioned with 20 μM TAU for 24 hr prior to ATP treatment. Neither age, however, increased NO release upon ATP stimulation even with TAU conditioning (**Figure 5-8B**). This suggests that age-specific phenotypes are either insensitive to environmental changes or resistant to short-term conditioning.

5.4 Discussion

Experiments in this chapter set out to investigate the age-specific differences in the activation of microglia. Using ATP as the activating agent, the results collectively showed that both basal and activated phenotypes of microglia vary as a function of age. While the basal production of microglial effectors generally increases with age, ATP-stimulated production of the effectors is higher in Em and Neo microglia than adults. This pattern correlates to that of both morphological features and their overall effects on neuronal survival. Additionally, it was found

that among adult age groups, microglia from the oldest group of 13-15 mo old rats showed more effector activation than microglia from the younger groups of adults. Finally, the conditioning experiments showed that age-specific differences in microglial activation have low plasticity and were resistant to short-term changes in the neurochemical milieu.

The results are in agreement with the study by Floden and Combs (2006), which also observed that adult microglia in culture are less reactive than Neo microglia. Although several reports have compared age-specific differences of microglia, this is the first study that takes Em microglia into account. It is worth noting a potential issue in the experimental design, that the 13-15 mo old microglia used in this study may not be old senescent microglia, since some strains of rats live to more than two years of age. However, this particular rat strain has a high incidence of renal failures after 17 mo of age (Keenan et al., 2000; Ling et al., 2009). To ensure the health of the animals, they were not aged beyond 15 mo. This may explain why the 13-15 mo old microglia, which are considered old microglia in this study, did not contain a high count of dystrophic- and senescent-looking microglia. Nonetheless, a few microglia at 13-15 mo did appear

senescent with broken processes (**Figure 5-1** arrow). Perhaps the most interesting finding of this study is that 13-15 mo old microglia are surprisingly more similar to Neo microglia than adult microglia of other ages both morphologically and phenotypically. Apart from the similarities, one discriminating feature between the two ages is the lack of NO upregulation in 13-15 mo old microglia, and this likely explains why ATP-activated 13-15 mo old microglia are not neurotoxic whereas Neo microglia are. Since LPS can induce NO upregulation in all adult age groups, the lack of NO increase in response to ATP is not due to an absence of the iNOS protein. The differences between 13-15 mo old and Neo microglia are more likely a result of differential expression and 'wiring' of signaling substrates in the ATP signaling system. There is circumstantial evidence in this study that supports this hypothesis. In the immunoblots of P2 receptors, it was observed that Neo microglia in response to ATP upregulate the expression of both P2YR2 and P2YR12 (**Figure 5-5**). Although this type of receptor 'rearrangement' in response to changes in ligand availability was not observed in the blots of other age groups, the antagonist experiments showed evidence of a similar phenomenon. When the P2 receptor antagonist RB2 was co-administered with ATP, glutamate uptake of both 9-11 mo old microglia and 13-15 mo old microglia was upregulated to a

level greater than that activated by ATP alone (**Figure 5-7**). Since ATP did not induce changes in P2 receptor expression for these two age groups, receptor rearrangement is likely not the case here. However, it is possible that rearrangement of downstream signaling substrates had occurred. Results from previous chapters showed that different P2 receptor subtypes regulate different mitogen-activated protein (MAP) kinases, so the differential activation of MAP kinases might play a role here. The observation that P2XR7 and P2YR12 expressions increase with age may explain why activation threshold also increases with age, but perhaps the more critical factor is how the purinergic receptors are coupled to downstream messengers. If the expression of P2 receptors can be experimentally manipulated, their role in the determination of age-specific microglial responses will be more clear. However, primary microglial cells are difficult to transfect, and genetic means may be required.

Another intriguing finding in this study is the lack of plasticity of age-specific phenotypes. **Chapter 4** showed that region-specific phenotypes of microglia can be readily altered and conditioned. The fact that age-specific phenotypes do not exhibit the same level of plasticity suggests that age-specific phenotypes originate

from more intrinsic mechanisms. There may be transcripts within microglia that alter as they age, and yet are insensitive to weaker neurochemical signals in the surrounding environment. In the region study, Neo microglia were used. As shown in this chapter, Neo microglia have a lower activation threshold for ATP compared to adult microglia; they may thus also have a lower threshold for conditioning. It is possible that adult microglia are too committed down the developmental path to have any degree of phenotypic plasticity.

In conclusion, the results in this chapter provide novel evidence supporting the notion that phenotypic and functional differences exist between activated microglia of each developmental age. This is the first study to show that phenotypic differences exist even between Em and Neo microglia. This study is also the first to show a striking similarity between the activated phenotypes of Neo microglia and old microglia. Additionally, the results suggest that age-specific phenotypes of microglia are relatively hardwired, which is in sharp contrast to the significant amount of plasticity in the region-specific phenotypes. Most importantly, the study complements the findings of the previous chapter and adds another intrinsic element involved in the overall determination of microglial

activation type.

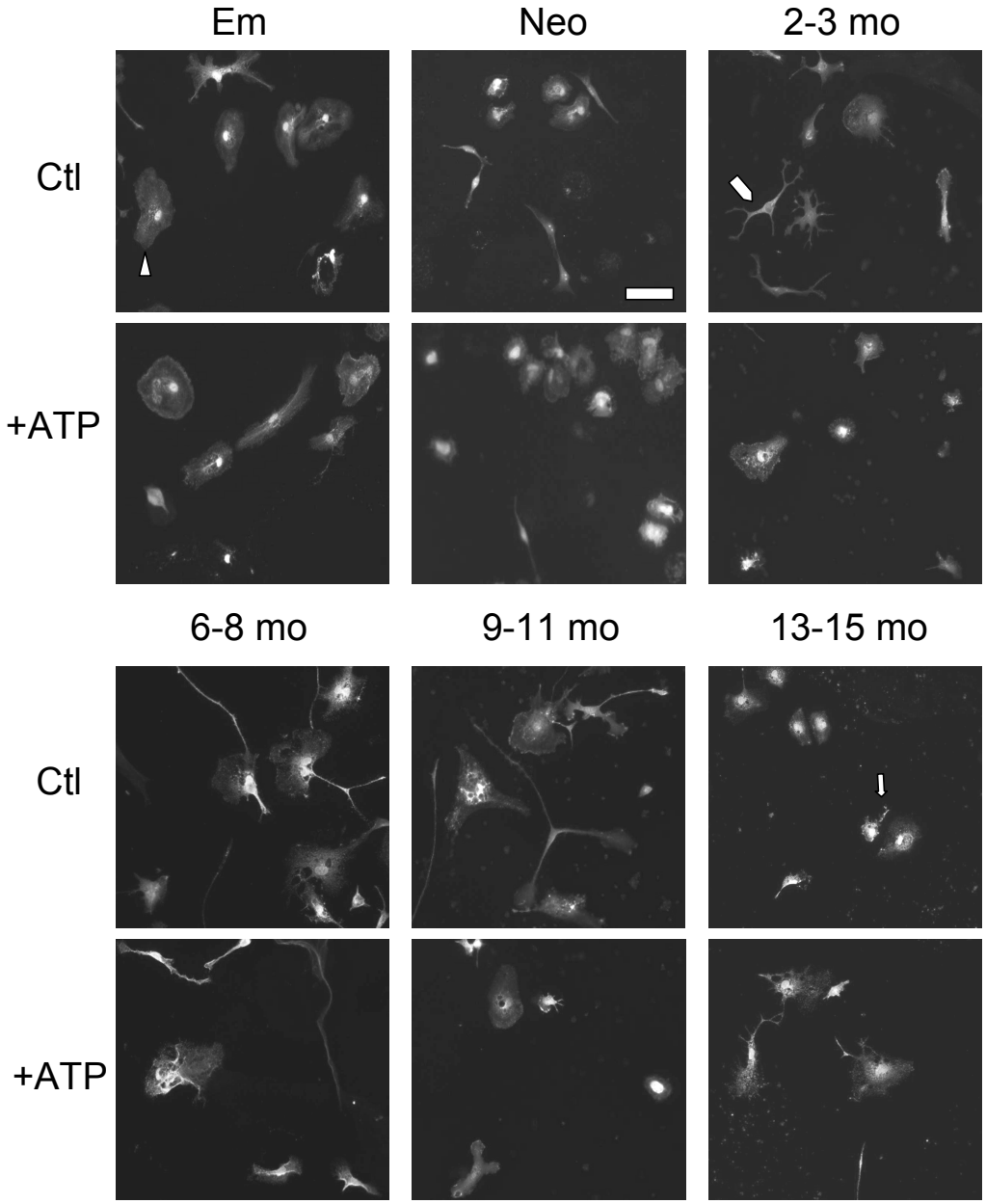

Figure 5-1 – Representative micrographs of untreated and ATP-stimulated microglia derived from various age groups. Total magnifcation = 200X. Scale bar = 20 μm. Arrow points to microglia with broken processes. Chevron points to microglia with a ramified morphology. Triangle points to microglia with an amoeboid morphology.

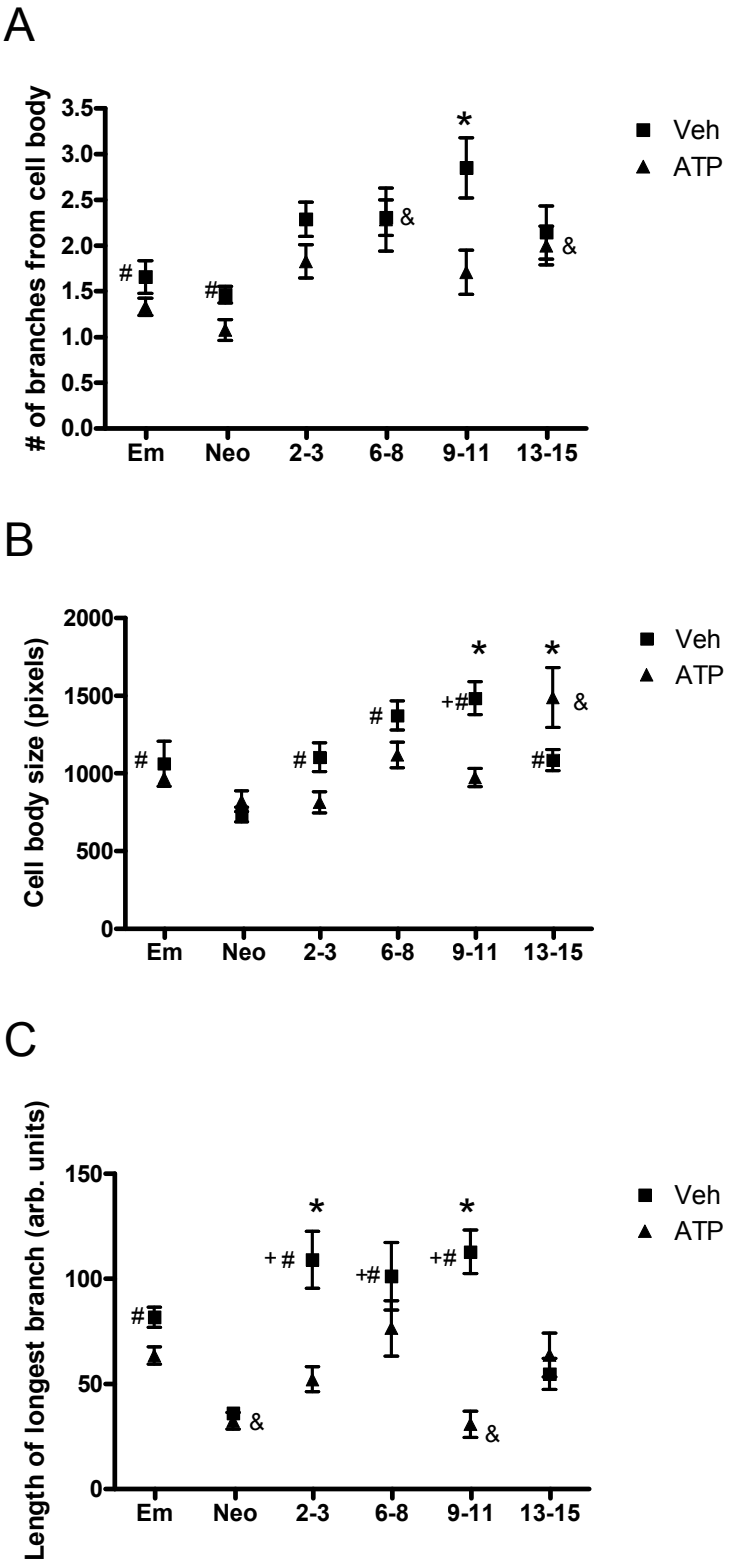

Figure 5-2 – Quantitative morphological analysis of untreated and

ATP-treated microglia derived from different ages. Three morphological features are analyzed: **A.** Total number of branches stemming from the cell body (n = 8 from two pooled experiments). **B.** Size of the cell body (n = 8 from two pooled experiments). **C.** Length of the longest branch (n = 8 from two pooled experiments). For all three parameters, * denotes significance of ATP treatment within one age group. # and + denote significance between the different untreated age groups. For **A**, # is relative to 9-11. For **B**, # is relative to Neo; + is relative to Em, 2-3, and 13-15. For **C**, # is relative to Neo; + is relative to a 13-15. & denotes significance between the different ATP-treated groups. For **A**, & is relative to Em and Neo. For **B**, & is relative to Em, Neo, 2-3, 6-8, and 9-11. For **C**, & is relative to Em, 6-8, and 13-15. Error bars represent standard errors for all figures.

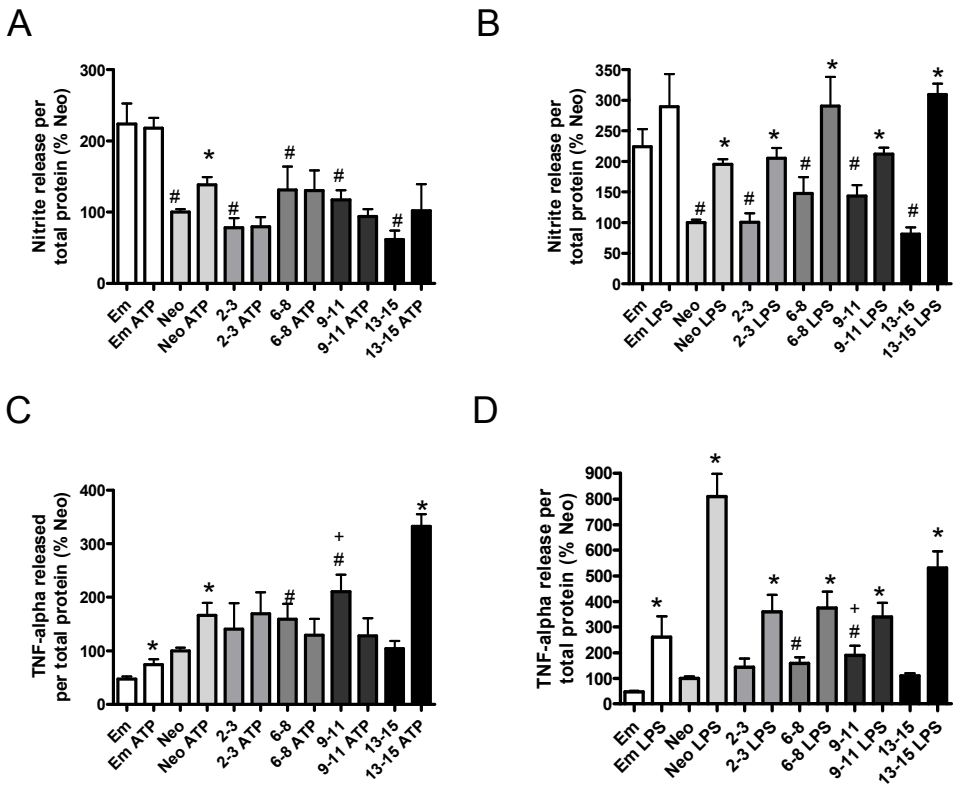

Figure 5-3 – Release of pro-inflammatory mediators by ATP- and LPS-treated microglia derived from different ages. A. ATP-stimulated NO release (n = 6 from three pooled experiments). # denotes significance relative to Em. **B.** LPS-stimulated NO release (n = 6 from two pooled experiments). # denotes significance relative to Em. **C.** ATP-stimulated TNF-α release (n = 6 from three pooled experiments). # denotes significance relative to Em. + denotes significance relative to Neo. **D.** LPS-stimulated TNF-α release (n = 6 from two pooled experiments). # denotes significance relative to Em. + denotes significance relative to Neo. For all of **A**, **B**, **C**, and **D**, * denotes significance of ATP or LPS treatment relative to the untreated control in the same age group.

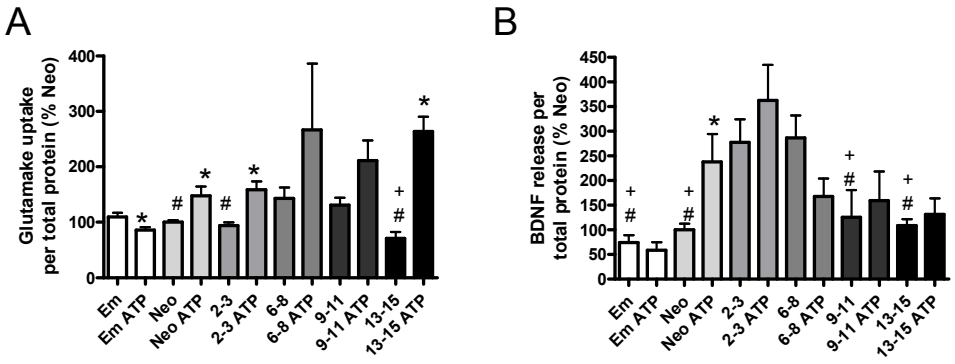

Figure 5-4 – Production of neuroprotective effectors by ATP-stimulated microglia of different ages. A. Glutamate uptake (n = 6 from three pooled experiments). # denotes significance relative to 6-8. + denotes significance relative to 9-11. **B.** BDNF release (n = 6 from three pooled experiments). # denotes significance relative to 2-3. + denotes significance relative to 6-8. For both **A** and **B**, * denotes significance of the ATP treatment relative to the untreated control in the same age group.

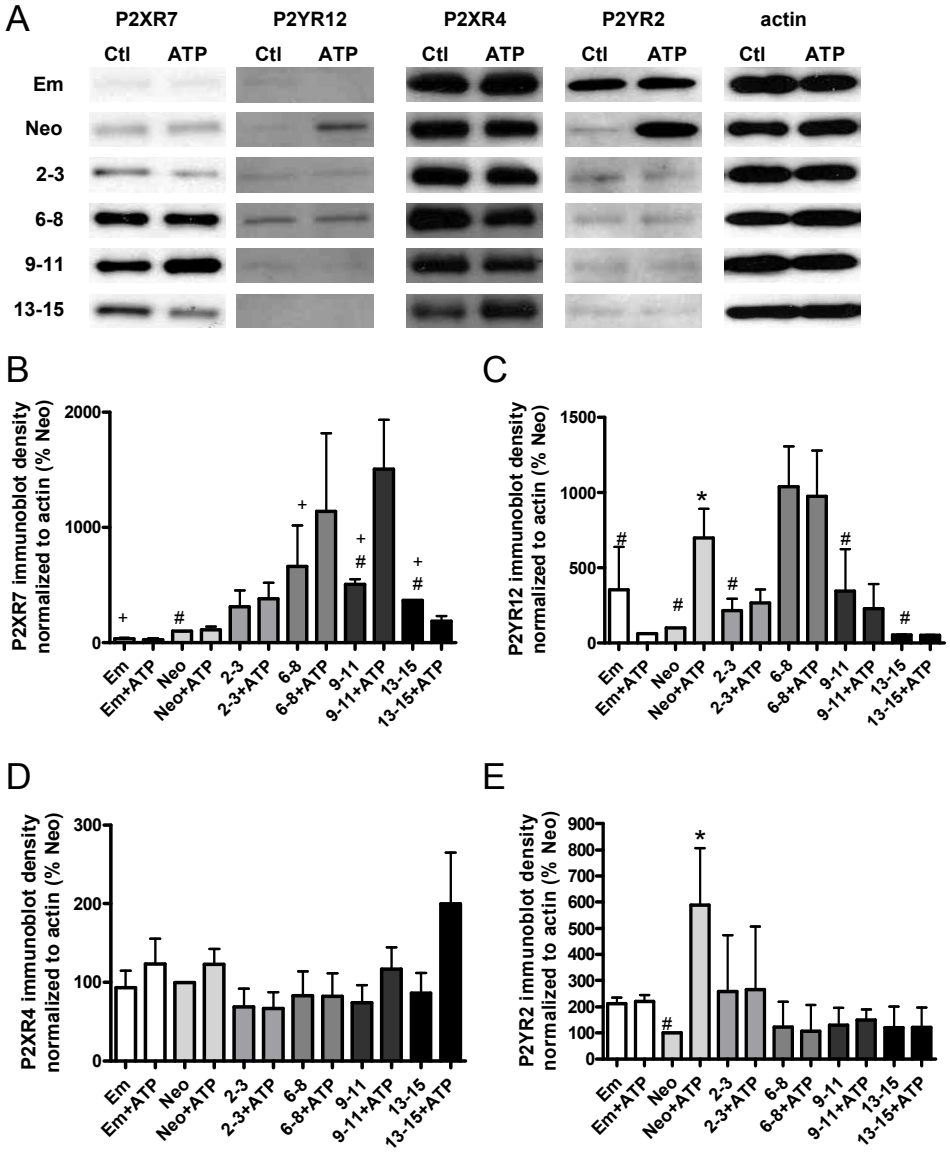

Figure 5-5 – Expression of purinergic receptors on microglia derived from different ages. A. Representive immunoblots of the P2X7, P2Y12, P2X4, and P2Y2 receptors as well as actin which is the loading control. **B.** Blot quantification of P2X7 receptor expression (n = 3 pooled from three experiments). # denotes significance relative to Em. + denotes significance relative to Neo. **C.** Blot quantification of P2Y12 receptor expression (n = 3 pooled from three experiments). # denotes significance relative to 6-8. **D.** Blot quantification of P2X4 receptor expression (n = 3 pooled from three experiments). **E.** Blot quantification of P2Y2 receptor expression (n = 3 pooled from three experiments). # denotes significance relative to Em. For all of **B**, **C**, **D**, and **E**, * denotes

significance of ATP treatment relative to the untreated control in the same age group.

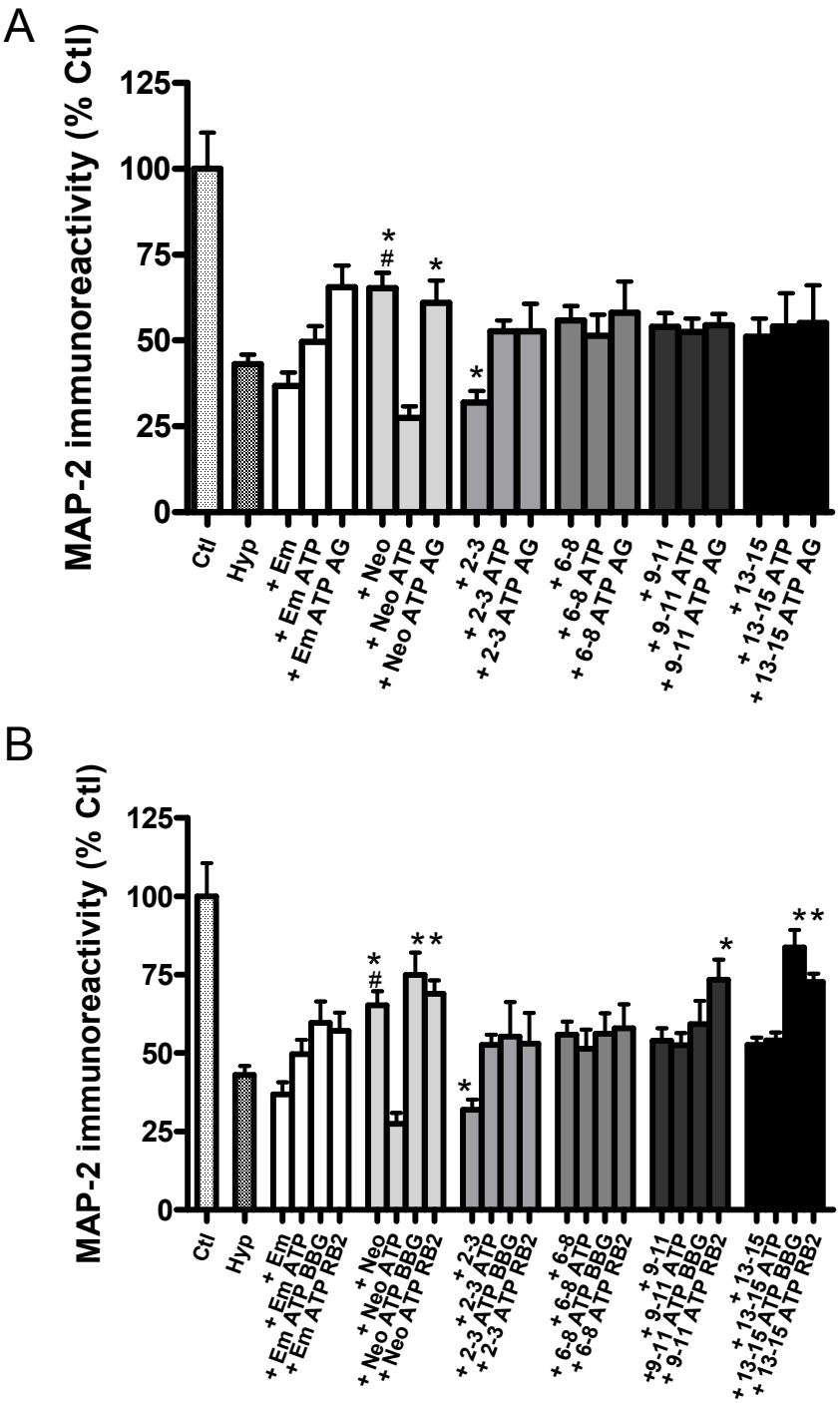

Figure 5-6 – Post-hypoxia neuronal survival measured by MAP-2 immuonreactivity is regulated by ATP-treated microglia in an age-dependent manner. A. Effects of the NOS2 inhibitor aminoguanidine (AG) on microglia-dependent regulation of neuronal survival. **B.** Effects of the P2X7 receptor antagonist Brilliant Blue G (BBG) and the non-selective P2Y receptor

antagonist Reactive Blue 2 (RB2) on microglia-dependent regulation of neuronal survival. For both **A** and **B**, * denotes significance relative to the ATP-treated condition of each age group. # denotes significance relative to Hyp. Results in **A** and **B** are from the same two experiments (pooled to n = 6).

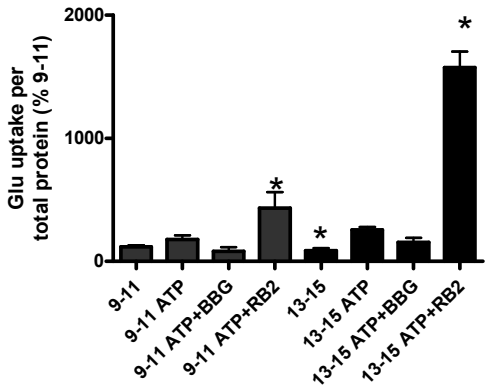

Figure 5-7 – Possible involvement of glutamate uptake in the microglia-dependent neuroprotection induced by P2 receptor antagonists. Glutamate uptake of 9-11 and 13-15 month old microglia is measured in response to ATP and P2 receptor antagonist treatments (n = 6 from two pooled experiments). * denotes significance relative to the ATP-treated condition in each age group.

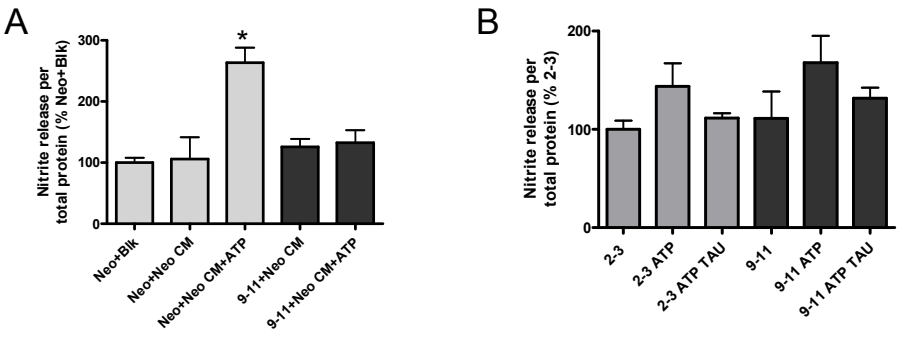

Figure 5-8 – Conditioning experiments to determine the plasticity of age-specific phenotypes in microglia. A. NO release from neonatal and 9-11 month old microglia pre-conditioned with CM from neonatal mixed cultures (n = 6 from two pooled experiments). **B.** NO release from 2-3 month old and 9-11 month old microglia pre-conditioned with TAU (n = 6 from two pooled experiments). * denotes significance relative to Neo+Blk.

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CHAPTER 6

General discussion

6.1 Summary of findings

The introduction of this thesis described microglial activation as having a multifaceted nature. There is a significant level of uncertainty as to how this multifaceted activation is regulated. Although several mechanisms have already been identified, there are other factors that could potentially have a role. The main aim of this thesis was to define and characterize unidentified factors that cause the differential activation of microglia. Using an *in vitro* approach, each of the four experimental chapters identified a distinct novel mechanism that controls microglial activation type. **Chapter 2** and **Chapter 3** focused on the role of neuronal signals. In **Chapter 2**, the severity of neuronal injury was shown to be a crucial determinant of microglial activation type. The results demonstrated that mild, moderate, and severe injuries induce neurons to release different amounts of the same signals into the culture media. Microglia exposed to this media were activated into different phenotypes depending on the severity of the neuronal injury. Moderate injury yielded a neuroprotective phenotype, while mild and severe injuries produce a neurotoxic phenotype. Inhibitor experiments showed that glutamate (GLU) and adenosine 5'-triphosphate (ATP), two molecules released by injured neurons, were both involved in this severity-dependent

regulation of activated microglia. The question remained as to whether the amount and concentration of a neuronal signal could dictate the phenotype of activated microglia. **Chapter 3** addresseed this question by studying ATP in isolation without interfering effects of other neuronal signals. It was found that a high concentration of ATP activated microglia into a neurotoxic phenotype, while a low concentration of ATP induced a neuroprotective phenotype. The results further showed that this differential response to varying strengths of ATP stimulation was due to activation of different purinergic (P) receptor 2 subtypes as well as activation of different mitogen-activated protein (MAP) kinases.

The last two experimental chapters examined differential microglial activation from another perspective. Whereas **Chapter 2** and **Chapter 3** characterized activator-dependent mechanisms, **Chapter 4** and **Chapter 5** hypothesized that different 'types' of microglial populations exist, and their phenotypic differences act as intrinsic mechanisms for differential activation. Results from **Chapter 4** showed that microglia derived from various brain regions were phenotypically distinct populations and were activated differentially by ATP. Specifically, microglia derived from cortex (Ctx) and hippocampus (Hip), as opposed to

microglia from other regions, adopted neurotoxic phenotypes when stimulated with ATP. Conditioning experiments showed that the regionality of microglial activation is not hardwired. Ectopic conditioning of microglia from one region with media from another region switched the phenotype to that of the latter region. From the conditioning experiments, it was also found that the amino acid taurine (TAU) may be one of the key molecules involved in establishing the regionality of microglia.

Chapter 5 also examined phenotypically distinct populations of microglia. Here, microglia derived from rats of various age groups were found to have age-specific activation responses to ATP. Neonatal (Neo) microglia were found to have the lowest threshold of activation compared to other age groups and were also the most neurotoxic when stimulated by ATP. 13-15 months (mo) old microglia were morphologically and phenotypically more similar to Neo microglia than to other adult ages. Unlike region-specific activation, however, age-specific responses to ATP were more hardwired, and were not altered by ectopic conditioning with media from another age group.

6.2 Overall significance of findings

6.2.1 Differential activation of microglia by varying signal strengths

The finding that varying strengths of ATP stimulation yield distinct activation phenotypes adds another level of complexity to what is currently known about the regulation of microglial activation. Prior to this finding, the most characterized mechanism of differential microglial activation is stimulation with distinct activators that act on distinct microglial receptors. The idea of the same family of receptors eliciting polarized responses due to varying stimulation strength is a novel concept in microglia. This phenomenon is not restricted to microglia, however, as similar polarized responses to ATP have been observed in astrocytic cultures (Kucher and Neary, 2005). In fact, even among neuronal populations, ATP acts as a neuromodulator when released in low concentrations extracellularly, but induces neurotoxic effects in higher concentrations (Amadio et al., 2005; Burnstock, 2009). P2 receptors are expressed by all cell types of the central nervous system (CNS) (Abbracchio et al., 2009), so it is not surprising that bi-directional responses to ATP are ubiquitous among most CNS cell types. ATP signaling is unique such that the same ligand elicits ionotropic and metabotropic signaling simultaneously (Abbrachio et al., 2009), and that the metabolites of ATP,

including adenosine 5'-diphosphate (ADP) and adenosine (ADO) are also known activators of microglia and contribute to the overall effects (Fiebich et al., 1996; Gebicke-Haerter et al., 1996; Heese et al., 1997; Honda et al., 2001; Chakfe et al., 2002; Min et al., 2008; Orr et al., 2009). The complexity of this signaling system likely contributes to its ability to mount polarized strength-specific responses in microglia. It remains a question whether the bi-directional responses to ATP can be extended to other activators of microglia. GLU is a probable candidate. Like ATP, GLU is released by injured neurons, and as shown in **Chapter 2**, the amount released correlates with the severity of neuronal injury, thus potentially serving as means by which injured neurons convey injury severity to microglia. Moreover, results from **Chapter 2** showed that both GLU and ATP are involved in the differential activation of microglia that is dependent on the injury severity of neurons. Another similarity between GLU and ATP is their receptor system. GLU receptors, like P2 receptors, consist of both ionotropic and metabotropic subtypes (Meldrum, 2000). Interestingly, microglia express both types of GLU receptors (Gottlieb and Matute, 1997; Pocock and Kettenmann, 2007). Therefore, the possibility exists such that GLU functions as a bi-directionally activating signal for microglia. This remains an open question for future studies to confirm.

From an *in vivo*, translational point of view, the finding that varying strengths of the same ligand differentially activate microglia also has implications. For instance, in animal models of stroke, pharmacological inhibition of the P2 receptors have yielded seemingly contradictory results from independent studies. In a model of focal ischemia with middle cerebral artery (MCA) unilateral and common carotid artery (CCA) bilateral occlusions, the non-specific P2 receptor antagonist suramin improved stroke outcome (Kharlamov et al., 2002). In a similar study using unilateral MCA occlusion, the broad spectrum P2Y receptor antagonist Reactive Blue 2 (RB2) also reduced stroke damage (Melani et al., 2006). However, in another study of MCA occlusion, the P2X7 receptor antagonist adenosine 5'-triphosphate-2',3'-dialdehyde (OxATP) yielded the opposite trend and exacerbated stroke damage (Yanagisawa et al., 2008). At first glance, these results are difficult to reconcile: It appears that the P2X7 receptor mediates neuroprotective processes in stroke, yet how does blocking other P2 receptors in addition to blocking the P2X7 receptor produce improved outcome? If the findings in **Chapter 3** are taken into consideration, one could better explain the results from these stroke studies. The findings of **Chapter 3** showed that P2Y

receptors respond to low concentrations of ATP while the P2X7 receptor is activated by high concentrations of ATP only. In a stroke model, high ATP concentrations would be present in areas that are closer to the ischemic core while low ATP concentrations would be in the penumbral areas. The ability of microglia to affect neuronal survival at the ischemic core is limited since the majority of brain tissue in this area is non-rescuable. Inhibition of the P2X7 receptor could interfere with scar formation and other damage control-related processes putatively mediated by microglia near the core, which would otherwise prevent secondary damage to the surrounding regions. This would then explain why P2X7 receptor inhibition worsens stroke outcome. On the other hand, in the penumbral areas, other subtypes of P2 receptors mediate the responses of microglia. Based on the findings of **Chapter 3**, one would expect these non-P2X7 subtypes to mediate a different set of microgilal functions, so rather than damage control, these functions could cumulatively promote inflammation and secondary damage. This would explain why RB2, which blocks P2Y receptors, improved stroke outcome. The fact that suramin, which blocks all P2 receptors including P2X7, improved stroke outcome suggests that the effects of blocking P2Y receptors outweigh that of blocking the P2X7 receptor. Interestingly, the *in vitro* findings of **Chapter 3**

showed a trend opposite to that of animal models, such that the P2X7-independent functions are neuroprotective while P2X7-dependent processes are neurotoxic. This discrepancy likely results from the presence of interacting variables in the *in vivo* setting. When microglial cultures are treated with ATP alone, cytokine release is increased, as shown by results from **Chapter 3**. However, in the presence of lipopolysaccharide (LPS), it has been shown that ATP treatment actually attenuates cytokine secretion (Ogata et al., 2003). Therefore, in an *in vivo* setting where an array of microglial activators is present, it is not surprising to observe a trend opposite to that observed in culture. Another contributing factor that is absent in culture but present *in vivo* is the effect of purinergic signaling in other cell types since most CNS cells express P2 receptors. Regardless of the contrasting patterns between *in vitro* and *in vivo* findings, the strength-dependent bi-directional nature of purinergic responses is a common theme in microglia. In addition to stroke, the roles of purinergic receptors are starting to receive attention in other CNS disorders such as experimental autoimmune encephalitis (EAE), amyotrophic lateral sclerosis, and neuropathic pain (Matute et al., 2007; Inoue, 2008; D'Ambrosi et al., 2009). The findings here may provide a mechanistic explanation for the patterns observed in these *in vivo* studies.

6.2.2 Differential activation of microglia by intrinsic variations

In contrast to **Chapter 2** and **Chapter 3**, the last two experimental chapters characterize mechanisms that are intrinsic within microglia and not related to the activating signal *per se*. **Chapter 4** presents evidence supporting the notion that microglia in different regions of the brain are phenotypically distinct populations that respond differentially to the same activator. Even until recently, the regionality of microglia has not been well documented. A review in 2007 stated that there is "little evidence for different microglial populations" (Hanisch and Kettenmann, 2007). Coincidentally, not long after the publication of this review, de Haas et al. (2008) published a seminal paper demonstrating that microglia isolated by flow-cytometry are indeed regionally distinct with regard to surface markers. **Chapter 4** extends this finding by exposing microglia from various regions to activating stimuli, showing that in addition to phenotypic differences, there are also functional differences between microglia of different regions. Perhaps the most significant implication of this finding is the added complexity to current *in vitro* models of microglia. The majority of primary microglial cultures have been prepared from whole brains. Whether the method of isolation is the

conventional shaking procedure or the more recently developed trypsin procedure, these cultures are first grown as mixed cultures consisting of most CNS cell types. As results from **Chapter 4** have shown, the regionality of microglial activation is not hardwired, so the region-specific phenotypes microglia have prior to culturing is likely conditioned into a 'mixed region' phenotype after growing in whole brain mixed cultures for over two weeks. Comparing the results of **Chapter 4** to that of other chapters (which use whole brain microglia), one could conclude that the 'mixed region' phenotype is different from each of the regional phenotypes observed in **Chapter 4**. Whole brain microglia are somewhat similar to microglia derived from Ctx and Hip in that all of them increase nitric oxide (NO), tumor necrosis factor-α (TNF-α), and brain-derived neurotrophic factor (BDNF) when stimulated by ATP. However, unlike whole brain microglia, Ctx and Hip microglia do not upregulate GLU uptake. As a matter of fact, increased microglial GLU uptake was not observed in any of the regions examined. The phenotypic differences between activated microglia derived regionally and those derived from whole brains raise some concerns with regard to modeling region-specific CNS disorders. For instance, in Parkinson's disease (PD), where pathology is localized to the substantia nigra (Lee et al., 2009), brainstem (Brs) microglia, or more

specifically, microglia derived from the nigral region, would be a more accurate *in vitro* microglia model than whole brain microglia. Another example is spinal cord disorders like neuropathic pain. A significant portion of mechanistic studies in neuropathic pain relies on eletrophysiological data derived from cultures of spinal cord neurons (Abdulla et al., 2003). To study microglial contributions in the context of spinal neurons, microglia derived from spinal cord are likely a better model than microglia derived from the brain. In this thesis, however, spinal cord microglia have not been examined in comparison with microglia from the various regions of the brain. It would be interesting for future studies to extend the findings of **Chapter 4** and put spinal microglia in perspective.

The issue regarding the accurate use of *in vitro* models is also implicated by the aging study in **Chapter 5**. The results here show that microglia of different ages have distinct activation responses to ATP. The majority of *in vitro* studies on microglia, however, use neonatal (Neo) cultures. As data from **Chapter 5** suggest, in response to more potent activators like LPS, age differences are less of a factor. However, age should be taken into consideration if the study uses an endogenous activator that generates relatively weaker effector responses, for example ATP.

Because Neo microglia have a lower activation threshold compared to adult microglia, using Neo microglia to model adult CNS disorders creates the danger of studying effector responses that otherwise would not have been present in adults. This concern has been repeatedly brought up in the microglia literature, as Neo cultures of microglia have been regarded as being more similar to infiltrating macrophages than resident parenchymal microglia based on immunological markers (Ransohoff and Perry, 2009). However, Neo microglia continue to be used because of their high yield. Isolating microglia from adult brains is more technically challenging due to the higher susceptibility of adult microglia to autolyse during the dissociation procedure (Cardona et al., 2006). The precise reason for this is unknown, although the presence of toxic myelin debris in the process of dissociating adult brains (which is not an issue in Neo cultures) is a possible explanation. **Chapter 5** used a Percoll-based method to isolate adult microglia and produced an adequate yield. The yield, however, was only a quarter of that isolated from the same number of Neo brains. The same procedure produces an even smaller yield of microglia when used on mouse brains, which is also an issue because this would limit the possibilities of genetic manipulations. The development of a high-yield method for isolating adult microglia would

negate the necessity to use Neo microglia as a model for adult disorders. As **Chapter 5** showed, even between the different age groups of adult microglia, the effector responses to ATP are age-specific. Microglia derived from old rats were found to have a low activation threshold similar to that of Neo microglia. For CNS disorders that have an old age onset, such as Alzheimer's disease (AD), cultures of old microglia would be a more accurate *in vitro* model. Using the current isolation protocol, however, the amount of time and investment required to generate enough old age microglia may outweigh their research merits. Again, developing a high-yield isolation method would help to overcome this technical obstacle.

6.3 Limitations of the model and future directions

Despite the aforementioned issues with age and region of origin, the *in vitro* models used in this thesis are able to address several important mechanistic questions regarding the regulation of microglial activation. In an *in vivo* setting, an endogenous activator like ATP would trigger an array of non-microglial responses, rendering it difficult to differentiate the cellular origin of the observed responses. Even if neuronal death and behavioral deficits are present, one cannot

conclusively attribute the effects to be microglial in origin. The culture model circumvents this problem by creating separate compartments for microglia and neurons. Another useful type of experiment limited to the culture setting is the conditioning experiment performed in **Chapter 4** and **Chapter 5**. The finding that Neo microglia derived from one particular region can be conditioned into a more neuroprotective phenotype resembling that of another region has therapeutic implications. One of the ultimate goals of microglial research is to selectively manipulate microglia in a way that their beneficial functions are amplified or preserved and their detrimental side effects are suppressed. However, to test this possibility, the culture model would not be sufficient. The *in vitro* model of microglia lacks cell-to-cell contacts with other CNS cells as well as continuous signaling with other cell types. Trends observed *in vitro* can therefore be opposite to those *in vivo*, as illustrated previously in the example of P2 receptor inhibition in stroke. Furthermore, for results to be transferable to the clinical context, behavioral data are also required. The main obstacle here is to find *in vivo* models that allow selective conditioning of microglia. One potential model is the intracerebral injection of cultured microglia. Previous reports have shown that injection of cultured microglia into post-stroke brains can improve stroke outcome

in both rats and gerbils (Kitamura et al., 2004; Hayashi et al., 2006; Imai et al., 2007). The mechanism of this phenomenon is unknown, but an explanation would be that these injected microglia have not been deprived of energy themselves and are thus more neuroprotective. There is, however, a limited time window for this injection to effectively improve neuronal survival. An interesting future study would be to condition the microglia into a more neuroprotective phenotype prior to their injection, and to see if the time window can be extended. It is possible that at later time points post-stroke, the pro-inflammatory effectors of microglia need to be suppressed so that secondary damage from pro-inflammatory responses do not negate the effects of the regenerative responses. The fact that microglia can be conditioned without genetic manipulations increases the potential of microglia injection/transplantation becoming an option in the clinical setting. Genetic manipulations of the injected microglia would nonetheless reveal the specific effector molecules involved. It would be also interesting to see if blood macrophages can be conditioned and manipulated the same way, since the injection of an individual's own macrophages would eliminate host rejection issues.

The same *in vivo* model of microglia injection can also be extended to CNS disorders other than ischemic stroke. For example, a hypothesis for the cause of AD is the senescence and change of function in old age microglia. A study that may strengthen this hypothesis is to inject Neo or young microglia into the brains of aged animals. Over a long term period, the injected microglia could compensate for the loss of function in senescent microglia. A methodological challenge of this experiment is the lack of a non-familial amyloid-generating animal AD model. Intracerebral injection of amyloid peptides is a viable alternative. Injection of amyloid peptides can induce tissue lesions, suppression of long term potentiation, as well as cognitive deficits, a pathology that somewhat resembles that of AD (Gonzalo-Ruiz et al., 2005; Gonzalo-Ruiz et al., 2006; Minogue et al., 2007; Haque et al., 2008). Compared to young animals, amyloid injections into aged animals induce larger lesion sizes and greater cognitive deficits (Gonzalo-Ruiz et al., 2006; Haque et al., 2008). If the increased lesion size due to aging is a result of microglial senescence, then injection of young microglia should decrease the lesion size. Another methodological issue of this experimental setup is the survival time of the transplanted microglia. Unlike the case in stroke, the time course of pathology development in the amyloid-injected

animals is longer and can last up to a month (Gonzalo-Ruiz et al., 2006; Haque et al., 2008). If the injected microglia cannot survive through that time period, then an alternative method is required. Genetic manipulations that specifically target microglia are a viable option. Microglia-specific promoters such as those for CD11b and macrophage cology-stimulating factor (M-CSF) can be used to express transgenes or knockouts in a cell type-specific manner (Siao et al., 2003; Gowing et al., 2006). The caveat of using transgenics for this study is that the molecular substrates that differentiate young and old microglia have not been characterized. Without putative targets, the effort towards generating transgenics becomes a risky investment. The data from **Chapter 5** suggest that P2 receptors are involved. However, the injection of amyloid peptide likely induces the release of several activators other than ATP. P2 receptors could represent only a small portion of the molecular substrates involved in the age-specific responses of microglia. Successful characterization of these molecular players *in vitro* would then help to identify putative targets for the *in vivo* transgenic studies.

6.4 Concluding statement

Overall, while several limitations of the model raise issues that require further

experimentation, the results presented by this thesis answered important questions regarding the regulation of microglial activation. There is strong evidence to support the hypothesis that microglial activation is regulated not only by extrinsic signals, but also by their inherent differences. As described in the general introduction, microglial activation is an integral part of CNS pathology, and gaining a better understanding of the mechanisms that regulate this process is critical not only towards grasping CNS pathology in general, but also towards developing better therapeutic strategies against pathological conditions of the brain.

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