Characterization of Spinal Cord Microglia

by

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Abstract

Microglia are the primary immune cells of the central nervous system (CNS). They are essential for normal brain structure and function at all stages of development and during adulthood. During development, they are involved in the removal of apoptotic neurons and establishment of proper neural circuity in the CNS. In the adult brain during normal physiology, microglia have a ramified morphology with long processes that constantly assess the local environment surrounding the neurons and glial cells of the CNS. In the event of perturbation or injury to the CNS, microglia migrate to site of injury and produce anti-inflammatory factors, pro-inflammatory factors, prostaglandins, cytokines, and super oxides that can be neurotrophic or neurotoxic depending on the severity of the injury. Previous studies suggest that the functional response properties of activated BM (brain microglia) varied according to the brain region they were derived from, suggesting that these activation profiles are a function of their local environment. The functional profiles of microglia derived from the spinal cord have not previously been investigated. Here, cell culture methods were used to probe the morphological and secretory response profiles of SCM (spinal cord microglia) in response to activating stimuli. In Chapter 1, activation of BM and SCM with lipopolysaccharide revealed that SCM released less of the pro-inflammatory factors NO (nitric oxide), TNF (tumour necrosis factor), and IL-1β (interleukin 1 beta) after activation relative to BM. Differences in morphology and reduced

phagocytosis in LPS activated SCM relative to BM were also observed, suggesting that SCM have a reduced inflammatory profile relative to BM when activated with a pathogen like stimulus. In Chapter 3, an improved protocol for isolation of SCM was optimized, then applied to studies of endogenous immunoactivators released during CNS injury. Insults to the CNS, such as ischaemic stroke or spinal cord injury, cause neuronal injury and death and increase the concentrations of neurotransmitters such as ATP (adenosine 5'-triphosphate) and glutamate in the extracellular environment near the injury. However, the consequences of physiological concentrations of these immunoactive molecules on BM and SCM has not been well characterized. In Chapter 4, physiologically relevant concentrations of ATP and glutamate were used to activate BM and SCM. Release of pro-inflammatory factors by BM and SCM was measured, and the data suggest that ATP and glutamate at these concentrations do not induce significant cytokine release and that there was no significant difference in the release profile of pro-inflammatory factors by BM and SCM in response to ATP. An overall reduction in NO and IL-1β release by SCM was observed in glutamate experiments. To test whether regional heterogeneity in microglia could be explained by differences in the regional extracellular milieu, BM were then exposed to conditioned media from either brain or spinal mixed glia and activated with LPS (lipopolysaccharide), ATP, or glutamate. It was found that BM exposed to the spinal cord mixed glia conditioned media shifted their responses to activation towards a more SCM-like

phenotype (overall reduction in IL-1β and increased IL6 release). This suggests that the immediate environment differs for SCM and BM and that this environment can modulate microglial function in response to known activators. Finally, in Chapter 5 the role of BM and SCM in an *in vitro* model of ischaemic injury (oxygen glucose deprivation, OGD) was tested. Despite differences in their responses to LPS activation, there were no significant differences in the pro- and anti-inflammatory factors released by BM and SCM on exposure to injured neurons. BM offered greater neuroprotection than SCM during moderate neuronal injury, while there was no difference in neuroprotection during severe injury. Together these results confirm that BM and SCM have different inflammatory response profiles under particular activation conditions, particularly when exposed to a pathogen like stimuli. However, physiological activators (ATP, glutamate, and OGD) and ischaemic injury did not induce significantly different inflammatory response profiles in SCM compared to BM, suggesting their overall function in the injured CNS is conserved between different brain regions.

Preface

This thesis is an original work done by Sam Joshva Baskar Jesudasan. Chapter 2 of this thesis is published in PLOS ONE as Baskar Jesudasan SJ, Todd KG, Winship IR "Reduced Inflammatory Phenotype in Microglia Derived from Neonatal Rat Spinal Cord versus Brain" PLoS ONE 9(6): e99443. I collected and analyzed the data and wrote the manuscripts and thesis chapters. Dr. Ian R. Winship and Kathryn G. Todd supervised experimental design and analyses and assisted with manuscript composition.

Dedication

For always roaming with a hungry heart……..

Much have I seen and known; cities of men…..

……. I am a part of all that I have met……..

…….. Forever and forever when I move……

…… To follow knowledge, like a sinking star,

Beyond the utmost bound of human thought…

…….. To strive, to seek, to find, and not to yield…

excerpts from Ulysses (between lines11 and 70)

Author Alfred Lord Tennyson (1809–1892)

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Table of Contents

List of Tables

List of Figures and Illustrations

List of Symbols, Abbreviations, and Nomenclature

µg	micrograms
µM	millimolar
µm	micrometres
A1, A2	P1 receptor sub types
AMPA	α-amin0-3-hydorxy-5 methyl-4 isoxazolepropionic acid
ANOVA	analysis of variance
AP-1	activator protein -1,
ATP	adenosine 5'-triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BM	brain microglia
BMix CM	Brain mixed-glia conditioned media
BSA	bovine serum albumin
CCR	chemokine (C-C) receptor
CD	cluster of differentiation
CL	chemokine ligand

CNS	central nervous system
CpG	A minimum of 200 base pairs rich (>50%) in cytosine and guanine
Ctl	control
DAB	3,3'-Diaminobenzidine
DAMP	damage associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagles's media
DMEM/F12	Dulbecco's modified Eagles's media with Ham's F12
DMSO	dimethyl sulfoxide
DR	death receptor
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene diamine tetra acetic acid
EGA	ethylene glycol-bis(β-aminoethyl ether)-N-N-N'-N' tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid

GDNF	glia-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLU	glutamate
GMSCF	granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptors
GTP	guanosine 5'-triphosphate
HBSS	Hanks's balanced salt solution
HCl	hydrochloric acid
HRP	horseradish peroxidase
hrs	hours
Iba1	ionised calcium binding adaptor molecule 1
IL	interleukin
KO	knock out
LPS	lipopolysaccharide
M	molar
MAPK	mitogen activated protein kinase
MCAo	middle cerebral artery occlusion
mg	milligram
mGLuR	metabotropic glutamate receptor

mins	minutes
mL	millilitres
mM	millimolar
mm	millimetres
MS	multiple sclerosis
MSCSF	macrophage colony stimulating factor
MTT	thiazolyl blue tetrazolium bromide
MyD88	myeloid differentiation primary response 88
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NT	neurotrophin
P1 receptor	adenosine receptor belonging to a class of G-protein coupled purinergic receptors

P_2X receptors	purinergic receptors (ATP ligand gated cationic channels)
P_2Y receptors	purinergic receptors (ATP ligand dependent metabotropic receptors)
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3 kinase
PRRs	pattern recognition receptors
R	receptor
rpm	rotations per minute
SCI	spinal cord injury
SCM	spinal microglia
SDS	sodium dodecyl sulphate
SMix CM	spinal mixed-glia conditioned media
TAB	TAK1 binding proteins
TAK	transforming growth factor-β (TGF-β)-activated kinase
TBI	traumatic brain injury
TGF-β	transforming growth factor-β
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor

TNF	tumour necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
TREM-2b	triggering receptor expressed on myeloid cells 2b
TrkR	tyrosine receptor kinase
U	unit of enzyme activity

Chapter One: Introduction

1.1 Microglia

The central nervous system (CNS) is composed of neurons and glial cells. The glia are further classified into astrocytes, ependymal cells, microglia, and oligodendrocytes. Microglia and oligodendrocytes were once considered a single population called the "Third Element" of the CNS. However, Dr. Pío del Río Hortega defined microglia as an unique glial populations in CNS (Del Rio Hortega, 1932; Giulian and Baker, 1986; Sierra et al., 2016). He reported that in the event of brain injury microglia migrate to site of injury and phagocytose debris (Giulian and Baker, 1986; Sierra et al., 2016). Dr. Hortega claims were very controversial at that time, as most other scientists were convinced that oligodendrocytes and microglia were the same type of cells (Del Rio Hortega, 1932; Sierra et al., 2016). In fact, the research community denied the existence microglia for decades after Dr. Hortega's postulates. However, in 1968, Blinziger and Kreutzberg rejuvenated interest in microglia by characterizing their role in the injured facial nerve and their capacity to phagocytose debris in the facial nucleus (Blinzinger and Kreutzberg, 1968). Kreutzberg estimated that microglia constitute approximately 20% of the total CNS glial population (Kreutzberg, 1995) and research since that time has shown that microglia are actively involved in all aspects of CNS homeostasis (Kettenmann et al., 2011). In normal physiology, microglia have a ramified morphology with long processes that are constantly assessing the extracellular environment surrounding the neurons and glial cells of the CNS (Davalos et al., 2005; Nimmerjahn, 2005; Wake et al., 2009). After perturbation, injury or disease in the CNS, microglia respond immediately by migrating to the site of injury or perturbation, releasing a repertoire of molecules that can exacerbate injury and promote repair as well upregulating surface receptors that sense and initiate a response to DAMPs (damage associated molecular

pattern) and PAMPs (pathogen associated molecular patter) (Kettenmann et al., 2011). Depending on degree of activation, microglia change their morphology to assume a ramified-amoeboid or circular shape (Kettenmann et al., 2011). Below, the current understanding of microglia and their role in CNS development, homeostasis, injury and infection will be discussed. Functional differences due to region of origin and extracellular environment will be highlighted.

1.2 Signalling in microglia

The repertoire of microglial receptors involved in signalling in CNS include neurotransmitter receptors (purinoceptors, glutamate receptors, GABA receptors , cholinergic receptors, adrenergic receptors, dopamine receptors), neurohormone and neuromodulators receptors, cytokine and chemokine receptors (including tumor necrosis factor receptors, interleukin receptors, macrophage colony-stimulating factor receptors, epidermal growth factor receptors, and many others) pattern-recognition receptors, toll-like receptors, calcium receptors, leukotriene receptors, notch receptors, complement receptors, thrombin receptors, CD200 receptors, lysophosphatidic acid receptors, formyl peptide receptors, and sigma receptors (Abbracchio et al., 2009; Burnstock, 2007; Kettenmann et al., 2011; Murugan et al., 2013; Niswender and Conn, 2010). This section will focus primarily on receptors and downstream signalling pathways of activators used in later chapters. Specifically, LPS, ATP (adenosine triphosphate), and glutamate as ligands and the TLRs (toll-like receptors), chemokine receptors, TNFR (tumour necrosis factor receptors), IL-Rs (interleukin receptors), purinoceptors, and glutamate receptors will be discussed.

1.2.1 Chemokines and chemokine receptors

Chemokine receptors induce cellular chemotaxis and are important for cellular migration during development and pathophysiology (Asensio and Campbell, 1999; Baggiolini et al., 1997; Sallusto and Baggiolini, 2008). These receptors are comprised of small proteins (8-12 kDa) that have up to four conserved cysteine residues, and are divided in to following several subgroups (C, CC, CXC and CX_3C) (Fernandez and Lolis, 2002; Laing and Secombes, 2004). The chemokines signal through GPCRs (G protein coupled receptors) classified as CCR, CXCR, CX_3CR (Kettenmann et al., 2011). Downstream signalling molecules include adenyl cyclases, phospholipases, GTPases (Rho, Rac, and Cdc42) and kinases such as MAPK and PI3K (phosphatidylinositol 3–kinase)(Kettenmann et al., 2011). Chemokines themselves are mostly released into the extracellular milieu where they generate a gradient that facilitates cellular migration (Kettenmann et al., 2011). However, there are also surface bound chemokines such as CX_3CL (fractalkine) expressed on the surface of neurons (Kettenmann et al., 2011). Chemokine expression can be constitutive or facultative suggesting that some are needed for normal physiology and others only in response to a pathophysiological condition (Kettenmann et al., 2011). In the CNS, chemokines are expressed and released by both neurons and microglia (Biber et al., 2008; de Haas et al., 2007). Activation of TLRs (Toll-like receptors described in the next section) on microglia can induce release of CCL2 and recruitment of other immune cells such as peripheral macrophages and other nearby microglia in an autocrine/paracrine fashion (Kettenmann et al., 2011). CX_3CL1 is constitutively expressed on surface of neurons and may provide a cue to keep the microglia from becoming activated, whereas CCL2, CCL21 and or

CX_3CL1 are released by injured neurons and recruit microglia to the site of injury (Mildner et al., 2007; Prinz and Priller, 2010; Wolf et al., 2013).

1.2.2 TLRs.

Toll-like receptors belong to a family of receptors known as PRRs (pattern recognition receptors) that are involved in innate immunity (a non-specific defence mechanism that is trigged by chemical properties of the foreign body/antigen). The PRRs detect pathogen-associated molecular patterns (PAMPs) such as viruses and bacteria (Akira and Takeda, 2004; Sims and Smith, 2010). The PPRs also capable of detecting with damage-associated molecular patterns (DAMPs), including endogenous factors that are released or exposed only by damage or injury to CNS (Akira and Takeda, 2004; Sims and Smith, 2010). The TLRs are related to the IL-1R (interleukin-1 receptor) by the presence homological TIR (Toll/IL-1R) cytoplasmic domains (Akira and Takeda, 2004; Sims and Smith, 2010). PAMPs and DAMPs are crucial components of TLR receptor mediated regulation of CNS homeostasis or response to a pathogen or injury (Akira and Takeda, 2004; Sims and Smith, 2010). In the CNS, TLRs are predominantly expressed by glia, however, some TLRs such as TLR-3 and TLR-4 are also expressed by neurons (Lafon et al., 2006; Leow-Dyke et al., 2012). TLRs such as 1/2, 6/2, 4 are hetero or homomeric integral transmembrane proteins on the surface of plasma membrane whereas TLR 3, 7, and 9 are intra-cellular and found only in endosomal compartments (Akira and Takeda, 2004). Thus, TLR such as 1/2, 6/2, and 4 are the first line of sensors that monitor the immediate CNS environment to detect DAMPs and PAMPs.

1.2.2.1 Toll-like (TLR/IL-1R) superfamily signalling cascade

TLRs/IL-1Rs dimerize upon binding ligand, thereby changing their confirmation and leading to recruitment of a variety of downstream signalling molecules including adaptor molecule MyD88 (myeloid differentiation primary-response protein 88), IRAKs (IL-1R associated kinases), TRAF6 [tumour-necrosis factor (TNF)-receptor-associated factor 6], TAK1 [transforming growth factor-β (TGF-β)-activated kinase], TAB1, and TAB2 (TAK1 binding proteins) (Akira and Takeda, 2004). These molecules signal either through transcription factor AP-1 (activator protein -1, primarily involved in cell proliferation and cell death) or nuclear translocation of transcription factor NF-κB (nuclear kappa-light-chain-enhancer of activated B cells, primarily involved in inflammation) (Akira and Takeda, 2004). Activation of TLRs induces the release of various cytokines and chemokines by microglia. TLR 2, 3 and 4 are the best characterized receptors for their role in inflammation (Akira and Takeda, 2004). For example, TLR2, upon activation by di-, and tri-acyl lipopeptides, lipoteichoic acid, or peptidoglycan (primary components on bacterial cell wall) induces the secretion of IL (interleukin) -6 and IL-10. TLR2 knock out mice lose the ability to express inducible nitric oxide synthase, TNF, IL6 and IL-1β, drastically altering their immune response (Kim et al., 2007). TLR3 is activated through contact with viral double-stranded RNA and induces the release of IL-6, IL-12, CXCL10 (chemokine ligand 10), TNF, and IFN-β (Akira and Takeda, 2004). TLR4 is activated by polysaccharides and viral proteins, particularly LPS (lipopolysaccharide), and activation induces release of IL-6, and TNF (Akira and Takeda, 2004). Similar to TLR2, in TLR4 knock out mice, microglia have reduced expression of pro-inflammatory factors including TNF, IL6 and IL-1β (Tanga et al., 2005). TLR9 recognizes particular sequences in DNA molecules (CpG sequences that act as

PAMPs) and induce NO (Nitric Oxide) and TNF release (Akira and Takeda, 2004; Kettenmann et al., 2011; McKimmie and Fazakerley, 2005). Cross talk between TLRs is common, and activation of TLR4 results in upregulation of TLR2 and 3 in microglia (Kielian et al., 2005; McKimmie and Fazakerley, 2005). This suggests that the TLR4 can prime microglia for TLR2 and TLR3 receptor signaling. Thus, TLRs induce microglial activation and production of pro-inflammatory factors, and are thereby key components of the CNS immune response.

1.2.3 Interleukins and Interleukin receptors (IL-R).

IL-R are transmembrane proteins with a homologous cytoplasmic TIR (toll/interleukin-1 region) domain and an immunoglobulin-like extracellular domain. The IL-1 receptors family consists of IL-1 type I receptor (IL-1R1), IL-1 type II receptor (IL-1RII), and IL-1 receptor accessory protein (IL-1RAcP) (Akdis et al., 2016; Kettenmann et al., 2011). The IL-6R complex also belong to the Il-1 type 1 receptor family (Mihara et al., 2012). IL-6Rα in tandem with IL6Rβ serve as receptors for IL6 (Mukaino et al., 2010). Microglia have low expression of IL-1RI, and high expression of IL-1RII. However, activation of microglia with LPS significantly increases the expression of IL-1RI, IL-1RII, IL-1RAcP, and IL-10R1 (IL-10 receptor R1) and release of IL-1β and IL-6) (Akdis et al., 2016; Kettenmann et al., 2011). *In vitro* IL-10 down-regulates the expression of TNF and IL-1β and thereby reduces the neurotoxicity mediated by these pro-inflammatory cytokines (Broderick et al., 2000). IL-10 administration reduced TNF, IL-1β and improved the behavioural outcome of rats after TBI (traumatic brain injury) (Knoblach and Faden, 1998). Similarly, IL-10 administration reduced TNF, and improved the behavioural outcome of rats after SCI (spinal cord injury)(Bethea et al., 1999). Thus, IL-10 mediated IL-10R

activation is anti-inflammatory and improves outcome of CNS injuries (Bethea et al., 1999; Knoblach and Faden, 1998; Moore et al., 2001). Activation of primary microglia *in vitro* with LPS upregulates IL-2 receptors and application of anti-mouse IL-2 receptor antibodies reduces microglia viability and proliferation (Sawada et al., 1995). IL-6Rα in tandem with IL6Rβ serve as receptor for IL6 (Mukaino et al., 2010). IL6 and IL-1β are most studied well characterized inflammatory factors released by microglia (Hill et al., 1999; Lambertsen et al., 2012; Nakamura et al., 1999; Sist et al., 2014; Yang et al., 2005). IL-6 has both pro- and anti-inflammatory actions (Erta et al., 2012; Leibinger et al., 2013). IL-6 *in vitro* down regulated TNF expression and increased axonal regeneration (Aderka et al., 1989; Leibinger et al., 2013). IL-6R block in SCI mice reduced the expression of chemokines (CCl2, CCL5, and CXCL10) involved in recruiting peripheral immune cells and increased GM-CSF (Granulocyte/Macrophage colony stimulating factor, promotes microglia proliferation) expression (Mukaino et al., 2010; Okada et al., 2004). Moreover, IL-6R inhibition in SCI mice resulted in reduced tissue damage and increased axonal sprouting, suggesting that IL-6 overexpression might be detrimental for recovery after SCI (Mukaino et al., 2010; Okada et al., 2004). Numerous studies suggest that IL-1β has neurotoxic effects, and inhibition of IL-1β rescues neurons from injury (Jin et al., 2010; Kettenmann et al., 2011; Lambertsen et al., 2009; Nakamura et al., 1999; Smith et al., 2012). The above studies suggest that IL-6 has dual roles in the progression of inflammation while IL-1β predominantly plays a role as a pro-inflammatory factor.

1.2.4 TNF receptors (TNFR)

The TNFRSF (TNFR superfamily) consists of at least 27 member (MacEwan, 2002). The TNFRs consist of an extra cellular domain (two to six repeats of cysteine rich motifs) and a cytosolic signalling domain that can either signal apoptosis through DD (death domain) or survival through TRAF (tumor necrosis factor receptor-associated factor) family of ubiquitin E3 ligases (Idriss and Naismith, 2000; Ward-Kavanagh et al., 2016). TNFR1, TNFR2 , DR3, $p75^{NTFR}$, CD40, CD95, Fn14 are expressed in the homeostatic brain (MacEwan, 2002; Twohig et al., 2011).

TNF is released in the brain and spinal cord after an injury or disease and can influence the outcome of injury/disease (Green and Nolan, 2012; Jin et al., 2010; MacEwan, 2002; Oshima et al., 2009; Sist et al., 2014; Sriram, 2002). TNF is a very complex ligand and exhibits both neurotoxic as well as neuroprotective actions after brain or spinal cord injury (Smith et al., 2012; Sriram, 2002). TNFR KO (knock out) mice exhibit exacerbated injury and epileptic seizures after focal cerebral ischaemic (Bruce et al., 1996). However, acute inhibition of TNFR reduces infarct volume after MCAo (middle cerebral artery occlusion) (Dawson et al., 1996). Interestingly, TNF knock out mice exhibit reduced axonal sprouting in the spinal cord after brain injury, suggesting that TNF plays role beyond neuronal death and survival and contributes to spinal plasticity that is essential for functional recovery (Oshima et al., 2009). *In vitro,* TNF increases phagocytic activity of microglia derived from neonatal mice (von Zahn et al., 1997). Thus, acute inflammation by TNF maybe beneficial after injury; however, prolonged loss of TNF signaling could be detrimental for outcome after the injury.

1.2.5 Neurotrophins and neurotrophin receptors

BDNF (brain-derived growth factor), NGF (nerve growth factor), and NT-3 and 4 (neurotrophins 3 and 4) are well-known neurotophins in the CNS (Kettenmann et al., 2011). BDNF, NGF and NTs signal through TrkR (tyrosine receptor kinase) and p75 NTR (p75 neurotophin receptor) (Kettenmann et al., 2011). BDNF is a pro-survival factor that is secreted by microglia (Fujita et al., 2008; Miwa et al., 1997; Trang et al., 2009), and BDNF expression is upregulated in microglia after neuronal injury (Lai and Todd, 2008). Activated microglia exposed to BDNF attenuate NO release, suggesting that TrkR signalling is anti-inflammatory (Mizoguchi et al., 2014). Moreover, BDNF is needed for the neuronal plasticity (axonal sprouting and rewiring) that follows CNS injury and contributes to recovery (Huang et al., 2016; Sist et al., 2014). Binding of BDNF induces proliferation of microglia (Gomes et al., 2013; Mizoguchi et al., 2014): Depending on the region of CNS this BDNF induced proliferation can be beneficial or detrimental. For example, BDNF-mediated proliferation could contribute to clearing of cellular debris from dying cells after CNS injury. However, extensive proliferation of microglia could also extend or enhance inflammation which could be deleterious, as has been observed in BDNF-mediated pain hypersensitivity (Hayashi et al., 2011; Zhou et al., 2011). Thus, BDNF released by microglia could be beneficial or detrimental dependent on the spatial and temporal profile of activation.

1.2.6 Purinoceptors.

In the CNS, the principal purinergic signalling molecules are ATP and its derivatives (Abbracchio et al., 2009; Burnstock and Verkhratsky, 2009; North and Verkhratsky, 2006). ATP can be released into the extra-cellular milieu in a controlled fashion by neurons via exocytosis, transporters or even through lysosomes (Abbracchio et al., 2009; Pankratov et al., 2007, 2006; Sperlágh and Illes, 2007; Zhang et al., 2007). ATP released into the extracellular milieu is rapidly degraded by extracellular ectonucleotidases into ATP derivatives such as adenosine, ADP, and AMP (Abbracchio et al., 2009; Zimmermann, 2006). However, injury to the CNS can result in large volume dump of ATP by injured and necrotic neurons, contributing to neurotoxicity and recruitment of microglia to the site of injury (Honda et al., 2001; Wu et al., 2007). Hence, uncontrolled ATP release is deemed a "damage" signal in the CNS (Burnstock and Verkhratsky, 2010; Qureshi et al., 2007, p. 4).

ATP and its derivatives signal via several classes of purinoceptors including the metabotropic P1 adenosine receptors (A1, $A2_A$, A2, and A3), the ionotropic P_2X purinoceptors (P_2X_1 - P_2X_7), and the metabotropic P2Y purinoceptors (P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, P_2Y_{11-14}) (Abbracchio, 2006; Burnstock, 2007; Fischer and Krügel, 2007; Fredholm et al., 2007, 2001; Khakh et al., 2001; North, 2002; Surprenant and North, 2009). ATP and ATP mimics have been show to induce differential morphological changes (e.g. extension of processes to lesion site, membrane raffling) and release of cytokines and chemokines by microglia via activation of these different receptors (Bianco et al., 2005; Davalos et al., 2005; Färber and Kettenmann, 2006; Ferrari et al., 1997; Honda et al., 2001; Nimmerjahn, 2005; Sanz and Virgilio, 2000; Wollmer et al., 2001).

1.2.6.1 P1 receptors (Adenosine receptors)

P1 receptors are a class of purinergic G protein-coupled receptors that are further sub-divided into A1, $A2_A$, $A2_B$ and A3 isoforms. A1 and A2 receptor-specific agonists induce NGF expression (Nerve growth factor, essential for neuronal survival) and prostaglandin E_2 expression (anti-inflammatory factor) in rat primary microglia (Fiebich et al., 1996; Heese et al., 1997). A1 can also induce proliferation of microglia *in vivo* (Haselkorn et al., 2010).

1.2.6.2 P_2X receptors

P_2X receptors belong to the P_2X super family of ligand gated cationic channels that have significant roles in normal CNS function (Khakh et al., 2001; Nicke et al., 1998; Roberts et al., 2006; Woolf and Mannion, 1999). Studies suggest that microglial purinergic signalling mainly is involved in pathological states such as neuropathic pain (Färber and Kettenmann, 2006; Inoue, 2006a, 2006b; Inoue et al., 2004; Trang et al., 2006). Notably, antagonists of P_2X_{1-4} attenuate neuropathic pain in rat models of spinal nerve injury (Tsuda et al., 2003), while inhibiting $P_2X_{1-3, 5,7}$ does not reduce neuropathic pain (Tsuda et al., 2003). Several studies have suggested increased expression of P_2X_4 by activated microglia suggesting they might have a role in initiation, and or instigation of neuropathic pain (Guo et al., 2005; Inoue et al., 2007; Tsuda et al., 2005, 2003).

P_2X_7 receptors have the capacity to be toxic or trophic depending on extent and duration of receptor stimulation. Systemic administration of LPS induced a reduced released of IL-1β, and TNF in P_2X_7 KO mice relative to wildtype mice, suggesting that P_2X_7 receptor activation could

prime the TLR4 receptor (Mingam et al., 2008). *In vitro* it was shown that combined treatment of P_2X_7 KO microglia with LPS and ATP did not induce release of IL-1β, whereas IL-1β was released by wildtype microglia (Mingam et al., 2008) However, *in vivo* after an ischaemic injury, inhibition of P_2X_7 exacerbated the injury to cerebral cortex (Yanagisawa et al., 2008). Thus, P_2X_4 and P_2X_7 well studied and characterized members of the P_2X superfamily that play a major role in pain regulation and inflammation in the CNS.

1.2.6.3 P_2Y receptors

The P_2Y receptors are metabotropic seven-transmembrane domain (GPCRs) G protein-coupled receptors sub divided into the $P_2Y_{1,2,4,6,11}$ and $P_2Y_{12,13,14}$ subtypes (Abbracchio, 2006; Fischer and Krügel, 2007). The $P_2Y_{1,2,4,6,11}$ signal through G_q/G_{11} protein and are involved in regulation of cytosolic Ca^{2+} movement by signalling through PLC (phospholipase C) or $InsP_3$. (Abbracchio, 2006; Fischer and Krügel, 2007). The $P_2Y_{12,13,14}$ signal through $G_{i/o}$ and hence primarily inhibits adenylyl cyclase (Abbracchio, 2006; Fischer and Krügel, 2007).

ATP-mediated P_2Y activation reduces inflammatory factors released *in vitro* in brain slices and SCM (spinal cord microglia) cultures (Boucsein et al., 2003; Ogata et al., 2003). ATP and P_2Y agonists also induce chemotaxis by microglia *in vitro* while P_2Y receptor antagonists inhibit chemotaxis by microglia (Wu et al., 2007). In the spinal cord, P_2Y_{12} receptors are primarily expressed by microglia and play a role in inducing tactile allodynia (abnormal pain associated with touch) and hyperalgesia (increased sensitivity to pain) (Inoue and Tsuda, 2009; Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). Ablation of microglial P_2Y_{12} receptors

however, does not prevent microglia activation but does attenuate neuropathic pain (Tozaki-Saitoh et al., 2008).

1.2.7 Glutamate receptors.

1.2.7.1 Ionotropic receptors

AMPARs (α-amin0-3-hydorxy-5 methyl-4 isoxazolepropionic acid receptors) and NMDAR (N-methyl-D-aspartate receptors) are transmembrane ionotropic receptors of glutamate primarily studied for their roles in neuronal signalling that are also expressed on microglia (Kettenmann et al., 2011; Murugan et al., 2013). There are only a few studies that have examined the role of ionotropic glutamate receptors role in regulation of microglia functions. AMPAR signalling is implicated in chemotaxis of microglia (Liu et al., 2009). AMPAR is also implicated in glutamate mediated TNF release in primary microglia (Hagino et al., 2004). Similarly, NMDAR activation increased NO, TNF and IL-1β release by microglia (Kaindl et al., 2012; Tikka and Koistinaho, 2001). Since there is a limited number of published studies on microglial ionotropic glutamate receptors, a definitive role in inflammation has not been defined (Domercq et al., 2013).

1.2.7.2 Metabotropic receptors

The mGLuRs (metabotropic glutamate receptors) are GPCRs primarily involved in synaptic transmission in the CNS (Niswender and Conn, 2010). They are further classified in to group I (mGLuRs 1 and 5), group II (mGLuRs 2 and 3), and group III (mGLuRs4, 6, 7 and 8) based on

sequence homology, G-protein coupling and ligand selectivity (Niswender and Conn, 2010). Specific antagonists of group I mGluRs attenuate LPS-mediated release of NO and TNF by rat microglia (Byrnes et al., 2009; Farso et al., 2009). Activation of group II mGluRs by either agonists or glutamate that was released after an ischaemic injury induced release of TNF and neurotoxicity (Kaushal and Schlichter, 2008). The group II mGluRs induce microglia activation triggered NF-κB signalling and TNF release; however, unlike TLR4 receptors they do not invoke NO release (Kaushal and Schlichter, 2008; Taylor, 2005). Furthermore, it was found that group II mGluRs were neurotoxic and promoted TNF release, while group III attenuated microglia-mediated neurotoxicity (Taylor, 2005; Taylor et al., 2003, 2002). Overall, it has been postulated that mGluR groups I and II induce inflammatory factor release by microglia and drive them toward a neurotoxic phenotype, while mGluR group III activation can attenuate inflammatory factor release and neurotoxicity by microglia (Byrnes et al., 2009; Farso et al., 2009; Taylor, 2005; Taylor et al., 2003; Vincent and Maiese, 2000).

Table 1: List of well characterised receptors that microglia express and their functions in the CNS

Receptor	Ligand	Function	Reference
TLR2	di-, and tri-acyl lipopeptides, lipoteichoic acid, or peptidoglycan (primary components	NF-κB mediated inflammation (increased release of IL-1, IL-6, IL-8, and TNF)	(Kim et al., 2007; Mele and Madrenas, 2010).

	on bacterial cell wall)		
TLR 3	double-stranded RNA	NF-κB mediated inflammation by release of IL-6, IL-12, CXCL10 and IFN-β	(Akira and Takeda, 2004; Takeda and Akira, 2004).
TLR4	lipopolysaccharide	NF-κB mediated inflammation (increased release of IL-1β, IL-6, and TNF)	(Akira and Takeda, 2004; Takeda and Akira, 2004).
IL-1R	IL-α, IL-1β, IL-1ra and IL-18	IL-α and IL-1β induces inflammation (increased induction of COX2, iNOS, PGE2 IL-1ra blocks activity of IL-α or IL-1β	(Boraschi and Tagliabue, 2013; Dinarello, 2009).
IL-10R	IL-10	IL-10 is an anti-inflammatory factor that downregulates TNF and IL-1β) and improves behavioural outcome after TBI and SCI	(Bethea et al., 1999; Broderick et al., 2000; Diab et al., 1997; Knoblach and Faden, 1998).

IL-6R	IL-6	IL-6 is a bimodal inflammatory factor. that downregulates TNF. overexpression is detrimental in SCI	(Erta et al., 2012; Leibinger et al., 2013; Mukaino et al., 2010; Okada et al., 2004).
CCL2R	CCL2	Essential for recruitment of immune cells	(Kettenmann et al., 2011).
CX_3CR	CX_3CL	CX_3CR establishes microglia-neuron interactions and is anti-inflammatory, CX_3CL released by injured neurons recruit microglia to site of injury.	(Mildner et al., 2007; Prinz and Priller, 2010; Wolf et al., 2013).
TNFR	TNF	Pro-inflammatory factor, essential for progression of inflammation, phagocytosis, axonal sprouting, and better	(Green and Nolan, 2012; Jin et al., 2010; MacEwan, 2002; Oshima et al., 2009, 2009; Sist et al., 2014; Sriram,

		outcome of CNS injuries	2002; von Zahn et al., 1997).
TRkR and p75 NTR	BDNF, NGF, NT-3and 4	enhances axonal sprouting, neuronal plasticity and survival of neurons	(Fujita et al., 2008; Gomes et al., 2013; Huang et al., 2016; Kettenmann et al., 2011; Lai and Todd, 2008; Miwa et al., 1997; Mizoguchi et al., 2014; Sist et al., 2014; Trang et al., 2009).
P1 receptor	Adenosine	neuronal survival, microglia proliferation	(Fiebich et al., 1996; Haselkorn et al., 2010; Heese et al., 1997).
P_2X receptor	ATP	P_2X_4 involved in induction of neuropathic pain, P_2X_7 modulates other receptors such as	(Guo et al., 2005; Inoue et al., 2007; Tsuda et al., 2005, 2003).

		TLR4.	
P_2Y	ATP	regulates intracellular Ca^{2+} levels, inhibition of adenylyl cyclase. $P_2Y_{12}$is involved in induction of tactile allodynia and hyperalgesia	(Abbracchio, 2006; Fischer and Krügel, 2007). (Inoue and Tsuda, 2009; Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008).
AMPAR	Glutamate	implicated in TNF release and microglia chemotaxis	(Hagino et al., 2004; Liu et al., 2009).
NMDA	Glutamate	implicated in induction of inflammation (NO, TNF and IL-1β release by microglia)	(Kaindl et al., 2012; Tikka and Koistinaho, 2001).
mGLuR	Glutamate	group I (mGLuRs 1 and 5) group III (mGLuRs4, 6, 7 and 8) receptors are anti-inflammatory and group III are also	(Byrnes et al., 2009; Farso et al., 2009; Taylor et al., 2002; Vincent and Maiese, 2000).

	neuroprotective. group II (mGLuRs 2 and 3) receptors are pro-inflammatory and neurotoxic.	(Kaushal and Schlichter, 2008; Taylor, 2005)

1.3 Microglia origin, and role in development

Microglia are haematopoietic in origin. However, unlike peripheral macrophages, microglia are not derived from haematopoietic stem cells from bone marrow. Instead, microglia originate from erythromyeloid progenitors in the yolk sac that have capacity to differentiate into macrophages and microglia (Ginhoux et al., 2010; Kierdorf et al., 2013; Mizutani et al., 2012; Schulz et al., 2012). Microglia migrate through the circulatory system into the neuroectoderm in a fractalkine receptor (CX3CL1) and matrix metalloproteinase-8 and -9 dependent manner (Kierdorf et al., 2013). They have been found to have invaded all regions of developing mice brains at around E9.5 (Alliot et al., 1999). Microglia/macrophage present peaks at p8 in internal capsule, fornix, and at p12 in corpus callosum and cerebellar white matter in mice. Microglia/macrophage phenotypes are mostly ramified in the grey matter at this developmental age (Milligan et al., 1991). The microglia in the brain are actively involved in pruning of synapses during development as well phagocytosis of dying cells and debris (Paolicelli et al., 2011). In humans, microglia-like cells are found at around 3 weeks of gestation in the brain, and 9 weeks of gestation in the spinal cord. The migration of these cells closely follows the development of different CNS regions (Milligan et al., 1991; Rezaie and Male, 1999). The maturation of

microglia may take up to 35 weeks for humans post conception (Choi et al., 2016; Esiri et al., 1991).

Most regions in CNS during embryonic development undergo extensive remodelling (such as building and removal of the astrocyte scaffold involved in axon patterning) and apoptosis of neurons that have insufficient target interactions (Schafer et al., 2012; Wake et al., 2015). Microglia during CNS development are primarily involved in phagocytic activity to clear away the dying and degenerating neurons (Del Rio Hortega, 1932; Giulian and Baker, 1986; Paolicelli et al., 2011; Schafer et al., 2013, 2012; Sierra et al., 2016; Tremblay et al., 2010). They are also involved in synaptic pruning. Recently, it was shown that microglia engulf inactive pre-synaptic elements in a complement system (a system of proteins involved in phagocytosis and regulation of inflammation) dependent manner (Schafer et al., 2012). It was also shown that microglia play a role in remodelling and maintenance of synapses in the developing brain (Davalos et al., 2005; Nimmerjahn, 2005; Paolicelli et al., 2011; Schafer et al., 2013; Tremblay et al., 2010; Wake et al., 2009). Microglia are highly territorial and avoid contact with other microglia, while still constantly monitoring the immediate environment with their processes (Hanisch and Kettenmann, 2007). This territorial nature with the surrounding environment is considered as a necessity for structural and functional integrity of CNS architecture (Tremblay et al., 2010). Thus, microglia are critical for assisting in establishment of functional neural circuits during development and maintenance throughout adulthood, such that altered activity in microglia during this developmental stage could potentially results in diseases like autism or schizophrenia that affect the physiology of the CNS well into adulthood (Kulhara and Gupta, 2010).

1.4 Microglial role in normal CNS physiology

Housekeeping by microglia is a new area of research that has gained attention only relatively recently. Microglia are implicated in synapse and synaptic circuit maintenance in an experience-dependent manner (Tremblay et al., 2010; Wake et al., 2009). The fractalkine (CX3CL1) ligand is expressed on the surface of neurons and binds microglia through the chemokine receptor CX3CR1. CX3CR1 knockout mice show reduced microglia density and defective synaptic connectivity in postnatal hippocampus (Mizutani et al., 2012; Paolicelli et al., 2011), suggesting a microglial role in synapse maintenance in normal CNS. In vivo imaging experiments in mice suggest that at least one microglia in a given hour makes transient contact with axon terminals and dendritic spines (Wake et al., 2009). The contacts last for an average of 5 mins; however, injury or reduced neural activity results in retraction of microglia processes and reduces the number of microglia interactions to less than one per hour, without altering the basal motility rate of microglia (Wake et al., 2009). In mouse visual cortex, microglia contacted a small proportion of small dendritic spines which then disappeared if there was an absence of visual experience (Tremblay et al., 2010). This suggests that the microglia interact with the synapse during normal physiology (in absence of an insult), and might contribute to synaptic pruning, and rewiring in adults in an activity dependent manner (Tremblay et al., 2011). Furthermore, in healthy (naïve) young and aged adult mice, microglial processes containing structures akin to dendritic spines and axonal terminals have been observed, further suggesting that microglia regulate synaptic plasticity by phagocytizing these structures independent of inflammation not only during postnatal development, but throughout the entire lifespan (Tremblay et al., 2010).

1.5 Microglia role in ischaemic stroke and spinal cord injury

1.5.1 Ischaemic stroke

Ischaemic stroke is a devastating injury to the brain caused by a sudden reduction of cerebral blood flow, which results in a cascade of events that induces metabolic dysfunction, ionic imbalance, and excitotoxicity leading to apoptosis and necrosis neurons in the brain (Astrup et al., 1981; Danton and Dietrich, 2003; Dirnagl et al., 1999). Ischaemic stroke results in an infarct (permanent irreversible damage to brain) and penumbra (region surrounding the infarct that is metabolically unstable but recoverable) in the brain parenchyma (Astrup et al., 1981; Danton and Dietrich, 2003; Dirnagl et al., 1999). Immediately after ischaemia, microglial contacts with synapses are prolonged and followed by the disappearance of the presynaptic bouton (Wake et al., 2009). This suggests that microglia determine the fate of synapses at very early stages of ischaemia. Furthermore, damaged neurons in the infarct region release DAMPs into the extra cellular milieu (Gadani et al., 2015; Kim and Cho, 2016). These DAMPs include various classes of immunomodulatory molecules including proteins, nucleic acids, and metabolites (Gadani et al., 2015). DAMPs activate a repertoire of receptors on microglia such as Toll like receptors, purinergic receptors, metabotropic glutamate receptors, and scavenger receptors (involved in uptake of low-density lipoproteins) (Choi and Rothman, 1990; Gadani et al., 2015; Kettenmann et al., 2011; Kim and Cho, 2016; Murugan et al., 2013; Rodrigues et al., 2015; Vincent and Maiese, 2000). Microglia activated by DAMPs present at the site of injury release a wide range of pro- and anti-inflammatory factors that induce inflammation (Choi and Rothman, 1990; Clausen et al., 2008; Sist et al., 2014). NO, TNF, IL-6 and IL1-β are well

characterised pro-inflammatory factors released by activated microglia/macrophage during ischaemic injury (Clausen et al., 2008; Colton and Gilbert, 1987; Lambertsen et al., 2012; Sist et al., 2014; Tarkowski et al., 1995). ATP and glutamate are DAMPS that are released by injured neurons (Choi and Rothman, 1990; Davalos et al., 2005; Duan et al., 2009; Liu et al., 2009; Rodrigues et al., 2015). Numerous *in vitro* studies, and studies from peripheral immune system suggests they can be pro- and anti-inflammatory in a receptor dependent manner [6,24,26]. However, recent studies have shown that ATP and glutamate also induce chemotaxis of microglia to the site of ischaemic injury (core and penumbra) (Choi and Rothman, 1990; Davalos et al., 2005; Duan et al., 2009; Liu et al., 2009; Rodrigues et al., 2015). This raises the question whether ATP and glutamate alone are sufficient to act as DAMPs and induce inflammation through microglia.

Microglia and macrophages proliferate within the first three days post-stroke, and maintain their density and activated state for several weeks (Gelderblom et al., 2009). Studies suggest that acute inflammation triggered by microglia/macrophages is neuroprotective, and is needed for clearing the dead and dying debris (Faustino et al., 2011; Hayashi et al., 2006; Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hébert et al., 2007; Neumann et al., 2006). However, with greater severity of injury and prolonged inflammation, these processes could be neurotoxic/detrimental to outcome after ischaemic injury (Block et al., 2007; Jiang et al., 2012; Kaushal and Schlichter, 2008; Lai and Todd, 2008; Tang et al., 2014). Interestingly, ablation of microglia is equally detrimental and increases neuronal death (Hayashi et al., 2006; Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hébert et al., 2007; Neumann et al., 2006). TNF released by microglia highlights the dichotomy of inflammation. Inhibition of TNF released by microglia decreases infarct volume; however, total knock out of TNF exacerbates infarct volume (Bruce et

al., 1996; Dawson et al., 1996). This suggests that TNF is essential for protection of neurons after ischaemic injury but also contributes to toxic processes. Similarly, GMCSF (granulocyte macrophage colony stimulating factor), MCSFR (macrophage colony stimulating factor receptor), and TGFβ (Transforming growth factor beta) are factors that either expressed or released by microglia and are neuroprotective (Dhandapani and Brann, 2003; Kong et al., 2009; Mitrasinovic et al., 2005). Thus, microglia are important for progression of pathology and the processes that lead to recovery after ischaemic stroke.

1.5.2 Microglial role in diaschisis

Diaschisis or secondary dysfunction can result from distal inflammation and may occur after an initial insult such as ischemia or trauma to the brain (Basiri and Doucette, 2010; Nagamoto-Combs et al., 2007; Weishaupt et al., 2010). Secondary damage is due to activation of glial cells in regions distal from the injury (Nagamoto-Combs et al., 2007; Weishaupt et al., 2010). Previous studies have shown that SCM activation was seen in the cervical enlargement in rat spinal cord after a forelimb motor cortex ischaemic injury in the brain, as was degeneration of axons below the cervical enlargement whose cell bodies were not injured due to ischaemic (Weishaupt et al., 2010). The authors suggest that microglia in the spinal cord might be involved in secondary damage to the axons of neurons whose cell body is not injured (Weishaupt et al., 2010). However, the exact role of microglia recruited at these regions of secondary damage in spinal cord after an insult to brain remains largely unknown. After ischaemic stroke, there is increased plasticity, axonal rewiring, and remapping around the peri-infarct region as well as an increased plasticity in the spinal cord (Carmichael et al., 2005; Sist et al., 2014). It is possible

that microglia recruited to the regions of secondary damage in the spinal cord after cortical stroke may also play a role in plasticity and axonal rewiring in the brain and spinal cord as well as contributing to inflammation and secondary damage.

1.5.3 Microglia and spinal cord injury

Spinal cord injury (SCI) is commonly caused by a physical damage to the spine leading to laceration or compression of the spinal cord. SCI progression involves primary and secondary mechanisms of injury (Oyinbo, 2011). The primary mechanism involves initial damage to the spinal cord resulting in mechanical damage, haemorrhage and ischemia (Donnelly and Popovich, 2008; Prüss et al., 2011; Simon et al., 2009; Sroga et al., 2003). This primary damage causes the death of motor neurons, oligodendrocytes, astrocytes, and microglia at the site of injury (Gadani et al., 2015; Oyinbo, 2011). Primary mechanisms trigger secondary damage mechanisms through inflammation mediated by microglia and infiltrating macrophages (Donnelly and Popovich, 2008; Prüss et al., 2011; Simon et al., 2009; Sroga et al., 2003). Studies suggest that the extent and progression of secondary damage is determined by severity of primary injury (Dumont et al., 2001). Furthermore, it was also found that in spinal cord microlesions, blocking ATP and NO synthase reduced microglia process extension and migration to the site of injury (Dibaj et al., 2010), suggesting that NO and ATP play a role in recruiting microglia (Dibaj et al., 2010). Interestingly, it was also shown that a gradient of NO and ATP was required for recruitment of microglia (Dibaj et al., 2010). Microglia migrate to the site of primary injury within hours and generate pro-inflammatory factors including NO, TNF, IL1β, and IL6 (Ren et al., 2014). Notably, blocking TNF, IL6, and IL-1β reduces the neuronal death and demyelination

(potentially due to protection of oligodendrocytes or progenitor cells) suggesting that early activation of microglia/macrophage is detrimental in SCI (Ferguson et al., 2008; Mukaino et al., 2010; Nesic et al., 2001; Pineau and Lacroix, 2007). However, there are also potential beneficial roles for microglia in SCI. Inhibition of TGFβ and administration of GMCSF is neuroprotective after SCI (Ha et al., 2005; Kohta et al., 2009). Since microglia/macrophages are the primary cells that respond to TGFβ and GMCSF, this suggests that these factors induce a neuroprotective phenotype in microglia after SCI. The above findings suggest that early inflammation is detrimental to the outcome of SCI, but that microglia have a complex role in primary damage, secondary damage, and protection during SCI.

1.6 Heterogeneity of microglia in CNS

Neurons are a highly heterogenous cell population with varying morphology, surface receptors, and functions (Molyneaux et al., 2007; Temple, 2001). Similarly, astrocytes are heterogenous cells that have different morphology and function that is dependent on their location in CNS (Chaboub and Deneen, 2012). This suggests that microglia, known for their sensitivity to a variety of factors in their immediate environment, could be an equally heterogenous population of cells, and that the heterogeneity of neurons, astrocytes, and region specific differences in brain architecture and neurochemistry could have an impact on microglial phenotype. De Haas et.al. (2008) were the first to show heterogeneity in immunological receptor expression in microglia from cortex, striatum, hippocampus, cerebellum, and spinal cord of adult mice (de Haas et al., 2008). It was found that CD80 surface expression was highest in cortex, F4/80 surface expression was the highest in hippocampus; CD40, CXCR3, and TREM-2b expression was

highest in the cerebellum; and CD11b, CD86, and CCR9 surface expression was highest in SCM (de Haas et al., 2008). CD11b, CD40, and TREM-2b (Triggering receptor expressed on myeloid cells 2 b) surface expression was lowest in cortex; CD45, CD80, CXCR3,, and CCR9 surface expression was lowest in hippocampus; and CD86 and F4/80 surface expression was lowest in cerebellum (de Haas et al., 2008). More recently, it was shown *in vitro* that the cortical, and hippocampal microglia released higher levels of NO and TNF compared to microglia from striatum, thalamus, and brain stem on activation with ATP (Lai et al., 2011). This study further showed that the regional heterogeneity was not fixed, and that mimicking the environment of a specific region was enough change the phenotype of microglia from one region to another (Lai et al., 2011). This begs the question how different environments within the CNS affect SCM functions in spinal cord disease/injuries. Understanding the influence of region of origin and the spinal cord environment may help in discovering and developing better therapeutic interventions for spinal cord disorders.

1.7 Objectives of the research described in the thesis

As described above, the region of origin and the immediate environment, age and sex of the host animal contribute to microglial phenotype (Grabert et al., 2016a; Lai et al., 2013, 2011; Sorge et al., 2011). The functional characteristics of BM (brain microglia) have been well characterized in in *in vivo* and *in vitro* models of injury. However, microglia in the spinal cord (SCM, spinal cord microglia) are not well characterized. To address this knowledge gap, the following research objectives were defined to characterize and compare the functional differences between BM and SCM. Our primary objective was to define the differences in the inflammatory response profile

SCM under different activation paradigms. Within this primary objective, several specific objectives are addressed in this thesis. The research reported in Chapter 2 investigated if the inflammatory profile of SCM differs from BM *in vitro* after activation of the TLR4 signaling pathway via LPS. Our findings suggested that SCM have a reduced inflammatory profile after TLR4 activation. However, the mild trypsinizatoin methods used to isolate SCM resulted in formation of cellular contaminants. For this reason, in Chapter 3 we defined an improved protocol to achieve highly pure isolated SCM cultures. In Chapter 4, we used this improved isolation method to examine the functional response characteristics of SCM and BM after activation with the endogenous immunomodulators ATP and glutamate. Previously it had been shown through agonist and antagonist treatments that ATP and glutamate induce pro- and anti-inflammatory signaling in microglia (Domercq et al., 2013; Färber and Kettenmann, 2006; Sperlágh and Illes, 2007). However, those studies used concentrations that were non-physiological or induced constitutive receptor activation which does not mimic the stochastic activation of a receptor in normal physiology, injury or disease. Hence, in Chapter 4 we compared SCM and BM responses to physiological concentrations of glutamate and ATP then measured the pro-and anti-inflammatory factors released into the media. Additionally, because microglia are highly plastic cells that can adapt their phenotype to mimic microglia from other regions of the brain when exposed to mixed glia-conditioned media from those other regions, we also tested whether incubating BM in conditioned media from spinal glia mixed cultures would alter their inflammatory profile. Our data suggest that physiological concentrations of ATP and glutamate do not elicit significant cytokine release, and that BM will change their phenotype in response to conditioned media from spinal cultures. Finally, in Chapter 5 we investigated potential differences in neuroprotection and cytokine release by BM and SCM in neuronal co-

culture systems in a model of ischaemic neuronal injury (oxygen glucose deprivation, OGD). While subtle differences in culture viability between SCM and BM were observed after moderate OGD injury, our data suggest that the response of SCM and BM to this injury model is conserved among the different regions of origin.

Chapter Two: Reduced inflammatory phenotype in microglia derived from neonatal rat spinal cord versus brain

* a version of this chapter appears as a published manuscript in PLOS ONE (Baskar Jesudasan SJ, Todd KG, Winship IR (2014) Reduced Inflammatory Phenotype in Microglia Derived from Neonatal Rat Spinal Cord versus Brain. PLoS ONE 9(6): e99443. doi:10.1371/journal.pone.0099443)

2.1 Introduction

Microglia constitute 5 to 15% of the central nervous system (CNS) total cell population (Aloisi, 2001; Giulian and Baker, 1986; Rezaie and Male, 1999). In basal physiological conditions (i.e. prior to injury or activation), microglia have ramified morphology with long processes that monitor the extracellular environment surrounding the cells of the CNS (Wake et al., 2009). After brain trauma or infection, microglia become activated and withdraw their processes to assume amoeboid and spherical morphologies (Lai and Todd, 2006a). Amoeboid microglia are capable of secreting anti-inflammatory factors, pro-inflammatory factors, prostaglandins, cytokines, and reactive oxygen species (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a). These microglial effectors interact with surrounding neurons and other glial cells and can initiate trophic as well as toxic signaling pathways (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a). Upon further activation, microglia assume a spherical morphology and become predominantly phagocytic, acting to clear the CNS of dying cells and other debris (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a).

Investigations of the role of microglia in CNS injury such as stroke demonstrate that the microglial response to injury is complex (Castillo et al., 2000; Clausen et al., 2005; Hill et al., 1999; Kader et al., 1993; Lambertsen et al., 2009; Yenari et al., 2010). After injury to the brain, pro-inflammatory effectors such as NO, TNF, IL–6 and IL-1β are up-regulated within 24 hours and contribute to both neurotrophic and neurotoxic pathways (Castillo et al., 2000; Clausen et al., 2005; Hill et al., 1999; Kader et al., 1993; Lambertsen et al., 2009; Yenari et al., 2010). Similarly, there is an acute increase in TNF-α, IL-1β and IL-6 in the spinal cord after spinal cord injury that contributes to cell death and lesion expansion (Smith et al., 2012; Yang et al., 2005).

As a major source for TNF-α, IL-1β, IL-6, and trophic factors including brain-derived neurotrophic factor (BDNF), microglia are major contributors to increased levels of these cytokines after CNS injuries (Green and Nolan, 2012; Hill et al., 1999; Hua et al., 2012; Kader et al., 1993; Kettenmann et al., 2011; Lai et al., 2011; Lai and Todd, 2006b; Lambertsen et al., 2012; Matsui et al., 2010; Smith et al., 2012; Werry et al., 2011). The consequences of altering the microglial response to injury are difficult to predict, as microglial effectors can have both trophic and toxic consequences after injury (Green and Nolan, 2012; Hill et al., 1999; Hua et al., 2012; Kader et al., 1993; Kettenmann et al., 2011; Lai et al., 2011; Lai and Todd, 2006b; Lambertsen et al., 2012; Matsui et al., 2010; Smith et al., 2012; Werry et al., 2011). Nonetheless, several studies have examined the functional response profiles of microglia upon activation to define their secretory profile and morphological response to brain injury or infection (Green and Nolan, 2012; Hill et al., 1999; Hua et al., 2012; Kader et al., 1993; Kettenmann et al., 2011; Lai et al., 2011; Lai and Todd, 2006b; Lambertsen et al., 2012; Matsui et al., 2010; Smith et al., 2012; Werry et al., 2011).

Most studies investigating the functional activation profiles of microglia are done in BM. Data from these studies have confirmed that microglia can secrete a wide variety of cytokines, with the amount and type of effector released varying with the nature of the activating stimulus. Activation with lipopolysaccharide (LPS) increases the release of TNF-α, IL-1β, IL-6 and nitric oxide (NO) in neonatal BM (Lai et al., 2011; Nakamura et al., 1999). Recently, it was demonstrated *in vitro* that BM exposed to conditioned media collected from neurons subjected to moderate hypoxic injury were neuroprotective, but BM exposed to media collected from neurons subjected to severe hypoxic injury were neurotoxic (Kettenmann et al., 2011) These results and data showing severity dependent-expression of cytokines by SCM after SCI suggests that

functional microglial response properties are dependent on the severity of neuronal injury (Kettenmann et al., 2011; Smith et al., 2012).

In addition to a differential response to injury severity, microglia isolated from distinct regions of the brain have different effects on cell survival [18]. For example, activated microglia from cortex and hippocampus exhibit a neurotoxic profile relative to microglia from brainstem, striatum and thalamus (Lai et al., 2011). TNF release by the microglia from cortex and hippocampus is significantly higher than that of other brain regions after activation with ATP (Lai et al., 2011). Despite the observation that microglial effector profiles vary between regions in the brain, and the interest in understanding the role of microglia after spinal injury, very few studies have investigated activation profiles of SCM in culture (Matsui et al., 2010; Werry et al., 2011). In this study, we sought to characterize the morphological and secretory response profiles to an activating stimulus in SCM as compared to BM. To facilitate comparison, SCM and BM cultures were established from the same neonatal rat pups using a mild trypsinization protocol (Saura et al., 2003) The primary microglia cultures were activated with the bacterial endotoxin LPS, an established and well-characterized activator of microglia (Aderka et al., 1989; Broderick et al., 2000; Durafourt et al., 2012; Hill et al., 1999). The differences in the activation pattern of SCM and BM were then gauged by characterizing microglial morphology, cytokine release, NO release, and phagocytosis.

2.2 Materials and Methods

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta.

2.2.1 Primary brain and spinal cord microglia cultures

Mixed glia cultures were prepared from four brain and twenty spinal cord of post-natal Day 1 Sprague-Dawley rats (Lai and Todd, 2008; Siao and Tsirka, 2002). The meninges and blood vessels were removed from the brains and spinal cords, then tissue was finely minced and dissociated enzymatically by 0.25% Trypsin-EDTA for 20 mins t 37° C. Trypsin was inactivated with Dulbecco Modified Eagle Medium/Ham's F12 (DMEM/F12; Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 200 U/ml penicillin, 200 μg/ml streptomycin (P/S; Gibco). The brain and spinal cord tissues were triturated mechanically in DMEM/F12 in 10% FBS and 200 U/ml penicillin, 200 μg/ml streptomycin plated on poly-L-lysine coated 12 well plates at 1.7 x 10^7 cell/ml.

After 14-21 days *in vitro* (DIV), microglia were isolated by mild trypsinization (Saura et al., 2003). Briefly, 0.25% trypsin-EDTA was diluted in 1:3 ratio in DMEM/F12 and 200 U/ml penicillin, 200 μg/ml streptomycin media were added to brain/spinal mixed primary cultures and the mixture were incubated at 37°C in a humidified 5% CO_2 incubator for 20-50 mins. DMEM/F12 with 10% FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin were added after aspirating out the diluted trypsin-EDTA to the isolated microglia in the culture dish to inactivate trypsin and the culture dish was maintained at 37^0C for 5 min. DMEM/F12 with 10% FBS was removed after 5 minutes and DMEM/F12 containing 2% P/S was added to the isolated BM and SCM. Isolated BM and SCM were allowed to recover in DMEM/F12 containing 200 U/ml penicillin, and 200 μg/ml streptomycin overnight prior to any treatment. For LPS activation, BM and SCM were treated with 1μg/ml (final concentration in media) LPS for 24 hrs. At 24 hrs after

the LPS treatment the media from BM and SCM were assayed either for pro- and anti-inflammatory effectors released in the media, phagocytic activity or fixed with 10% formalin for morphometric analysis.

2.2.2 Immunocytochemistry

Immunocytochemistry was used to confirm culture purity and quantify microglia. Isolated brain and spinal microglia cultures were washed three times with phosphate buffered saline (PBS) and fixed with 10% formalin for 20 mins. After three washes with PBS the cultured cells were permeabilized and blocked for 40 mins with 0.1% Triton X-100 and 10% normal horse serum. Iba1 (ionized calcium binding adaptor molecule 1), a marker for microglia, was used to identify SCM and BM. Isolated brain and spinal microglia were incubated with a rabbit anti-Iba1 primary antibody (1:1000, Wako) at 4°C overnight. For immunofluorescent visualization of culture purity, the cultures dishes were then washed three times with PBS and incubated with an anti-rabbit Alexa 647 secondary antibody (1:200) for 1 hr at room temperature. After three washes, DAPI in vectashield mounting medium was applied and a cover slip was mounted on top of cells. Negative controls without primary antibody were used to rule out non-specific labeling by the secondary antibody. Epifluorescent images were acquired using Leica DMI 6000B microscope mounted with Leica DFC365 FX monochrome camera at 20X.

Microglia number was quantified by counting DAB (3,3'-diaminobenzidine)-positive cells. The procedure is similar to fluorescent immunocytochemistry up to the primary antibody incubation. After overnight incubation with primary antibody (rabbit anti-Iba1, 1:1000), the cultures were washed three times with PBS and labeled with a secondary antibody, biotinylated

donkey anti-rabbit IgG (Santa Cruz, 1:200) for 1 hr at room temperature. After three washes the cultures were incubated with avidin-biotin complex (ABC staining kit, Thermo Scientific) for 30 minutes at room temperature. DAB was prepared in PBS containing 3% H_2O_2 and cultures were treated according to manufacturer protocols. Iba1-immunopositive cells after DAB visualization were counted in a field area of 0.785 mm^2 under 20x magnification and bright field illumination. Average SCM and BM counts (in four independent experiments) were determined from the average count in six such field areas in each of four wells of a 12 well culture plate. The total number of Iba1-immunopositive cells per well was counted using the following formula,

Total number of SCM per well = Average SCM counts per 0.785mm^2 X 483

where 483 times the area of a field view is the total surface area of a single well in a 12 well culture dish. An unbiased observer verified SCM counts.

For morphology analysis, control and LPS activated Iba1 labeled microglia were counted from 6 fields acquired from three independent experiments. The images were acquired with DMI6000B Leica inverted microscope mounted with Leica DFC365 FX monochrome camera.

2.2.3 Nitric Oxide (NO) assay

NO in the media was assessed indirectly via its stable metabolite nitrite. 1 ml of media was collected after 24 hours after treatment or no treatment control. 100µL of LPS treated or no treatment control media was added per well of 96-wellplate with 2 replicates per condition. 50µL of 1% sulfanilamide (in 3 M HCl) was then added to the wells, followed by the equal volumes of

0.02% N-naphthylethylenediamine. The final mixture was read on a microplate reader at 540 nm (Molecular Devices SpectraMax M5).

2.2.4 Enzyme Linked Immunosorbent assays (ELISA)

A commercial ELISA kit was used to measure IL-1β and IL-6 in media (DuoSet, R&D Systems Minneapolis, USA)). ELISA procedures were carried out according to manufacturer protocols. For BDNF ELISA, a competitive ELISA protocol was used. In brief, media and recombinant BDNF standards diluted in PBS were mixed 1:1 with goat anti BDNF (1:500, Santa Cruz, Texas, USA) and incubated with 96-well plates in the cold room overnight. This mixture was aspirated following incubation and the plates were washed three times with PBS. The plates were then blocked with the ELISA diluent (1% bovine serum albumin in PBS) for 1 h. After blocking, the plates were washed 3 times with PBS and incubated with anti-goat IgG antibody conjugated to horseradish peroxidase (1:2000 in ELISA diluent, Santa Cruz, Texas, USA) for 30 min. The chromagen tetramethylbenzidine (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 sulfuric acid was added to stop the reaction, and the plates were read on microplate reader at 450 nm (Molecular Devices SpectraMax M5). All procedures were carried out at room temperature unless otherwise specified.

2.2.5 Multiplex bead based immunoassays for cytokines.

Rat TNFα and IL-10 were quantified using BD Cytometric Bead Array kit (BD Biosciences, New Jersey, USA). Samples were prepared according to the manufactures protocol.

Briefly, 50 μL of control or LPS treated microglia culture media were added to 50 μL of multiplex capture beads (TNF-α, IL-10) in a v-bottom 96 well plate. The v-bottom 96 well plate was placed in an orbital shaker for 5 min at 500 rpm and then incubated at room temperature for 1 hr. 50μL of phycoerythrin detection reagent was added to each sample in v-bottom 96 well plate and placed in an orbital shaker for 5 min at 500 rpm. The plate was then incubated at room temperature for 2 hr. The v-bottom 96 well plate was then centrifuged at 2000g and supernatant was aspirated out. The multiplex capture beads in v-bottom 96 well plates were re-suspended in 150μL of wash buffer and placed in a orbital shaker for 5min at 500 rpm. The multiplex capture bead in wash buffer where then analyzed by BD FACSCantoII flow cytometer. Data analysis was done using FCAP Array software from BD Biosciences.

2.2.6 Phagocytosis Assay

Phagocytosis was measured using a fluorescent bead uptake assay (Hua et al., 2012) Briefly, microglia were activated for 24 hours with LPS (1μg/ml), and 2.5 x 10^6 carboxylate-modified 1 μm beads (per well, Sigma, Missouri, USA) were added to the LPS activated or control microglia for an hour. After incubation with these beads, the cells were washed with Hank's Balanced Salt Solution to wash away beads that were not phagocytized. BM and SCM were lysed in 1% Triton-X detergent and lysate was transferred to 96-well plate. Fluorescence emitted by cell lysate was measured using a microplate reader (Molecular Devices SpectraMax M5). The excitation and emission spectra of plate reader were set to 470nm and 505 nm respectively. Negative controls were treated as above with the exception that cells with beads were maintained at 4 degrees Celsius.

2.2.7 Viability assay

Viability of microglia was assessed via a colorimetric assay for metabolic activity (mitochondrial activity). MTT can be reduced to an insoluble formazan dye by NAD(P)H-dependent cellular oxidoreductase enzymes. The absorbance of light by the formazan dye in solution can be measured at 540 nm by an UV spectrometer. BM and SCM were incubated in 0.05mg/ml of MTT in PBS for 2 hrs in CO_2 incubator. After 2 hrs, the MTT solution was aspirated and the cells were lysed in 200 microliters of DMSO. The absorbance of lysates was measured at 540 nm by an UV spectrometer

2.2.8 Statistical Analysis

Results are represented as percentage of total population (morphology analyses), raw values normalized to total protein (NO, cytokines and trophic factors), and percentage over relative control (phagocytosis assay) ± standard error of mean. One-way ANOVA was used to determine main effect of treatment on BM and SCM followed by the Newman-Keuls *post hoc* to test for significance between treatment groups. One-sample t-tests were performed to analyse significant changes in phagocytic activity between treatments and controls (as phagocytosis was normalized to control levels). n represents a single independent experiment (i.e. an independent culture preparation) with a minimum of three technical replicates. Each technical replicate is a well in a 12 well culture plate, except for morphology studies where n represents a single well of an independent experiment. * represents $p < 0.05$. All statistical analyses were done using Graphpad Prism version 5.

2.3 Results

2.3.1 Spinal cord microglia can be isolated by mild trypsinization

Mild trypsinization has been used successfully to isolate BM from neonatal mice (Saura et al., 2003), but its efficacy in isolating SCM had not been determined. To determine if mild trypsinization can isolate SCM from neonatal rat primary mixed glial populations, we applied 0.08% trypsin-EDTA to 2-3 wk old primary mixed glial cultures derived from brain and spinal cord. The optimal incubation time required to isolate SCM was determined from mild trypsinization durations of 20 mins, 30 mins, 40 mins, and 50 mins. Twenty min of mild trypsinization produced the highest yield of SCM (Figure 2-1). SCM count decreased with increased duration in trypsin and at 40 min there was nearly a 60% loss of SCM compared to the 20 mins time point. The purity of BM and SCM cultures were assessed by immune labeling against Iba1 (a microglia/macrophage marker) and was found to be ≥ 95% for BM and SCM (n = 3 independent experiments, see Figure 2-1A representative micrograph of SCM and BM).

2.3.2 Morphology of BM and SCM on activation with LPS (lipopolysacharide)

Lipopolysaccharide (LPS) is an endotoxin from gram-negative bacteria commonly used to activate immune cells. To evaluate if SCM and BM assume different morphology after activation, microglia were incubated overnight in 1ug/ml LPS in media (DMEM with 2% P/S) for 24 hrs. Ramified, amoeboid, and spherical morphological states were apparent in control (media alone) and activated microglia cultures from brain and spinal cord. Consistent with previous studies (Lai and Todd, 2008), morphological status was assigned based on the number

of microglia processes: Microglia that have more than two primary processes (processes stemming from cell body of microglia are considered primary) were considered ramified, those with one process were considered amoeboid and those with no process extension were considered spherical. A significant effect of treatment group (BM control, BM LPS, SCM control, SCM LPS) was observed for the percentage of amoeboid and spherical morphologies ($F_{(3,8)}$ = 14.32, p = 0.0014, $F_{(3,8)}$ = 8.125 p = 0.0082, respectively), while a main effect on percentage of ramified microglia of BM and SCM narrowly missed statistical criterion for significance ($F_{(3,8)}$ = 3.893 p = 0.0551) (Figure 2-2). Newman-Keuls post-hoc tests showed that LPS induced a significant increase in spherical morphology in BM (Figure 2-2C, LPS 55.27% ± 4.12 vs. Control 27.99% ± 9.15, respectively $p < 0.05$) and SCM (Figure 2-2C. LPS 60.72% ± 6.88 vs Control 28.27% ± 0.80 $p < 0.05$), but the percentage of spherical microglia in basal or LPS-activated states was not significantly different between BM and SCM. Interestingly, LPS induced significantly less amoeboid morphology in SCM compared to SCM control microglia as well as LPS-activated BM (Figure 2-2B, SCM LPS 16.18% ± 0.86, SCM Control 37.50% ± 4.47, BM LPS 34.32% ± 3.04, $p < 0.05$).

2.3.3 Secretion of pro-inflammatory and anti-inflammatory effectors by BM and SCM

TNF, IL-1β and NO are well-characterized pro-inflammatory effectors with established roles in inflammation and neurotoxicity after an injury to the CNS, and release of these inflammatory effectors by microglia varies with brain region of origin (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a). To determine if the release of inflammatory effectors differed between SCM and BM, microglia were activated with LPS (1μg/ml) for 24 h. Relative changes

in secreted levels of TNF, IL-1β and NO in media were measured using multiplex bead based flow cytometry for TNF, ELISA for IL-1β and the Greiss reaction assay for NO. One-way ANOVA identified a significant main effect of LPS treatment on the release of TNF($F_{(3,16)}$ = 5.201, p = 0.0107), IL-1β ($F_{(3,16)}$ = 4.21, p = 0.0225) and NO ($F_{(3,24)}$ = 15.76, p = 0.0001) (Figure 2.5.3). Newman-Keuls post-hoc tests revealed that TNF, IL-1β and NO release by LPS-activated BM were significantly higher than basal release (Figure 2-3A, Figure 2-3B, Figure 2-3C, TNF p <0.05; IL-1β p <0.05; NO p <0.001). LPS activated SCM showed a non-significant trend towards an increase in release of TNF, and TNF release from SCM was significantly less than BM (p <0.01). LPS did not induce significant release of IL-1β from SCM, and LPS-induced release was significantly reduced relative to BM ($p < 0.05$). NO release was significantly increased in LPS activated SCM compared to SCM control ($p < 0.05$); though it was still significantly less than LPS activated BM ($p < 0.01$). No differences were found in the basal release of TNF, IL-1β or NO by BM vs. SCM. Notably, reduced release by SCM could not be explained by differences in cell viability after LPS treatment. Tetrazolium dye (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced to insoluble formazan only in cells with functional mitochondria, was used to assay the viability of microglia in culture. There was no significant difference in microglial viability between treatment groups (Figure 2-6).

BM are also capable of releasing IL-6, IL-10, and BDNF, well known mediators of inflammation and cell survival in CNS injury or disease (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a). Here, SCM and BM were treated with LPS (1μg/ml in media) for 24 hours to determine if there are any differences in their release of IL-6, IL-10 and BDNF. Cytokine levels were evaluated by bead based flow cytometry for IL-10, ELISA for IL-6 and competitive ELISA

for BDNF. One-way ANOVA showed a trend towards a main effect of treatment group on IL-6 release ($F_{(3,8)} = 3.632$, p = 0.0642) but not IL-10 ($F_{(3,16)} = 0.6086$, p = 0.619) or BDNF ($F_{(3, 8)} = 0.142$, p = 0.932) (Figure 2-4).

2.3.4 LPS mediated phagocytosis in BM and SCM

Microglia can assume a phagocytic phenotype in response to activating stimuli. CNS diseases or injuries result in dying neurons and debris, which are cleared in part by phagocytic microglia (Lai and Todd, 2008; Rezaie and Male, 1999). Pro-inflammatory cytokines such as TNF have been shown to increase phagocytosis by microglia (Rezaie and Male, 1999)], and amoeboid and spherical microglial morphologies have been associated with phagocytic status (Hu et al., 2012; Rezaie and Male, 1999). LPS activation has been shown to increase phagocytic activity in previous studies of cultured BM. In this study, we compared the phagocytic activity of BM and SCM on activation with LPS (1μg/ml in media) for 24 hours (Figure 2.5.5). We show that LPS significantly increased the phagocytic activity of BM ($t_{(7)} = 2.815$, $p < 0.05$) but not SCM ($t_{(2)} = 1.336$, p = 0.3132) (Figure 2-5).

2.4 Discussion

2.4.1 Summary

In this study, we showed that SCM can be isolated from primary mixed glial cultures by mild trypsinization. Additionally, we showed that the LPS-activated phenotype of SCM is different from that of BM. SCM microglia exhibited a significantly less amoeboid morphology relative to BM after LPS activation. In BM, LPS significantly increased the release of NO, TNF

and IL-1β. Interestingly, LPS did not induce a statistically significant increase in the release TNF and IL-1β in SCM, and levels of all three of these pro-inflammatory cytokines in media from activated SCM were significantly reduced relative to BM. Conversely IL-6 and IL-10 release by LPS-activated SCM exhibited a non-significant trend towards higher release than BM. LPS activation was also associated with an increase in phagocytic activity of BM but not SCM. Overall, our findings suggest that SCM exhibit less inflammatory and phagocytic phenotype than BM in response to activation with LPS. Interestingly, a recent *in vivo* study using fluorescence-assisted cell sorting demonstrated that expression levels of the surface receptors CD45 and CD11b are higher in spinal microglia than brain microglia in naïve mice (Lai et al., 2011; Olson, 2010). In cells sorted three days after viral infection with Theiler's murine encephalomyelitis virus, surface receptors (including CD45, CD11b, CD40, CD80, CD86) were upregulated in spinal microglia compared to brain microglia (Lai et al., 2011; Olson, 2010). Combined with our findings, these data suggest that the activating stimulus and local environment interact to determine the phenotypic response of microglia (Lai et al., 2011; Olson, 2010).

2.4.2 Isolation of spinal microglial culture via mild trypsinization

Regional heterogeneity (in morphology and cytokine release) of microglia within the CNS has been observed both *in vitro* and *in vivo* based on region of origin (Hua et al., 2012; Lai et al., 2011; Smith et al., 2012). For example, microglia from neonatal cortex and hippocampus are more neurotoxic than those from striatum, thalami or brainstem (Lai et al., 2011). Notably, microglia from neonatal striatum have a more ramified morphology (presence of extensive processes) and reduced release of pro-inflammatory effectors relative to cortical microglia.

However, the morphological and functional heterogeneity of BM vs. SCM has not previously been assessed. Given that SCM can affect the fate of neurons during development and after spinal cord injury, an understanding of the functional response properties of SCM is important. Primary SCM cultures are not common in the literature and have not been characterized to the extent of BM primary cultures (Durafourt et al., 2012; Gingras et al., 2007; Kettenmann et al., 2011; Olson and Miller, 2004; Yip et al., 2009). Mild trypsinization (0.05% - 0.12% trypsin EDTA) is an established protocol for isolation of primary BM, with higher yields than isolation via shaking, but has not been well validated for use in SCM. Here, we demonstrate that mild trypsinization can also be used to isolate microglia from mixed glial cultures derived from neonatal rat spinal cord tissue.

2.4.3 Morphology and phagocytic activity of BM and SCM

In the undisturbed CNS, microglia typically assume a ramified morphology, with extensive processes extending from their soma (Kettenmann et al., 2011; Lai and Todd, 2008, 2006a). These processes contain membrane sensors to detect activating agents in the surrounding microenvironment. Activation of microglia typically results in a transition to an amoeboid morphology and expression of genes regulating cytokine (inflammatory, anti-inflammatory, and trophic) expression. Continued or extreme activation may induce a spherical and phagocytic morphology [34]. Previously studies have demonstrated that there is region-specific heterogeneity in BM morphology and in the secretory profile of microglia isolated from different brain regions (Lai et al., 2011). We did not find any significant differences between morphology of SCM and BM under basal conditions (Figure 2-2). However, SCM exhibit less amoeboid

morphology after LPS activation and a trend towards a persistent ramified morphology relative to BM. These morphological differences correlate with phagocytic activity of SCM and BM, where we see an increase in phagocytic activity in BM with increase in amoeboid and spherical form (Figure 2.5.2B, 2C and Figure 2-5). Previous studies showed that TNF can increase the phagocytic activity of microglia, while IL-10 can decrease phagocytic activity (Aderka et al., 1989; von Zahn et al., 1997). We show that TNF release is increased to a greater extent in LPS activated BM relative to SCM (IL-10 release is comparable between SCM and BM, (Figure 2-3 and Figure 2-4). These effector profiles are consistent with the reduced phagocytic activity of SCM (Figure 2-5).

2.4.4 Cytokine Profile of BM and SCM

Cytokines play a major role in determining the outcome of CNS injuries and diseases and microglia are one of the primary sources of cytokines in the CNS. Moreover, microglia are a dynamic population of cell capable of being neurotoxic and or neurotropic depending on the severity of the injury (Diab et al., 1997; Hill et al., 1999; Hu et al., 2012; Jin et al., 2010; Kader et al., 1993; Kettenmann et al., 2011; Lai et al., 2011; Lai and Todd, 2008; Lambertsen et al., 2012; Rezaie and Male, 1999; Smith et al., 2012). It has been shown that TNF-α, IL-1β, NO, and IL-6 are up-regulated after CNS injury (Castillo et al., 2000; Clausen et al., 2005; Green and Nolan, 2012; Hill et al., 1999; Jin et al., 2010; Lambertsen et al., 2012, 2009; Nakamura et al., 1999; Tarkowski et al., 1995; Yang et al., 2005; Yenari et al., 2010). However, the consequences of increased cytokine release are multifactorial. TNF exhibits both neurotoxic as well as neuroprotective actions after brain or spinal injury (Smith et al., 2012; Sriram, 2002). While up-

regulation of TNF can exacerbate neuronal injury, (Oshima et al., 2009; Sriram, 2002; Takeuchi et al., 2006; Taoufik et al., 2007; Taylor, 2005; Wang, 2004) genetic knockout of TNF or interference with TNF or TNF binding sites can increase neuronal tissue loss and reduce compensatory neuroplasticity after CNS injury (Araki et al., 2001; Centonze et al., 2009; Hill et al., 1999). IL-1β has a more typical inflammatory and neurotoxic profile, and inhibition of IL-1β reduces neuronal tissue damage after brain injury (Jin et al., 2010; Kettenmann et al., 2011; Lambertsen et al., 2009; Nakamura et al., 1999; Smith et al., 2012). Similarly, NO is an inflammatory and neurotoxic mediator known to contribute the lesion growth in spinal cord injury, stroke, and brain injury by the reactive metabolite perioxynitrite (Clausen et al., 2005; Huang et al., 1994; Kader et al., 1993; Kettenmann et al., 2011; Lambertsen et al., 2009). As such, the relative reduction in the release of pro-inflammatory effectors TNF, IL-1β and NO) by SCM relative to BM suggests that SCM might have a less neurotoxic profile after activation by LPS *in vitro*.

It is well known that IL-6 and IL-10 are upregulated after an injury or disease in the CNS (Diab et al., 1997; Lambertsen et al., 2012; Woodroofe et al., 1991; Yang et al., 2005) and that microglia are capable of releasing IL-6 and IL-10 (Aderka et al., 1989; Cafferty, 2004; Green and Nolan, 2012; Ma and Zhu, 2011; Werry et al., 2011). IL-6 has both pro- and anti-inflammatory actions, and is a major mediator of the brain's immune response to trauma or infection. (Bethea et al., 1999; Knoblach and Faden, 1998; Leibinger et al., 2013). Conversely, IL-10 is associated with a primarily protective role after CNS injury (Aderka et al., 1989; Armstrong et al., 1996; Jin et al., 2010; Ledeboer et al., 2002). IL-6 and IL-10 both down-regulate the expression of TNF and IL-1β and thereby reduce the neurotoxicity mediated by these pro-inflammatory cytokines (Broderick et al., 2000; Diab et al., 1997). Our data suggest

that release of these mediators by BM and SCM is comparable, with a trend towards increased LPS mediated release of IL-10 and IL-6 by SCM relative to BM (Figure 2-4). These data provide further support for the postulate that SCM exhibit a less inflammatory phenotype than BM after activation with LPS *in vitro*.

LPS has been associated primarily with a pro-inflammatory function due to its interactions with Toll like receptor 4 (TLR4). TLR4 is an essential component of the immune response and is known to up-regulate inflammatory cytokines via both myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways (Erta et al., 2012). Activation of the TLR4 MyD88-dependent pathway induces the production of TNF and IL-6 (Erta et al., 2012). However, the TLR4 MyD88-independent pathway, induces the production type 1 Interferon (IFN) gene products, but not TNF or IL-6, and mice deficient in0 yD88 did not produce TNF and IL-6 (Erta et al., 2012). Differences in the release of TNF by LPS activated SCM relative to BM (Figure 3A) may therefore suggests differential weighting of the MyD88-dependent and -independent pathways in BM and SCM.

2.4.5 Limitations

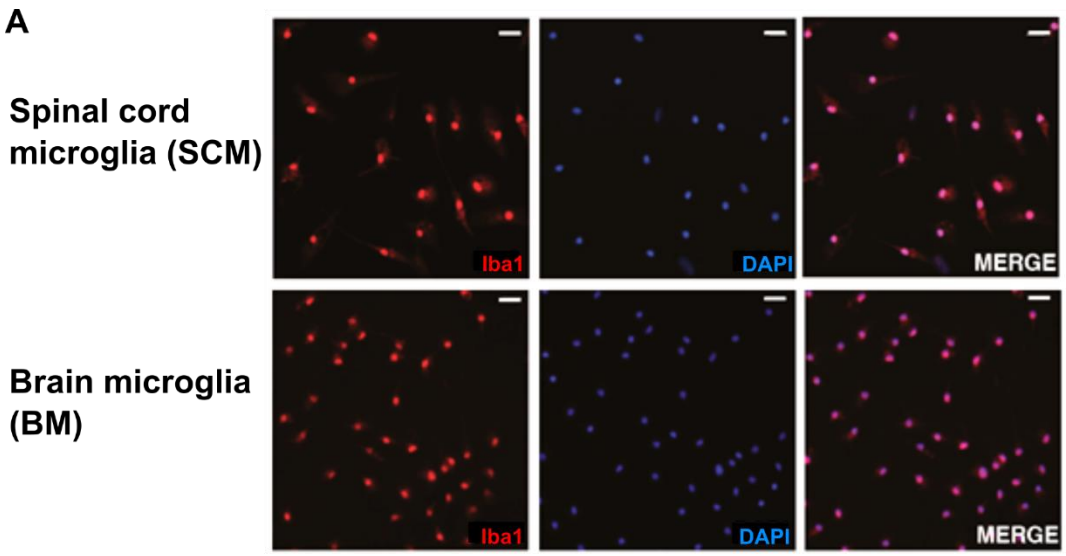
In vitro culture systems are a powerful reductionist model to study microglia in isolation. Commonly used activators such as LPS can act on receptors on other CNS cells including neurons and astrocytes. Hence, isolated microglia cultures improve resolution of microglial activation profiles without the interference from other CNS constituents. Nonetheless, in *vitro* models only mimic injury or inflammation found *in vivo*, and these simplified models *in vitro* may not reflect the whole spectrum of effectors involved *in vivo.* Notably, myelination is

incomplete in day one neonatal rats and cultures, and this environmental difference prior to isolation may influence microglia phenotypes (Downes and Mullins, 2014). Similarly, recent studies show increased expression of purinergic receptors involved chemotaxis and phagocytosis in adult microglia relative to neonates (Butovsky et al., 2013; Haynes et al., 2006; Kurpius et al., 2007; Lai et al., 2013). Genes regulating factors such as cell adhesion, proliferation, migration, complement system activity, and transcription regulation also exhibited differential expression in adult vs. neonatal microglia (Butovsky et al., 2013). Hence, microglia isolated from neonatal mixed glia preparation may not account for variances in response properties due to developmental state in microglia from different brain regions (Butovsky et al., 2013; Lai et al., 2013).

2.4.6 Conclusions

CNS microglia are a heterogeneous population of cells whose immune function is determined by local environment, severity of injury and age of the animal (Lai et al., 2013, 2011; Lai and Todd, 2008; Yang et al., 2005). Our results suggest that SCM may be less pro-inflammatory on activation through the TLR4 pathway. These differences between microglial activation pathways in SCM as compared to BM are an important consideration in rational design of immune-modulatory or anti-inflammatory therapies specific for spinal cord and brain injuries.

2.5 Figures


A
Spinal cord microglia (SCM)
Brain microglia (BM)
Iba1
DAPI
MERGE
Iba1
DAPI
MERGE

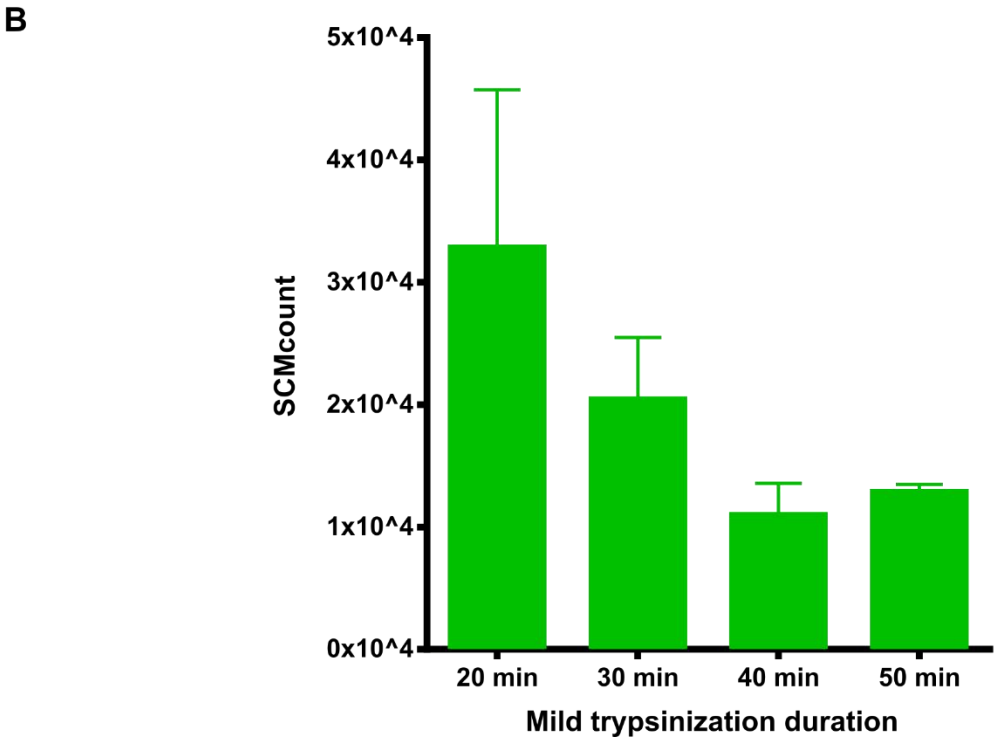

B
5x10^4
4x10^4
3x10^4
2x10^4
1x10^4
0x10^4
SCMcount
20 min
30 min
40 min
50 min
Mild trypsinization duration

Figure 2-1 SCM isolation by mild trypsinization.

(A) Representative micrograph of Iba1-labeled microglia derived from BM or SCM. Scale = 50μm. (B) 20 minutes of mild trypsinization produced the highest yield of SCM. SCM counts were quantified from an average of 6 fields each from four wells of 12 well cell culture plate (n = 4 where n represents the number of independent experiments, where an independent experiment is a separate microglia preparation).

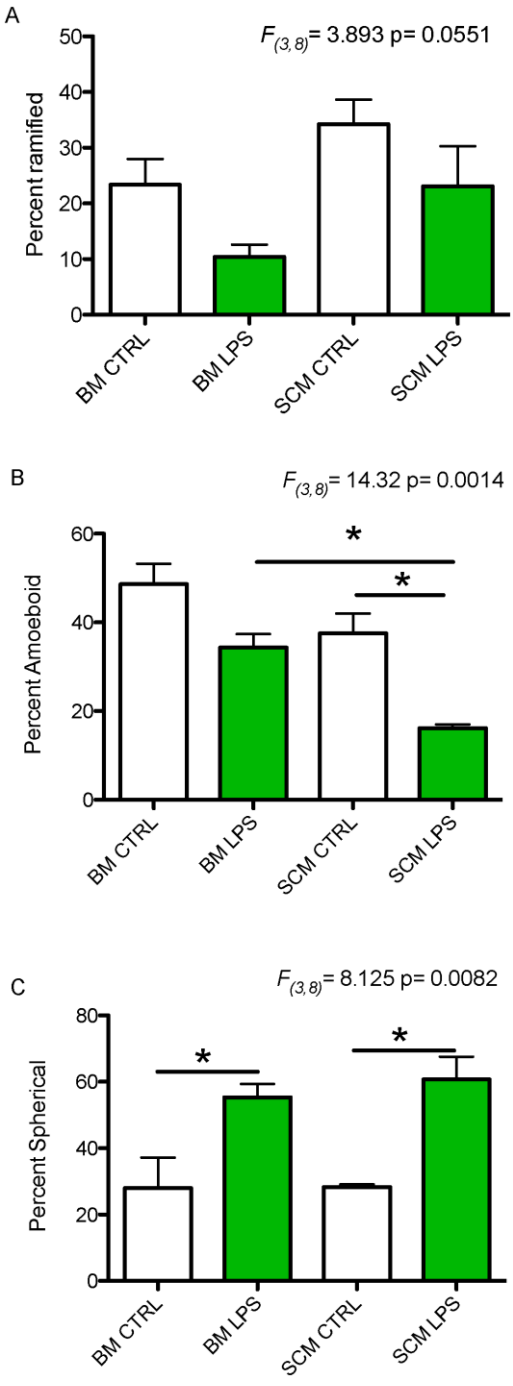

A
$F_{(3,8)}$= 3.893 p= 0.0551
Percent ramified
50
40
30
20
10
0
BM CTRL
BM LPS
SCM CTRL
SCM LPS
B
$F_{(3,8)}$= 14.32 p= 0.0014
Percent Amoeboid
60
40
20
0
*
*
BM CTRL
BM LPS
SCM CTRL
SCM LPS
C
$F_{(3,8)}$= 8.125 p= 0.0082
Percent Spherical
80
60
40
20
0
*
*
BM CTRL
BM LPS
SCM CTRL
SCM LPS

Figure 2-2 LPS mediated changes in morphology of SCM and BM.

BM and SCM were activated with LPS to compare the differences in ramified, amoeboid and spherical morphologies. (A) A main effect of treatment group (BM control, BM LPS, SCM control, or SCM LPS) on the proportion of microglia with ramified morphology was not significant (ANOVA, $F_{(3,8)} = 3.893$, $p = 0.0551$). (B) A significant main effect of treatment on the percentage of amoeboid cells was found (ANOVA, $F_{(3,8)} = 14.32$, $p = 0.0014$). Notably, LPS activated SCM had significant lower percentage of amoeboid cells relative to SCM controls and LPS activated BM (Newman-Keuls *post hoc* test, $n = 3$, $p < 0.05$). (C) Treatment significantly altered the percentage of spherical cells (ANOVA, $F_{(3,8)} = 8.125$, $p = 0.00821$). LPS-activated BM and SCM had a significant increase in spherical morphology relative to control conditions (Newman-Keul *post hoc* test, $n = 3$, $p < 0.05$). n represents the number of independent experiment with a minimum of three replicates. Bars represent percent cells ± s.e.m

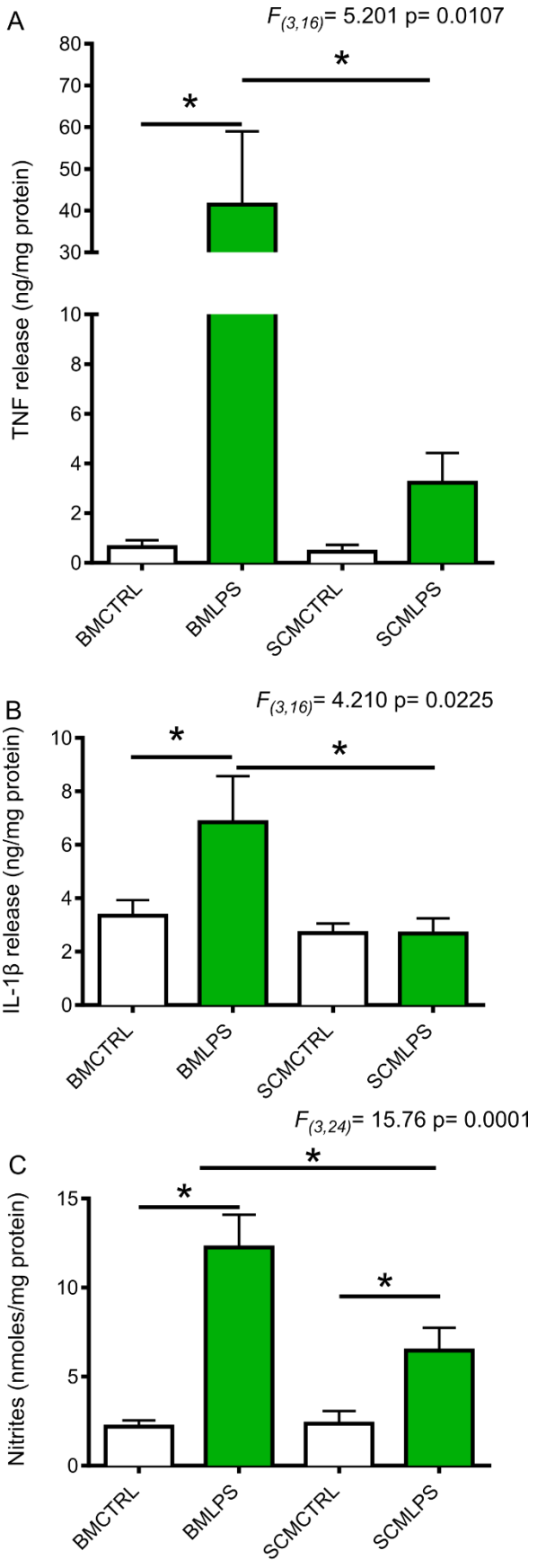
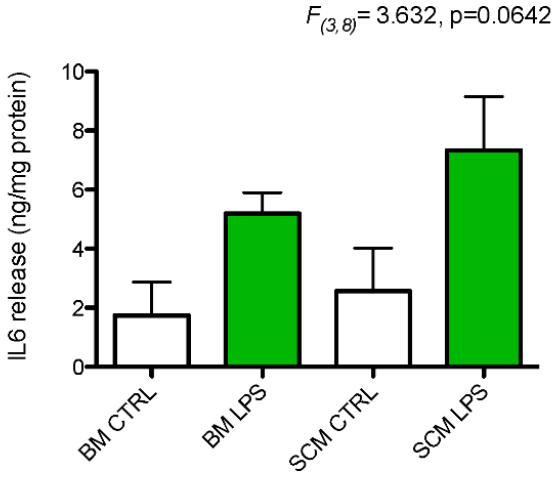

A
$F_{(3,16)}$= 5.201 p= 0.0107
TNF release (ng/mg protein)
80
70
60
50
40
30
10
8
6
4
2
0
*
*
BMCTRL
BMLPS
SCMCTRL
SCMLPS
B
$F_{(3,16)}$= 4.210 p= 0.0225
IL-1β release (ng/mg protein)
10
8
6
4
2
0
*
*
BMCTRL
BMLPS
SCMCTRL
SCMLPS
$F_{(3,24)}$= 15.76 p= 0.0001
C
Nitrites (nmoles/mg protein)
15
10
5
0
*
*
*
BMCTRL
BMLPS
SCMCTRL
SCMLPS

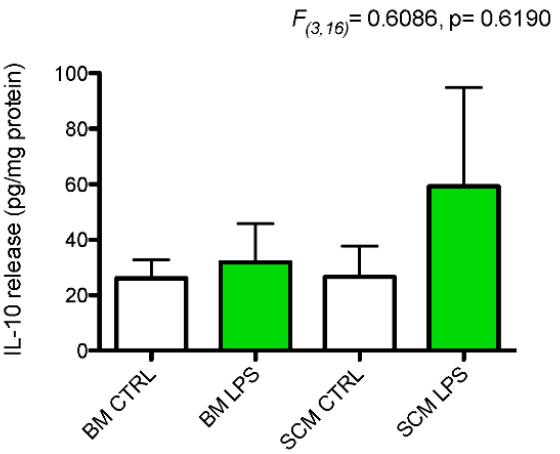
Figure 2-3 Secretion and release of the pro-inflammatory effectors TNF, IL-1β and NO by SCM and BM activated by LPS.

BM and SCM were activated with LPS to measure the difference in their release of pro-inflammatory molecules. (A) A significant effect of treatment on TNFsecretion between groups was observed (ANOVA, $F_{(3,16)} = 5.201$, $p = 0.0107$). LPS activated BM had significantly higher release of TNF compared to BM control and LPS activated SCM (Neuwman-Keuls *post hoc* test $n = 5$, $p < 0.05$). SCM had a trend towards increase in TNFon activation with LPS. (B) A significant effect of treatment was also observed for IL-1β secretion (ANOVA, $F_{(3,16)} = 4.210$, $p = 0.0225$). LPS-activated BM had a significantly higher release of IL-1β compared to BM control and LPS-activated SCM (Newman-Keul *post hoc* test $n = 5$, $p < 0.05$). We did not see any change in IL-1β release between LPS activated SCM and SCM control (Newman-Keul *post hoc*, $n = 5$, $p > 0.05$). (C) A significant main effect of treatment group on NO release was observed (ANOVA, $F_{(3,24)} = 15.76$, $p = 0.0001$). LPS induced a significant increase in release of NO by BM compared to BM control and LPS activated SCM (Newman-Keul *post hoc*, $n = 7$, $p < 0.05$). NO release by LPS-activated SCM was significantly higher than that of SCM and BM controls (Newman-Keul *post hoc*, $n = 7$, $p<0.05$). However, NO release by LPS activated SCM was significantly less than that of LPS activated BM (Newman-Keul *post hoc*, $n=7$, $p<0.05$). n represents the number of independent experiment with a minimum of three replicates. Bars represent mean ± s.e.m

A


$F_{(3,8)}$= 3.632, p=0.0642
IL6 release (ng/mg protein)
0
2
4
6
8
10
BM CTRL
BM LPS
SCM CTRL
SCM LPS

B


$F_{(3,16)}$= 0.6086, p= 0.6190
IL-10 release (pg/mg protein)
0
20
40
60
80
100
BM CTRL
BM LPS
SCM CTRL
SCM LPS

C

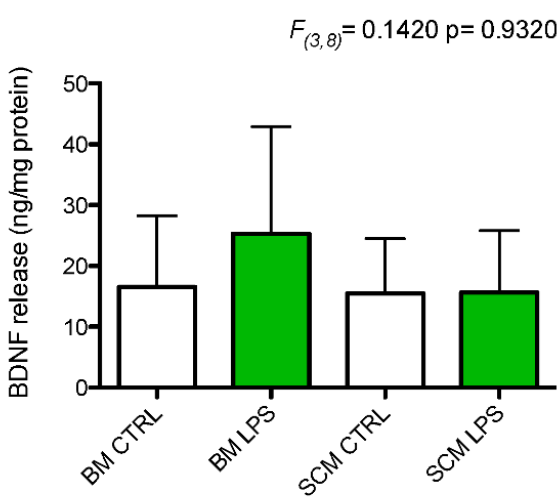

$F_{(3,8)}$= 0.1420 p= 0.9320
BDNF release (ng/mg protein)
0
10
20
30
40
50
BM CTRL
BM LPS
SCM CTRL
SCM LPS

Figure 2-4 Secretion of effectors IL-6, IL-10, and BDNF by LPS activated SCM and BM.

BM and SCM were activated with LPS to measure the difference in their release of these cytokines and trophic factors. (A) There was no significant main effect of treatment group on IL-6 release (ANOVA, $F_{(3,8)} = 3.632$, $p = 0.0642$, $n = 3$). LPS-activated BM and SCM exhibited a trend towards increased release of IL-6 compared to their respective controls. A significant main effect of treatment was not observed for either IL-10 release (B, ANOVA, $F_{(3,16)} = 0.6086$, $p = 0.6190$, $n = 5$) or BDNF release (C, ANOVA, $F_{(3,8)} = 0.1420$, $p = 0.9320$, $n = 3$). n represents the number of independent experiments with a minimum of three replicates. Bars represent mean ± s.e.m

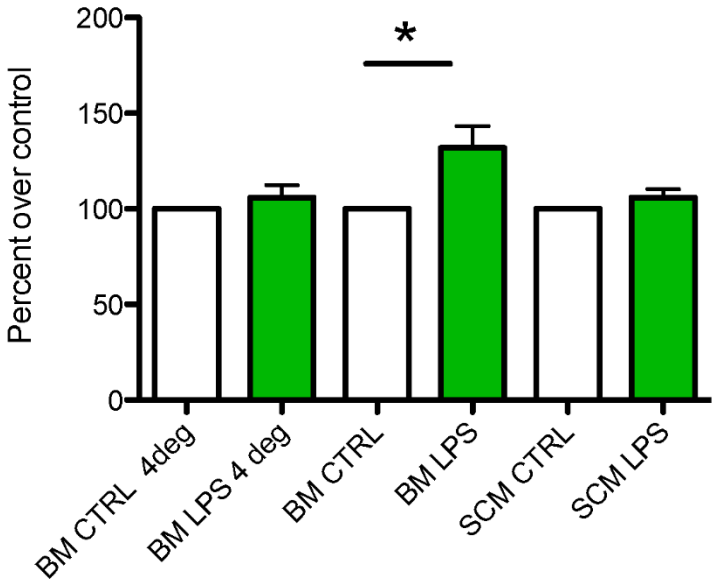


Figure 2-5 LPS mediated phagocytosis in SCM and BM.

Phagocytosis was measured according to fluorescent intensity of cell lysates from control or LPS-activated BM or SCM. Within each independent experiment, fluorescence was normalized to mean control values. LPS induced a significant increase in phagocytic activity of BM relative to control ($n = 8$, one sample t-test, $p < 0.05$) but did not induce any change in the phagocytic activity of SCM ($n = 3$, one sample t-test, $p > 0.05$). Phagocytic assays were performed at 4 degrees Celsius as negative controls (i.e. a reduced phagocytosis condition) to confirm assay validity ($n = 5$). n represents the number of independent experiment with a minimum of three replicates. Bars represent mean ± s.e.m

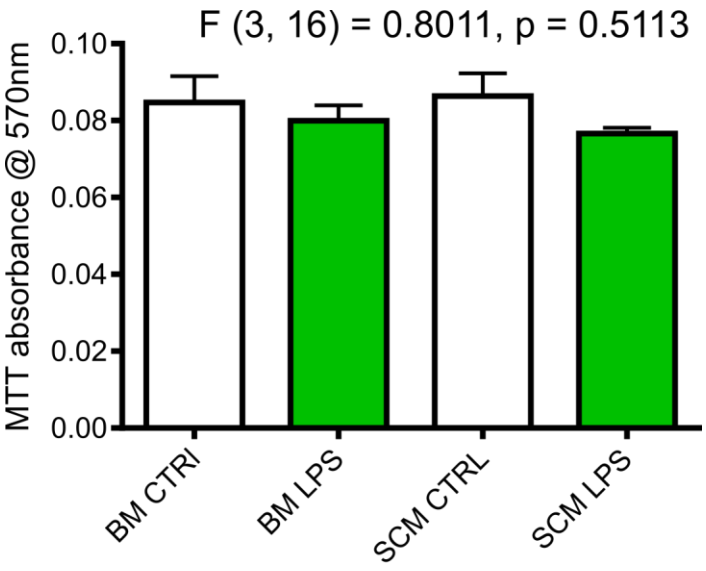


Figure 2-6 BM SCM viability after LPS treatment.

Microglial viability was assessed via the MTT assay in four treatment groups (BM control, BM LPS, SCM control, or SCM LPS). There was no main effect of treatment group on microglial viability ($F_{(3,16)} = 0.8011$, $p = 0.5113$). n = 5 independent microglia culture preparations. Bars represent mean ± s.e.m

Chapter Three: A robust method to isolate functional spinal cord microglia from rat primary mixed glia cultures

3.1 Introduction

Microglia are involved in a myriad of developmental and homeostatic roles such as clearing apoptotic neurons, synaptic pruning, and synapse maintenance during development and adulthood in the central nervous system (CNS) (Morsch et al., 2015; Paolicelli et al., 2011; Schafer et al., 2012; Sokolowski et al., 2014; Upender and Naegele, 1999; Wake et al., 2009). Additionally, microglia are the first responders in the event of a perturbation or injury to the CNS (Nayak et al., 2014; Saijo and Glass, 2011). As the primary immune cells of the CNS microglia are responsible for acute as well as chronic inflammation due to injury or disease in the CNS (Saijo and Glass, 2011). The capacity for microglia to be involved in this myriad of CNS functions is due to their ability to produce and release a repertoire of factors such as cytokines, chemokines, prostaglandins, reactive oxygen species and trophic factors (Kettenmann et al., 2011). Depending on the injury or disease as well as severity, microglia can be neuroprotective or neurotoxic (David and Kroner, 2011; Lai and Todd, 2008; Lambertsen et al., 2009). Microglia communicate extensively with other glia in the CNS as well as the peripheral immune system and play a major role in deciding the outcome of an injury or disease to the CNS (Tian et al., 2012).

Several studies have demonstrated that brain microglia function and response to perturbation to CNS are influenced by region of origin (Lai et al., 2011), severity of injury (Lai and Todd, 2008) and age of the organism (Ferrazzano et al., 2013; Floden and Combs, 2011; Lai et al., 2013). These studies suggest functional heterogeneity of microglial populations within the brain. Less studied is the heterogeneity between microglia derived from brain and spinal cord. Recent studies have also found significant functional differences between microglia derived from

brain and spinal cord (Baskar Jesudasan et al., 2014; Olson, 2010) suggesting microglia in spinal cord represent a functionally unique population. These previous studies of SCM employed a technique introduced by Saura et al., (2003) to isolate primary SCM from mixed glial cultures, considered the current gold standard for *in vitro* work and extensively used to study the functions of microglia (Daniele et al., 2014; Garcia et al., 2014; Gingras et al., 2007; Saura et al., 2003; Tamashiro et al., 2012). However, differences in the composition of spinal cord mixed glia cultures significantly reduces the frequency of achieving microglial isolation of sufficient (≥ 95%) purity using this method. Here, we present a method of isolating microglia in culture using gentle shaking after lidocaine HCl pre-treatment (Lai and Todd, 2008; Siao and Tsirka, 2002) that consistently yields isolated cell cultures of SCM with excellent purity (≥ 95%). Notably, the microglia (BM and SCM) isolated by this technique maintain established functional characteristics.

3.2 Material and Methods

3.2.1 Media and reagents

Hanks Balanced Saline solutions (HBSS), Dulbecco's Modified Eagle Medium – Hams'F12 nutrient mixture (DMEM-F12), DMEM-F12 with HEPES (DMEM-F12/HEPES), 0.25%trypsin-EDTA, FBS, normal horse serum (NHS), and Penicillin-Streptomycin (P/S) were from Gibco (Thermo-Fisher Scientific, Burlington, ON). Lidocaine HCl, Triton X-100, lipopolysaccharide (LPS), and sodium nitrite standard solution were from Sigma (Oakville, ON). VectaShield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) was from Vector Labs (Burlington, ON). Primary antibodies anti-rabbit Iba1 (019−19741) and mouse anti-beta-Actin

were purchased from Wako (Osaka, Japan) and Sigma (Oakville, ON) respectively. Secondary antibodies Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 647 donkey anti-mouse, and Hoechst 33342 were purchased from Molecular Probes (Thermo-Fisher Scientific, Ottawa, ON). Rhodamine Red donkey anti-rabbit was purchased from Jackson ImmunoResearch (Cedarlane, Burlington, Ontario).

3.2.2 Primary mixed glia preparation

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. Brain and spinal cord for establishing primary mixed glial cultures were obtained from postnatal day one or two male Sprague-Dawley (SD) rat pups as previously described (Churchward and Todd, 2014; Lai and Todd, 2008). The SD rat pups were euthanized, their brains (four) and spinal cords (twenty) were dissected and placed in dissection buffer (HBSS with and 200 U/ml penicillin, 200 μg/ml streptomycin). Meninges and blood vessels were removed under a dissection microscope. Tissues were cut into small pieces and incubated in 0.25% trypsin-EDTA for 25 mins at 37°C, and collected by centrifugation (2000 x g, 2 min). Trypsin -EDTAwas inactivated with maintenance media (DMEM/F12 supplemented with 10% FBS and 200 U/ml penicillin, 200 μg/ml streptomycin) and tissues were dissociated by trituration in maintenance media and centrifuged at 2000 x g for 2 mins. The brain and spinal cord cell pellet was re-suspended in maintenance media and seeded at equal density into cell culture treated T75 flasks coated with poly-L-lysine. Cells were maintained in a 37°C, 5% CO_2 humidified incubator with maintenance media replaced twice weekly.

3.2.3 Microglia isolation by Lidocaine HCl method

Microglia were isolated from primary mixed glial cultures at 21 days *in vitro* by modification of the lidocaine HCl shaking method (Lai and Todd, 2008; Siao and Tsirka, 2002). At 24-hrs prior to isolation cultures were refreshed with DMEM/F-12 supplemented with 10% FBS, and immediately before isolation this media was collected, filtered (0.22 um), and diluted 1:1 with DMEM/F-12 to make conditioned media. Brain and spinal mixed glial cultures at 21 days *in vitro* were refreshed with DMEM-F12/HEPES with 10% FBS at 37℃ 30 mins before lidocaine HCl treatment. Lidocaine HCl was added to the primary mixed glial culture to a final concentration of 15 mM in the media, the cultures were incubated for 3 mins and shaken for 7 mins in an orbital shaker at 50 rpm at 37℃. Microglia were collected from the media by centrifugation (2000 x g, 2 mins at room temperature). Cell pellets were gently re-suspended in 1 mL of conditioned media using 1 mL pipette tip. Cell suspensions were washed by adding 9 mL of their respective conditioned media and centrifuged at 2000 x g for 2 minutes at room temperature. The cell pellets were again re-suspended by trituration in their respective conditioned media and the cells were counted and seed at a density of $1x10^5$ cell/mL in 48 or 24 well poly-L-lysine coated polystyrene plates. The microglia were allowed to settle in the plates for 10 mins at 37℃ in a 5% CO2 incubator after which non-adherent cells were washed gently with DMEM at 37℃ and respective conditioned media was added to the brain and spinal microglia which were allowed to recover overnight. Conditioned medium was replaced with fresh DMEM/F-12 immediately prior to treatments.

3.2.4 Immunohistochemistry

Cells were washed 1x with HBSS and fixed in 5% PBS buffered formalin solution for 10 mins at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 10% NHS in PBS for 40 mins at room temperature. The fixed and permeabilized cells were sequentially labelled with primary antibodies and secondary antibodies prepared in PBS with 1% NHS for 1 hour at room temperature or overnight at 4ºC at the concentrations indicated in table 1.

Table 2 Chapter 3 Antibodies used

Primary Antibody	**Host**	**Concentration**	**Manufacture**
CNPase	Mouse	1:1000	SIgma
beta-Actin	Mouse	1:1000	Sigma
GFAP	Mouse	1:1000	Sigma
Iba1	Rabbit	1:1000	Wako
Secondary Antibody	**Host**	**Concentration**	**Manufacture**
Anti-rabbit Alexa Fluor 488	Donkey	1:500	Molecular Probes
Anti-mouse Alexa Fluor 647	Donkey	1:500	Molecular Probes

3.2.5 Nitric Oxide (NO)

NO release was measured indirectly by quantifying the stable metabolite nitrite in culture media using a method described by Griess (1879). Media was collected 24 hours after treatment and 100 µL of LPS treated or treatment control media and 50 µL each of 1% sulphanilamide (in 3N HCl) followed by 0.02% N-napthylethylenediamine were added per well of 96-well plate in duplicates. Absorbance was read at 540 nm and the amount of nitrite metabolite was interpolated from a set of standards measured in parallel.

3.2.6 Statistics

Results are represented as percentage of total population for cell purity and raw values normalized to total protein ± standard error of mean for NO assay. Statistical analyses were carried out using two-way ANOVA followed by Sidak's methods to test for significance between treatment groups. n represents a single independent experiment (i.e an independent culture preparation) with a minimum of three technical replicates. Each technical replicate is a well in a 48 or 24 well culture plate, except for immunocytochemistry studies where n represents a single well of an independent experiment. All statistical analyses were done using Graphpad Prism version 7.

3.3 Results

3.3.1 Presence of fibroblast like cells in SCM

Three-week old mixed brain and spinal cord glial cultures consist mainly of astrocytes, microglia, and a small population of oligodendrocytes, labelled with GFAP, Iba1 and CNPase respectively (Figure 3-1). Consistent with previous studies, spinal mixed glia consists primarily of fibrous astrocytes as compared to brain mixed glia, which consist predominantly of protoplasmic astrocytes (Figure 3-1) (Kerstetter and Miller, 2012). Previously, we have shown that mild trypsinization technique can be used to isolate spinal cord microglia (SCM) (Figure 3-1) (Baskar Jesudasan et al., 2014). However, over the course of 27 independent culture experiments, each consisting of four 12-well plates per brain or spinal culture preparation, only 78% of SCM isolated by mild trypsinization yielded sufficiently pure SCM (purity >95%). This reduced purity hampers the experimental utility and cost-effectiveness of this isolation method. In cases where high purity was not achieved, fibroblast-like cells (labelled with beta-Actin) were found in mild trypsin isolation cultures (Figure 3-2B and Figure 3-3). Based on these cellular contaminants, a better method of isolation to significantly reduce fibroblast contamination and achieve high purity SCM cultures was investigated.

3.3.2 Isolation of microglia by lidocaine HCl increases purity of SCM compared to the mild-trypsinization isolation method

Previous studies have shown that lidocaine HCl can be used to isolate highly pure BM (Lai and Todd, 2008; Siao and Tsirka, 2002), but the ability of this approach to reduce fibroblast

contamination in SCM cultures was not known. Our data show that lidocaine HCl isolation can be used to isolate SCM and that this technique effectively reduced fibroblast-like cells in SCM. Microglia were isolated in 15 independent brain and spinal culture preparations with at least 12 technical repeats for SCM and BM (each technical repeat is a well of a 48-well plate). Notably, for all cultures (total of 360 wells) purity was assessed using phase contrast microscopy and less than 5% contaminating fibroblast like cells were observed in all wells. The purity of BM and SCM (≥ 95% microglia) cultures was confirmed by immunocytochemistry (Figure 3-4).

3.3.3 Microglia isolated by lidocaine HCl are functionally similar to those isolated by mild-trypsinization

To confirm that BM and SCM maintain established functional properties whether by mild trypsin or lidocaine HCl protocols, release of nitric oxide (NO) after treatment with LPS (1μg/mL) was assessed. As expected, a significant main effect of LPS was observed in all microglial preparations ($F_{(1,16)} = 40.29$, $p < 0.0001$) (Figure 3-5). However, there was no overall main effect of isolation method ($F_{(3,16)} = 1.87$, $p = 0.18$) in three independent experiments (Figure 3-5), confirming that isolation with lidocaine HCl improves purity (relative to mild trypsinization) without altering functional properties in response to known activators.

3.4 Discussion

3.4.1 Highly pure SCM can be isolated by lidocaine HCl isolation method

Microglia play an important role in deciding the outcome of CNS injuries and disease (Kettenmann et al., 2011; Saijo and Glass, 2011). Factors released by microglia induce neurotoxic or neurotrophic processes in the CNS (Kettenmann et al., 2011; Saijo and Glass, 2011). Recent literature suggests that microglial phenotype is significantly altered by the region of origin, age, and severity of injury (Baskar Jesudasan et al., 2014; Ferrazzano et al., 2013; Floden and Combs, 2011; Lai et al., 2013, 2011; Lai and Todd, 2008). Hence, an improved understanding of the functional differences between brain and spinal cord microglia can further our understanding of their unique contributions to pathologies in the CNS and potentially guide therapeutic interventions targeting the inflammatory component of CNS disease and injury. SCM can be isolated from spinal cord mixed glia cultures by mild trypsinization (Chapter 2); however, SCM cultures isolated using this approach frequently contain contaminating fibroblast-like cells, limiting the utility of this approach (Figure 3-3). Consistent high purity SCM would improve throughput for studies examining the unique functional properties of these cells and their role in spinal physiology and pathology. This study demonstrates that SCM can be isolated from spinal cord mixed glia using a protocol including lidocaine HCl pre-treatment and gentle shaking. The purity of SCM and BM was greater than or equal to 95% in all wells and experiments (n=12 wells in 15 different cultures for BM and SCM respectively), whereas only 78% of SCM isolation using mild trypsinizaiton offered comparable purity.

3.4.2 Benefits of lidocaine isolation relative to mild trypsinization protocols

Previous studies have shown that lidocaine can attenuate TLR-4R expression and upregulation of inducible nitric oxide synthase by macrophages (Huang et al., 2006; Lee et al., 2008). However, our data suggest that lidocaine isolation and overnight recovery in conditioned media mitigated the effects of lidocaine on TLR-4R. This demonstrated in Figure 3-5 where lidocaine isolated microglia activated by LPS had NO levels comparable to that of mild trypsinization condition microglia. Microglial response properties do not appear to differ between isolation methods. Lidocaine HCl isolation is therefore a better protocol (as compared to standard approach using mild trypsinization) for studies using SCM. Beyond improved purity, this lidocaine HCl isolation method also improved the ability to control the seeding density between SCM and BM to allow for more accurate comparisons and is a faster isolation process than mild trypsinization or conventional shaking techniques. The ability to lift off microglia and control seeding density of microglia broadens the applications for the cultures, such as for incorporation into co-culture models, 3D cell cultures, and implantation into *in vivo* models where seeding density has to be consistent to make meaningful comparisons. The current understanding of SCM role in CNS disease or injury models is limited due to the near exclusive usage of BM, bone marrow-derived macrophages, or mix of brain-and spinal cord microglia to study spinal cord injury/disease models (Greenhalgh and David, 2014; Hynds et al., 2004; Sato et al., 2012; Yu et al., 2009). Given the demonstrated functional differences between BM and SCM (Chapter 2), an improved method to isolate SCM will allow more accurate investigations of their role in injury and recovery processes in the spinal cord and more effective tools to manipulate their activity locally in the cor

3.5 Figures

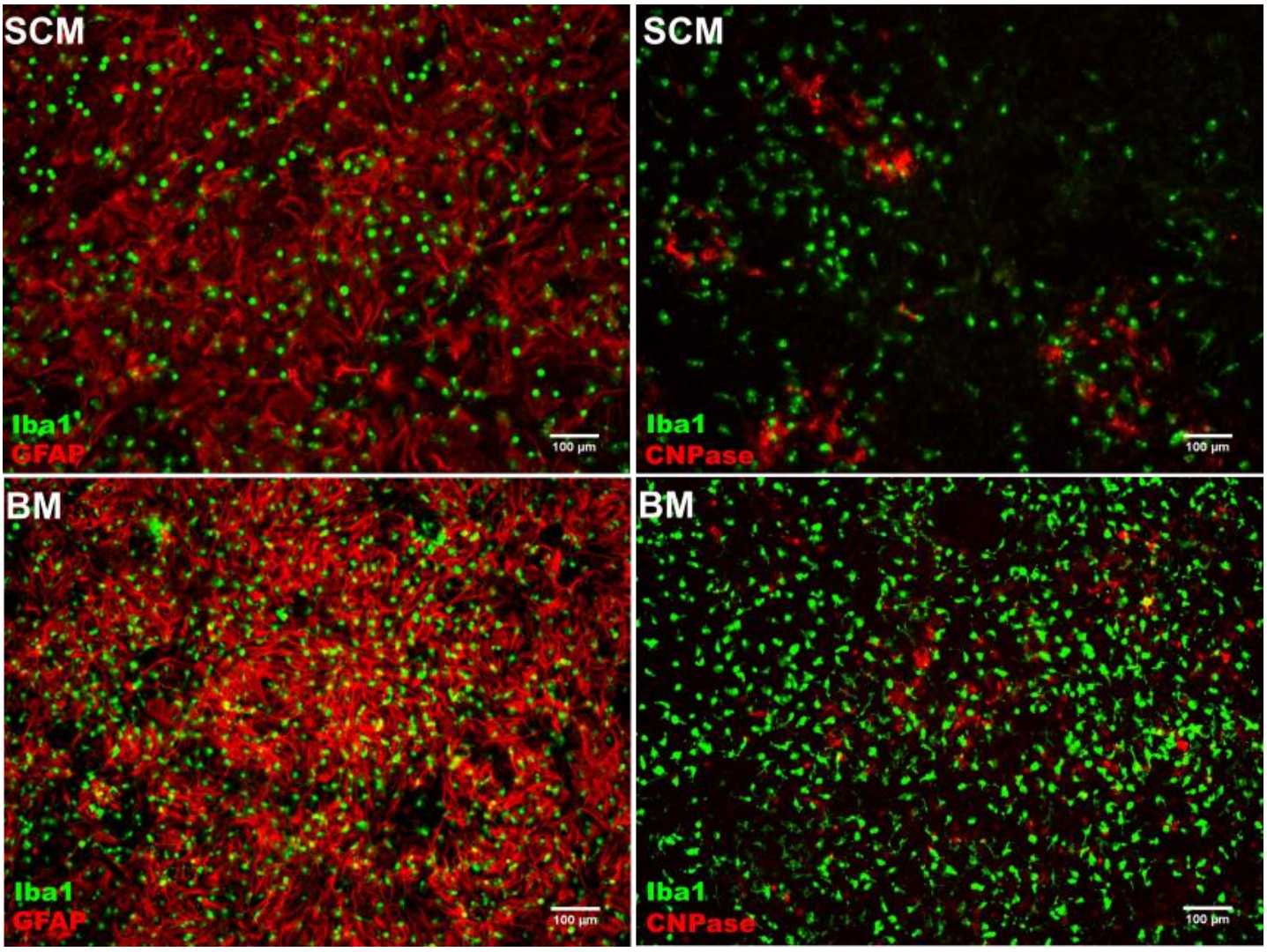
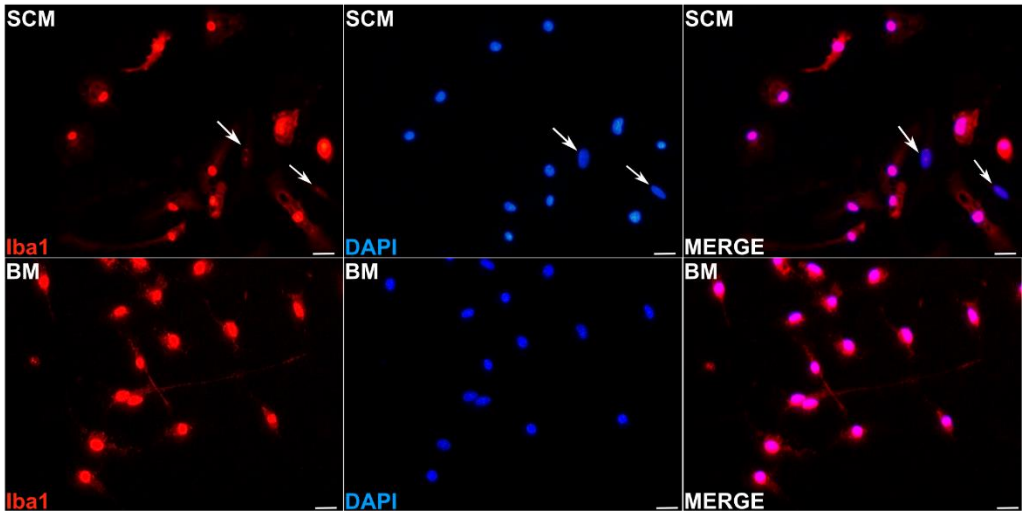


Figure 3-1 Brain and spinal cord mixed glia culture.

Representative photomicrographs of brain and spinal cord mixed glial cultures labeled for microglia (Iba1), astrocytes (GFAP), oligodendrocytes (CNPase). The spinal cord astrocytes were mostly fibrous in nature compared to brain astrocytes. Scale = 100 µm.

A


SCM
SCM
SCM
Iba1
DAPI
MERGE
BM
BM
BM
Iba1
DAPI
MERGE

B

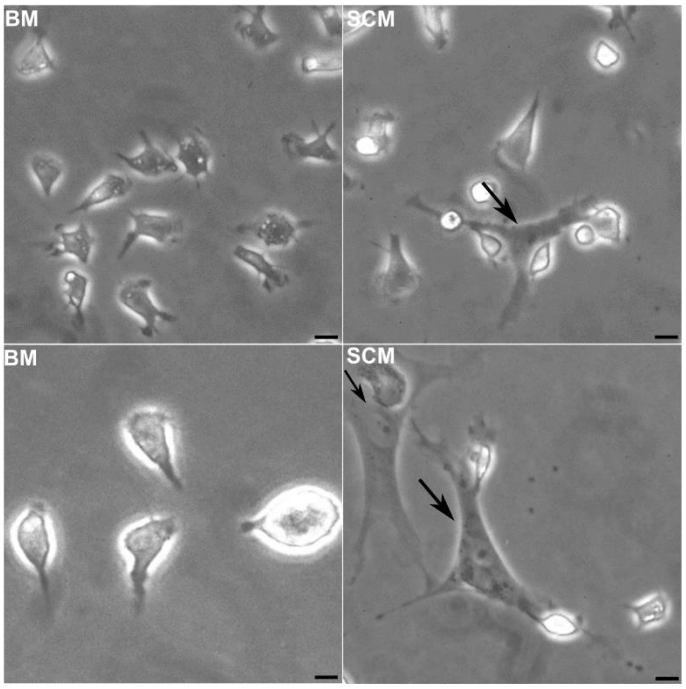

BM
SCM
BM
SCM

Figure 3-2 Representative photomicrographs of brain and spinal cord microglia isolated by mild trypsinization.

A) Brain and spinal microglia (BM and SCM) isolated by mild trypsinization. Note white arrows point to nuclei that were not labelled with Iba1. Such cells were only observed in SCM cultures. Scale = 50 μm.

B) Representative phase contrast images with fibroblast-like cells in SCM (black arrows) but not BM. The lower panel shows a higher magnification view of fibroblast-like cells (black arrows). Scale = 50 and 25 μm, respectively.

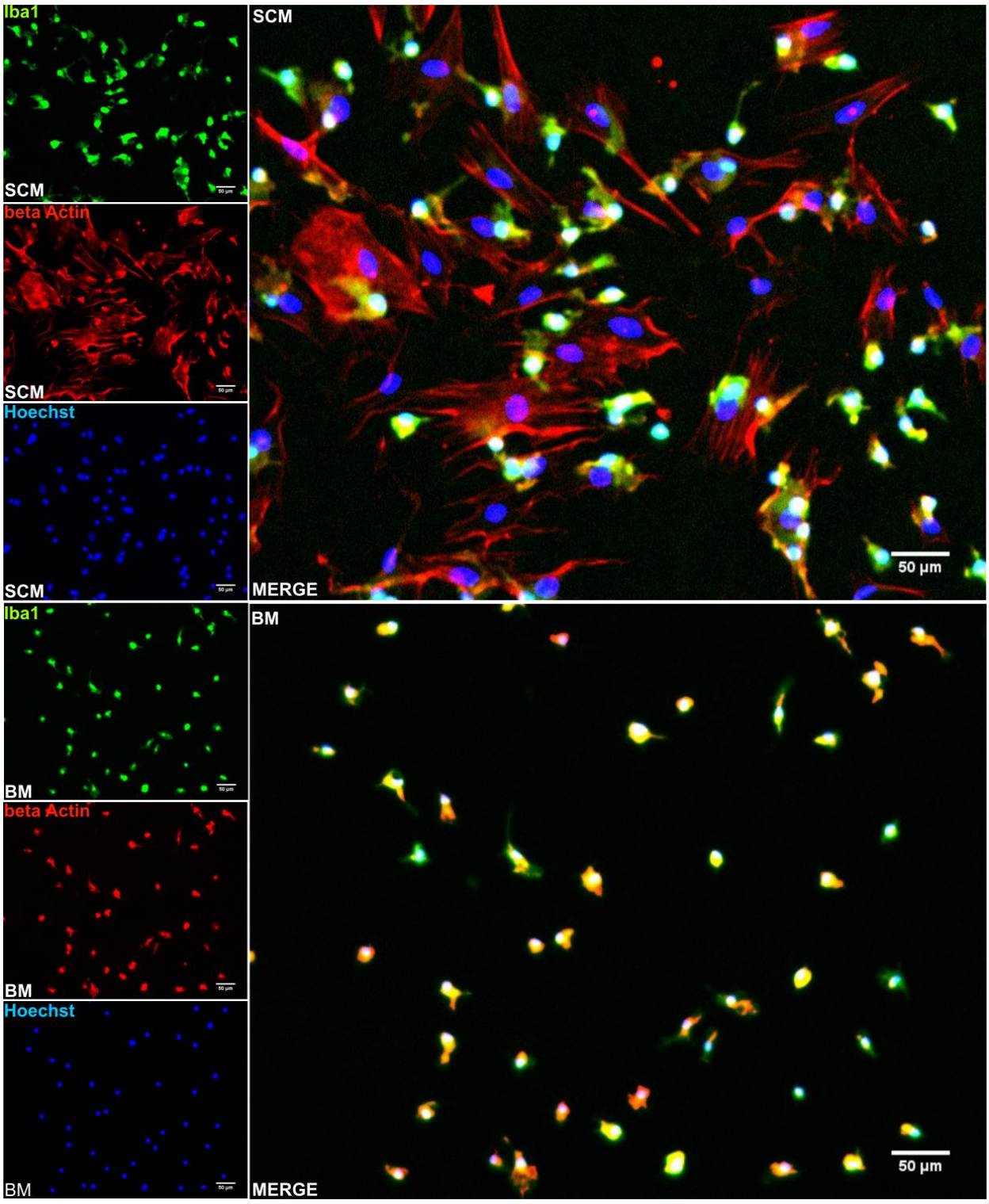

Iba1
SCM
beta Actin
SCM
Hoechst
SCM
SCM
MERGE
50 µm
Iba1
BM
beta Actin
BM
Hoechst
BM
BM
MERGE
50 µm

Figure 3-3 Fibroblast-like cells in spinal cord culture.

Fibroblast-like cells in spinal cord microglia cultures. Actin labelling distinguishes fibroblast-like cells from microglia in cultures isolated using mild-trypsinization method. 22% of isolations using this method had to be discarded due to contaminating cells. Scale = 50 μm

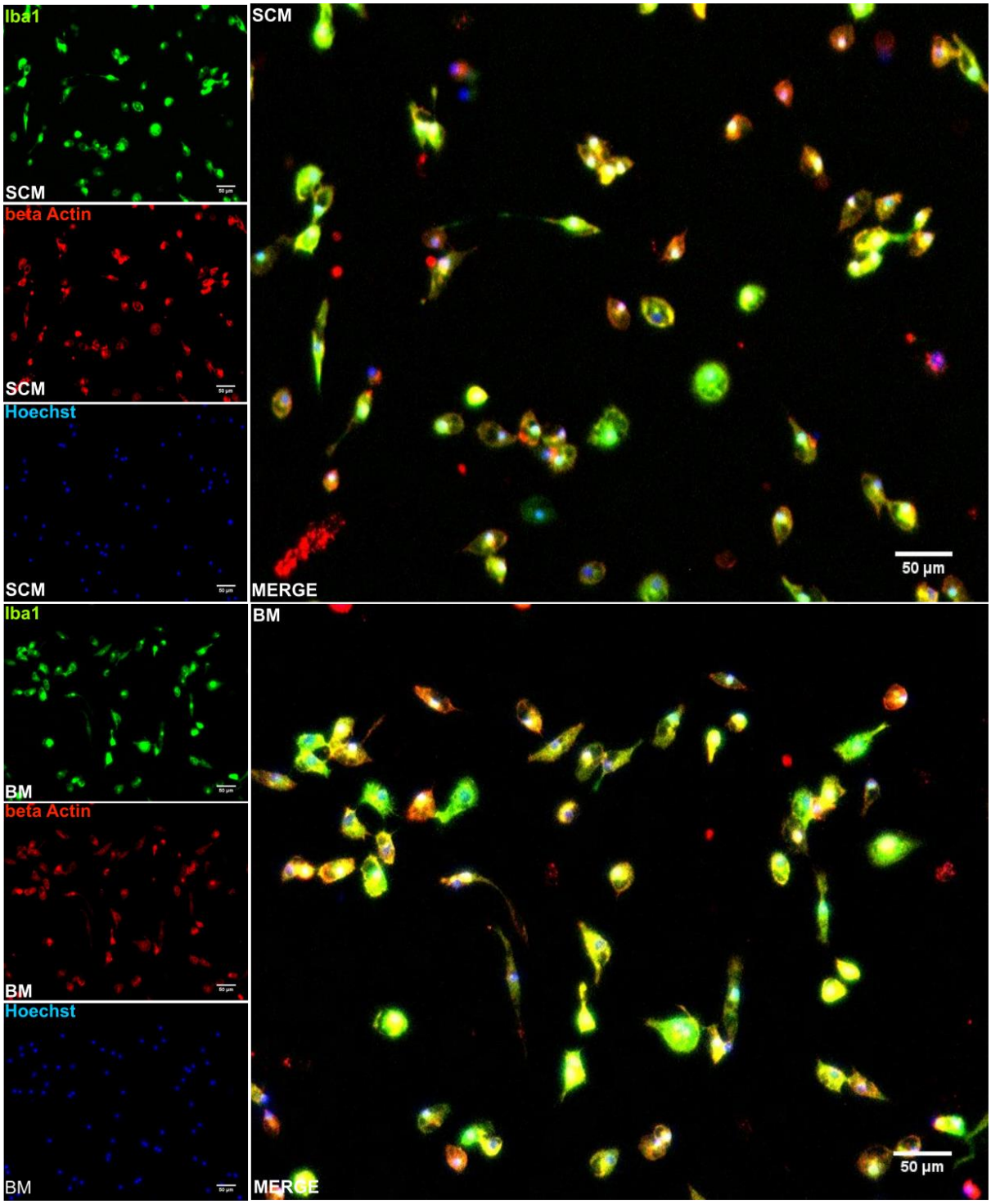

Iba1
SCM
beta Actin
SCM
Hoechst
SCM
SCM
MERGE
50 µm
Iba1
BM
beta Actin
BM
Hoechst
BM
BM
MERGE
50 µm

Figure 3-4 Representative lidocaine HCl isolated brain and spinal cord microglia.
Representative photomicrographs of brain and spinal microglia labelled with Iba1 for microglia and beta actin to label non-microglial cells. The purity of all BM and SCM cultures was ≥95%, N= 3, Scale = 50 μm

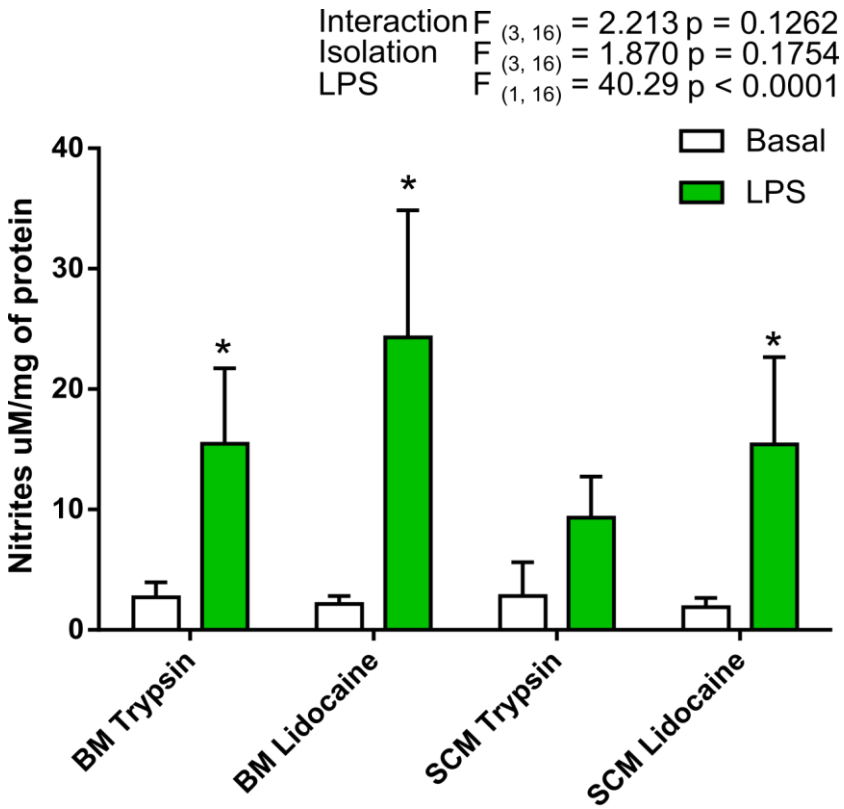


Figure 3-5 Release of the pro-inflammatory effector NO by mild trypsinization and lidocaine HCl isolated microglia

BM and SCM were treated with LPS (1µg/mL) to measure differences in NO release due to method of isolation and region of origin. NO release of BM and SCM isolated by mild trypsin and lidocaine HCl had a significant main effect for LPS treatment ($F_{(1,16)}$ =40.29, $p < 0.0001$). However, isolation method did not have a significant effect on NO release ($F_{(3,16)}$ = 1.87, p = 0.18). N=3, * represent $p < 0.05$.

Chapter Four: The effect of ATP, glutamate and conditioned media on brain and spinal microglia

4.1 Introduction

Microglia have the capacity to respond to pathogens, insults, and injuries that disrupt the homeostasis of the CNS (Kettenmann et al., 2011). They are able to respond to a wide variety of environmental stimuli due to their repertoire of receptors that can sense damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) in the central nervous system (CNS) (Gadani et al., 2015; Kigerl et al., 2014). Adenosine triphosphate (ATP) and glutamate are examples of DAMPs that are released into the extra cellular milieu in response to various injuries or perturbations of CNS, including cell death due to stroke, spinal cord injury, traumatic brain injury (Gadani et al., 2015; Kigerl et al., 2014).

ATP and glutamate are well known for their role as chemotactic agents that recruit microglia to the site of injury (Duan et al., 2009; Liu et al., 2009). ATP has also been shown to induce upregulation of pro-inflammatory factors such as TNF (tumor necrosis factor), IL1β (interleukin 1 beta) and NO (nitric oxide) by microglia through purinergic receptor pathways (Kettenmann et al., 2011; Rodrigues et al., 2015). Similarly, glutamate has been shown to induce pro-inflammatory factors such as TNF, IL1β, and NO in microglia through glutamate receptors (Kettenmann et al., 2011; Murugan et al., 2013; Pocock and Kettenmann, 2007). However, most studies that investigated these DAMPs used specific receptor agonists or antagonists or used non-physiological concentrations (10 fold above the physiological concentrations) to determine their effects on microglia in culture (Dai et al., 2010; Vincent and Maiese, 2000).

ATP and glutamate can activate ionotropic (ATP: $P_2X_{1\text{-}7}$, glutamate: AMPA(Glu2/3), kainate (Glu5)) and metabotropic receptors (ATP: $P_2Y_{1\text{-}6,11\text{-}14,}$ glutamate: $mGlu_{1\text{-}8}$) on microglia (for more details on types of receptors and signaling refer to chapter 1). Previously, it was shown

that 1mM glutamate induces release of TNF through AMPA (GluR2-4) and kainate (GluR5) receptors (Noda et al., 2000). The Group II mGlurR2 and 3 specific agonist DCG-IV also induces TNF release by microglia (Taylor, 2005). Interestingly, selective inhibition of group II mGlu5 reduces TNF release by LPS-activated microglia (Byrnes et al., 2009). In the present study, physiologically relevant concentrations of ATP and glutamate that mimic injury were used to test if they have a differential effect on BM and SCM. Previous studies in models of rat ischaemia and TBI have shown that a 10 μM concentration of glutamate occurs in the uninjured CNS extracellular milieu, and that this concentration is increased to 30 μM after ischaemic injury or TBI (Dai et al., 2010; Hinzman et al., 2010; Marini and Paul, 1992; Ueda et al., 1992). Glutamate concentrations of more than 100 μM induce neurotoxicity (Marini and Paul, 1992). While there is no general consensus on ATP concentration in the extra cellular milieu, it has been suggested that after an injury a 500μM concentration and above occurs (Burnstock, 2006; Seeland et al., 2015), and several studies have utilized a 1mM concentration of ATP to measure the effect of ATP on microglia (Ehrlich et al., 1987; Harada et al., 2011; Kawamura, 2004; Lai, 2010; Verderio and Matteoli, 2001)

Microglial phenotypes are dependent on region of origin (Lai et al., 2011), age, sex and environment (Grabert et al., 2016b; Lai et al., 2013; Sorge et al., 2011). Notably, spinal cord microglia (SCM) have a reduced inflammatory profile in response to activation by lipopolysaccharide (LPS) relative to microglia derived from the brain (BM) (Baskar Jesudasan et al., 2014). However, the responses of SCM (relative to BM) to physiological stimulation with ATP and glutamate have not been investigated. Given that previous data suggest a reduced inflammatory phenotype in SCM, this study tested the hypotheses that physiological activators such as ATP and glutamate would induce a reduced inflammatory profile in SCM compared to

BM. Previously, we have shown that microglia from different regions of brain (hippocampus and thalamus) that are exposed to conditioned media from striatum acquired an inflammatory profile similar to that of microglia originally derived from the striatum (Lai, 2010). This suggests that microglia are a highly plastic population of cells. Hence, we also hypothesized that regional heterogeneity is not fixed and that BM exposed to SCM condition media would move toward an inflammatory profile similar to that of SCM.

To test the first hypothesis, BM and SCM from one-day or two-day old Sprague-Dawley rat pups were activated *in vitro* with ATP (1mM, concentration *in vitro* as previously established (Lai, 2010) and glutamate (10 μM to model physiological concentration in rat brain parenchyma, 30 μM to mimic the concentration measured *in vivo* in rat brain parenchyma after ischaemic or TBI injury, and 100 μM to mimic excitotoxic injury) (Choi and Rothman, 1990; Dai et al., 2010; Hinzman et al., 2010; Marini and Paul, 1992; Ueda et al., 1992). To test the second hypothesis, BM were incubated in conditioned media from brain and spinal cord mixed glia (BMix CM and SMix CM, respectively) to replicate the environment in which BM and SCM were cultured. BM conditioned in BMix cm and SMix cm were activated with ATP, glutamate (10 μM, 30 μM,100 μM) and LPS (1μg/ml) and pro-inflammatory factors released were measured.

4.2 Methods

4.2.1 Material and Methods

4.2.2 Media and reagents

Hanks Balanced Saline solutions (HBSS), Dulbecco's Modified Eagle Medium – Hams'F12 nutrient mixture (DMEM-F12), DMEM-F12 with HEPES (DMEM-F12/HEPES), 0.25%trypsin-EDTA, feal bovine serum (FBS), and Penicillin-Streptomycin (P/S) were from Gibco (Thermo-Fisher Scientific, Burlington, ON). ATP, glutamate, lidocaine HCl, Triton X-100, LPS, and sodium nitrite standard solution were from Sigma (Oakville, ON).

4.2.3 Primary mixed glia preparation

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. Brains and spinal cords for establishing primary mixed glial cultures were obtained from postnatal day one or two male Sprague-Dawley (SD) rat pups as previously described (Churchward and Todd, 2014; Lai and Todd, 2008). The SD rat pups were euthanized, their brains (four) and spinal cords (twenty) were dissected and placed in dissection buffer (HBSS with 200 U/ml penicillin, 200 μg/ml streptomycin). Meninges and blood vessels were removed under a dissection microscope. Tissues were cut into small pieces and incubated in 0.25% Trypsin-EDTA for 25 min at 37°C, and collected by centrifugation (2000 x g, 2 min). trypsin was inactivated with maintenance media (DMEM/F12 supplemented with 10% FBS and 200 U/ml penicillin, 200 μg/ml streptomycin) and tissues were dissociated by trituration in

maintenance media and centrifuged at 2000 x g for 2 mins. The brain and spinal cord cell pellets were re-suspended in maintenance media and seeded at equal density into cell culture-treated T75 flasks coated with poly-L-lysine. Cells were maintained in a 37°C, 5% CO_2 humidified incubator with maintenance media replaced twice weekly.

4.2.4 Microglia isolation by lidocaine HCl isolation method

Microglia were isolated from primary mixed glial cultures at 21 days *in vitro* by modification of the lidocaine HCl shaking method as described in Chapter 3 (Lai and Todd, 2008; Siao and Tsirka, 2002). At 24-hours prior to isolation cultures were refreshed with DMEM/F-12 supplemented with 10% FBS, and immediately before isolation this medium was collected, filtered (0.22 um), and diluted 1:1 with DMEM/F-12 to make conditioned medium. Brain and spinal mixed glial cultures at 21 days *in vitro* were refreshed with DMEM-F12/HEPES with 10% FBS at 37℃ 30 mins before lidocaine HCl treatment. Lidocaine HCl was added to the primary mixed glial culture to a final concentration of 15 mM in the media, the cultures were incubated for 3 mins and shaken for 7 mins in an orbital shaker at 50 rpm at 37℃. Microglia were collected from the media by centrifugation (2000 x g, 2 minutes at room temperature). Cell pellets were gently re-suspended in 1 mL of conditioned media using 1 mL pipette tip. Cell suspensions were washed by adding 9 mL of their respective conditioned media and centrifuged at 2000 x g for 2 mins at room temperature. The cell pellets were again re-suspended by trituration in their respective conditioned media and the cells were counted and seed at a density of $1x10^5$ cell/mL in 48 or 24 well poly-L-lysine coated polystyrene plates. The microglia were allowed to settle in the plates for 10 mins at 37℃ in a 5% CO2 incubator after which non-

adherent cells were washed gently with DMEM at 37℃ and respective conditioned media were added to the BM and SCM and the mixture were allowed to recover overnight. Conditioned media were replaced with fresh DMEM/F-12 immediately prior to treatments.

4.2.5 Nitric Oxide (NO)

NO release was measured indirectly by quantifying the stable metabolite nitrite in culture media using a method described by Griess, (1879)). Media were collected 24 hours after treatment, 100 µL of treatment or control media were added to a 96-well plate in duplicates followed by 50 µL each of 1% sulphanilamide (in 3N HCl) and 0.02% N-napthylethylenediamine per well. Absorbance was read at 540 nm and the amount of nitrite metabolite was interpolated from a set of standards measured in parallel.

4.2.6 Enzyme Linked Immunosorbent assays (ELISA)

Commercial ELISA kits were used to measure TNF, IL-1β, IL6 and IL-10 in media (DuoSet, R&D Systems Minneapolis, USA). ELISA procedures were carried out according to manufacturer protocols.

4.2.7 Statistics

Statistical analyses were carried out using two-way ANOVA followed by Sidak's methods to test for significance between treatment groups. n represents a single independent experiment (i.e. an independent culture preparation) with a minimum of three technical replicates. Each technical

replicate represents a well in a 12, 24 or 48 well culture plate. All statistical analyses were done using Graphpad Prism version 6.

4.3 Results

4.3.1 Secretion of pro-inflammatory effectors by BM and SCM exposed to ATP

ATP is commonly released in the extracellular milieu after injury to the CNS (Gadani et al., 2015; Kettenmann et al., 2011; Rodrigues et al., 2015). To investigate phenotypic differences between SCM and BM in response to this endogenous activator, isolated SCM and BM microglia were treated with ATP. Previous studies suggest that ATP concentrations of 1 mM are sufficient to induced a pro-inflammatory profile in BM (Ehrlich et al., 1987; Harada et al., 2011; Kawamura, 2004; Lai, 2010; Verderio and Matteoli, 2001). BM and SCM were therefore treated with 1 mM ATP, and pro-inflammatory factors (NO, TNF, IL6, IL-1β) released into cell culture media were measured using the Greiss assay (NO) and ELISAs (TNF, IL6, IL-1β). Two-away ANOVA identified a significant main effect of treatment and a significant interaction between microglia and treatment for release of NO ($F_{(2,24)} = 4.119$, $p = 0.029$), TNF ($F_{(2,42)} = 20.00$, $p < 0.0001$), IL-1β ($F_{(2,30)} = 9.951$, $p < 0.0005$) (Figure 4-1,Figure 4-2A, Figure 4-3). A significant main effect of microglia was also observed for TNF ($F_{(1,42)} = 23.20$, $p < 0.0001$), IL6 ($F_{(1,30)} = 7.531$, $p < 0.010$) and IL-1β ($F_{(1,30)} = 26.05$, $p < 0.0001$). (Treatment: NO, $F_{(2,24)} = 14.58$, $p < 0.0001$; TNF, $F_{(2,42)} = 78.35$, $p < 0.0001$; IL6, $F_{(2,30)} = 17.82$, $p < 0.0001$; IL-1β, $F_{(2,30)} = 3.372$, $p = 0.0478$; Interaction: NO, $F_{(2,24)} = 4.119$, $p = 0.029$; TNF, $F_{(2,42)} = 20.00$, $p < 0.0001$; IL-1β, $F_{(2,30)} = 9.951$, $p < 0.0005$). A significant main effect of microglia was also observed for TNF ($F_{(1,42)} = 23.20$, $p < 0.0001$), IL6 ($F_{(1,30)} = 7.531$, $p < 0.010$) and IL-1β ($F_{(1,30)} = 26.05$, $p < 0.0001$).

Sidak *post-hoc* test revealed that NO, TNF and IL-1β released by LPS (1 μg/ml) activated SCM LPS was significantly less that of BM LPS (BM LPS vs SCM LPS: NO $p = 0.042$, TNF $p < 0.001$, IL-1β $p < 0.0001$). No other significant comparisons were found, and group differences appear to result primarily from response to LPS activation.

4.3.2 Secretion of proinflammatory effectors by BM and SCM exposed to glutamate

Glutamate agonists have been shown to induce pro- or anti-inflammatory profile in a receptor dependent manner in microglia (Dai et al., 2010; Mead et al., 2012; Vincent and Maiese, 2000). However, direct study of microglia phenotype in response to physiological concentrations of glutamate has not been frequently investigated. Here, 10 μM (representing physiological levels of glutamate in CNS extra cellular milieu), 30 μM (levels present in *in vivo* ischaemic injury), 100 μM (levels present at excitotoxicity injury sites) (Choi and Rothman, 1990; Ueda et al., 1992) were selected as concentrations for treatment of BM and SCM. Two-way ANOVA identified significant interaction between microglia and treatment for NO ($F_{(4,20)}=13.13$, $p <0.0001$) (Figure 4-4). There was also a significant main effect of microglia ($F_{(1,20)} =15.67$, $p = 0.0008$) and treatment ($F_{(4,20)} =50.43$, $p < 0.0001$) (Figure 4.5.4.). Furthermore, Sidak *post-hoc* analysis revealed that NO released by SCM LPS was significantly less than that of LPS treated BM (BM LPS vs SCM LPS $p < 0.0001$) (Figure 4-4). Two-way ANOVA also revealed a significant main effect of treatment for TNF ($F_{(4,30)} = 38.13$ $p < 0.0001$), IL6 ($F_{(4,40)} =16.87$, $p < 0.0001$), and IL-1β ($F_{(4,40)} = 20.54$ $p < 0.0001$) (Figure 4-5 and Figure 4-6). A significant main effect of microglia for IL-1β was detected ($F_{(1,40)} = 7.232$ $p = 0.0104$). Sidak *post-hoc* analysis revealed that LPS mediated IL6 and IL-1β release by BM was significantly higher than that of

SCM LPS condition (BM LPS vs SCM LPS $p < 0.05$) (Figure 4-5B and Figure 4-6). As with ATP studies above, *post-hoc* comparisons did not identify statistically significant differences in cytokine release between BM and SCM in response to different glutamate concentrations.

4.3.3 Secretion of proinflammatory effectors by BM exposed to BMix CM and SMix CM

In addition to regional heterogeneity in microglia isolated from different areas of the brain (Lai et al., 2011), it has been shown that the microglia phenotypes differ with age and that phenotypes from different age groups are not fixed. Notably, conditioned media from microglia derived from one age group as well as a different region can be used to alter the phenotype of microglia derived from animals of a different age group or region of brain (Lai, 2010). This suggests that the BM are highly plastic cells capable of adapting to immediate environment and their phenotype is dictated not only by genetics but also their immediate environment. Therefore, the pro-inflammatory profile of SCM may be due to the conditioning by spinal cord mixed glia culture media. We hypothesized that conditioning BM with SMix CM (spinal cord mixed glia conditioned media) would alter the concentrations of inflammatory factors released by BM. To test the hypothesis, BM were incubated in SMix CM or BMix CM (brain mixed glia conditioned media) prior to treatment with ATP, glutamate, or LPS. BM incubated in BMix CM and SMix CM were activated with glutamate (10 μM, 30 μM, 100 μM), ATP (1 mM), LPS (1μg/ml) and the release of IL6 and IL-1β was measured. Two-way ANOVA that conditioned media ($F_{(1, 24)} = 12.98$, $p = 0.001$) and treatment $F_{(5, 24)} = 92.45$, $p < 0.0001$) had a significant main effect for IL-6 release (Figure 4-7), and suggested a trend towards a significant interaction for IL6 release ($F_{(1, 24)} = 2.487$, $p = 0.058$). Sidak *post-hoc* tests suggest that IL-6 released in response to LPS was

significantly greater in BM incubated in SMix CM (BMix CM BM LPS vs SMix CM BM LPS, $p < 0.0032$) (Figure 4-7). Moreover, BM release of IL6 in response to 100 μM glutamate was significantly greater when incubated in SMix CM (BMix CM BM 100 μM vs SMix CM BM 100 μM, $p < 0.0480$ respectively) (Figure 4-7). Similarly, significant main effects of conditioned media ($F_{(1, 24)} = 6.965$, $p < 0.05$) and treatment ($F_{(5, 24)} = 12.37$, $p < 0.001$) were detected for IL-1β release by BM, and suggested an overall reduction in IL-1β release with SMix CM (Figure 4-8). However, the Sidak *post-hoc* test did not reveal any significant comparisons between BMix CM and SMix CM groups (Figure 4-8).

4.4 Discussion

4.4.1 ATP and glutamate activation of microglia

Insults to the CNS, such as ischaemic stroke and spinal cord injury, cause neuronal injury and lead to release of neurotransmitters such as ATP and glutamate into the extracellular milieu (Kettenmann et al., 2011; Pocock and Kettenmann, 2007; Rodrigues et al., 2015; Ueda et al., 1992). Increased glutamate concentration in the extracellular milieu induces excitotoxicity injury to neurons and chemotaxis of microglia to the site of injury (Kettenmann et al., 2011; Liu et al., 2009; Pocock and Kettenmann, 2007). Similarly, ATP released in the extracellular milieu induces microglial activation as well as chemotaxis of microglia to the site of injury (Li et al., 2013; Rodrigues et al., 2015). However, the response of microglia to known activators and to perturbations in the CNS are also dependent on their immediate environment (Lai and Todd, 2008).

4.4.2 ATP mediated cytokine profile of BM and SCM

Previous studies suggest that SCM have a reduced inflammatory phenotype in response to LPS exposure relative to BM. In this study, we found no differences in the release of the pro-inflammatory factors NO, TNF, IL-6 or IL-1β between BM and SCM treated with ATP (1mM) (Figure 4-1, Figure 4-2, and Figure 4-3). This data suggests regional heterogeneity (i.e. microglia from brain vs microglia from spinal cord) does not influence ATP (purinergic receptors) mediated activation of microglia, unlike LPS where the SCM inflammatory profile was reduced compared to that of BM (Chapter 2). This data suggests that ATP by itself may have a primary role as a chemoattractant rather than an immunomodulator (Honda et al., 2001; Wu et al., 2007). Moreover, previous studies have also shown that combination of DAMPs (ATP+LPS) induces a much quicker induction (peaked at five hours post treatment) of inflammatory cytokine IL-1β than just LPS (peaked at 25 hours) (Sanz and Virgilio, 2000). This suggests that ATP may act in combination with other DAMPs to evoke quick induction of inflammatory processes after an injury, in addition to a role as a chemoattractant.

4.4.3 Glutamate mediated cytokine profile of BM and SCM

Previous studies shown that 1mM glutamate induced release of TNF through AMPA (GluR2-4) and kainate (GluR5) receptors (Noda et al., 2000). Similarly, Group II m GlurR2 and GlurR3 specific agonist DCG-IV induce TNF release by microglia (Taylor, 2005). However, 1mM glutamate does not mimic the concentration of glutamate at the site of an ischaemic injury or TBI (Dai et al., 2010; Hinzman et al., 2010; Marini and Paul, 1992; Ueda et al., 1992). The purpose of this glutamate study was to test if glutamate can induce the release of inflammatory

factors at physiological concentrations mimicking ischaemic and excitotoxicity injury and to test if glutamate could induce differential release of inflammatory factors by BM and SCM. Glutamate at physiological concentrations did not induce the release of pro-inflammatory factors (TNF and IL6) and there was no significant difference between BM and SCM release of these factors (Figure 4-5). However, an overall reduction in NO and IL-1β release by SCM was observed in glutamate experiments (Figure 4-4 and Figure 4-6). Interestingly, it has been shown that glutamate can induce chemotaxis in microglia (Liu et al., 2009). Thus, glutamate at physiological concentrations may also play a primary role as a chemoattractant in addition to its role as mediator of inflammation.

4.4.4 Conditioned media-mediated cytokine profile of BM and SCM

All microglia in the CNS originate from the yolk sac during embryogenesis. This suggests that all microglia in CNS are genetically very similar (Ginhoux and Prinz, 2015). However, microglia are very versatile and studies have shown that they can have different phenotypes (surface receptor expression, pro-, and anti- inflammatory factors release) in distinct regions of brain (Lai, 2010). For example, the P_2XR_7 and P_2YR_{12} expression is higher in striatum than in other regions of the brain (Lai, 2010). This suggests that microglia adapt to their immediate environment, potentially due to paracrine signaling from neighbouring cells that alters the extracellular milieu.

The conditioned media experiments were designed to answer the question whether the BM exposed to SMix CM would adopt a SCM-like phenotype compared to BM exposed to BMix CM. Notably, SMix CM significantly increased release of IL-6 (Figure 4-7), suggesting

that the environmental milieu can significantly affect phenotype for certain inflammatory molecules. This increase in IL6 mimics a trend observed after LPS activation in Chapter 2. Similarly, an overall reduction in IL-1β release was observed after incubation of BM with SMix CM, again similar to differences observed between BM and SCM in Chapter 2. The conditioned media experiments therefore provide further evidence that BM phenotype is plastic and can be modified by exposure to SMix CM, thus supporting the hypothesis that not only the region of origin but also the immediate environment determine the phenotype of microglia. This further supports the postulate that brain as well as SCM phenotype is not fixed by region of origin and that immediate environment plays a crucial role in microglial phenotype after perturbation or injury to CNS. The data also aligns well with previous studies that demonstrated that the inflammatory profile of microglia is dependent on the severity of injury, where microglia were beneficial to outcome of mild injury (Lai et al., 2013; Lai and Todd, 2008).

4.4.5 Summary

Chapter 4 investigated whether ATP and glutamate induce a differential inflammatory profile in SCM vs. BM, and addressed whether these mediators at physiological concentrations induce the release of inflammatory cytokines by BM and SCM. Our study is one of the few studies to examine the effects of physiological concentrations of glutamate on microglia. Our results suggest that ATP and glutamate do not induce significant release of pro-inflammatory factors such as NO, TNF, IL6 and IL-1β. However, our data do not preclude a role for these activators in the inflammatory response to brain injury. Both ATP and glutamate are involved in chemotaxis of microglia and can potentially recruit microglia to the site of injury or perturbation

(Honda et al., 2001; Liu et al., 2009; Wu et al., 2007). Moreover, it was found that ATP in combination with LPS induces faster maturation and release of intracellularly accumulated IL-1β (Sanz and Virgilio, 2000). The consequences of glutamate treatment on the inflammatory profile of LPS are not defined. Previous studies have shown that exposing microglia from thalamus and hippocampus to conditioned media from striatum induces a striatum-like phenotype in the microglia (Lai, 2010). Similarly, we found that SMix CM altered the response profiles of BM in BMix CM, increasing release of IL6 release and reducing IL-1β in response to LPS activation (Figure 4-7 and Figure 4-8). Overall, our data suggest that ATP and glutamate at physiological concentration do not induce an inflammatory profile in BM and SCM, at least with respect to cytokine release. Thus, the data provide further support that the phenotype of BM as well as SCM *in vitro* is determined by both region of origin and immediate environment in addition to other factors such as age and sex (Grabert et al., 2016a; Lai et al., 2013; Sorge et al., 2011).

4.5 Figures

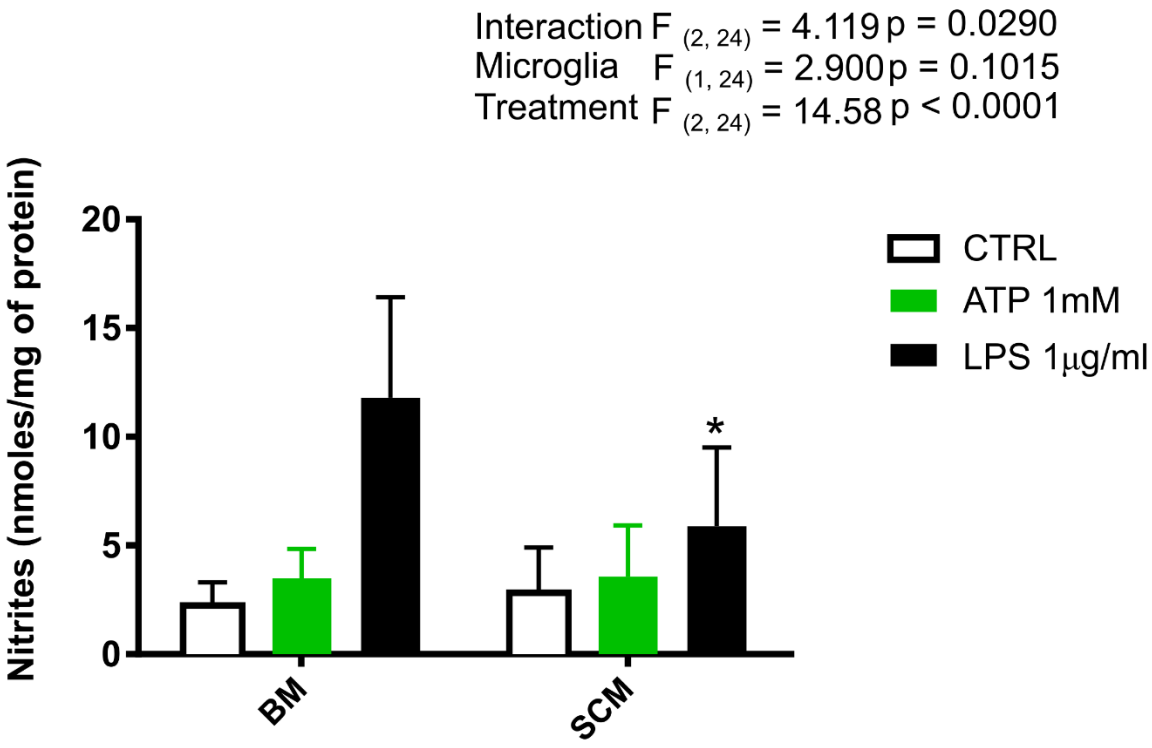
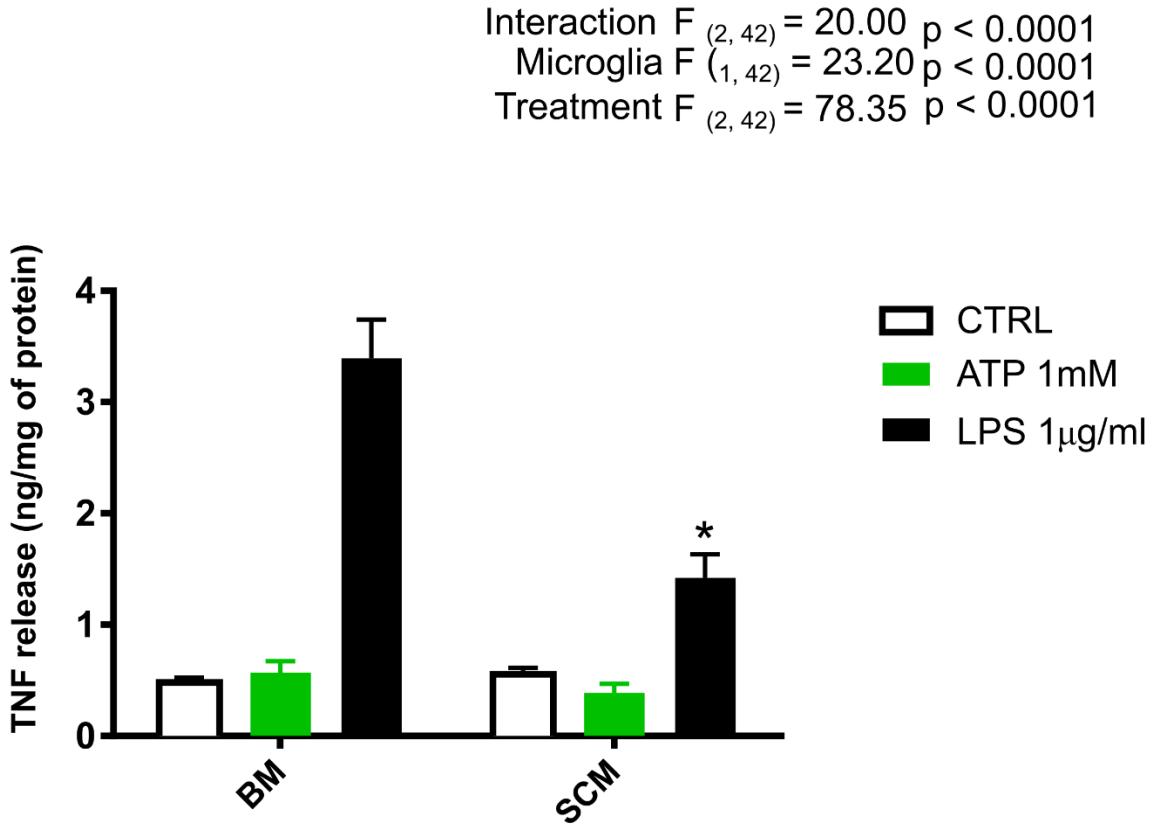

Interaction F (2, 24) = 4.119 p = 0.0290
Microglia F (1, 24) = 2.900 p = 0.1015
Treatment F (2, 24) = 14.58 p < 0.0001
Nitrites (nmoles/mg of protein)
20
15
10
5
0
*
BM
SCM
CTRL
ATP 1mM
LPS 1μg/ml

Figure 4-1 Release of the pro-inflammatory effector NO by ATP activated BM and SCM.

Two-away ANOVA identified significant interaction between microglia and treatment for NO ($F_{(2,24)} = 4.119$, $p = 0.0290$) and Two-way ANOVA identified a main effect of treatment for NO ($F_{(2,24)} = 14.58$, $p < 0.0001$). ATP treatment did not induce a significant difference in the release of inflammatory factor NO between BM and SCM ATP treatments. However, Sidak *post-hoc* test revealed a significant difference between BM LPS and SCM LPS, * represents $p < 0.05$, $n = 5$ where n represents the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

A


Interaction F (2, 42) = 20.00 p < 0.0001
Microglia F (1, 42) = 23.20 p < 0.0001
Treatment F (2, 42) = 78.35 p < 0.0001
TNF release (ng/mg of protein)
0
1
2
3
4
*
BM
SCM
CTRL
ATP 1mM
LPS 1μg/ml

B

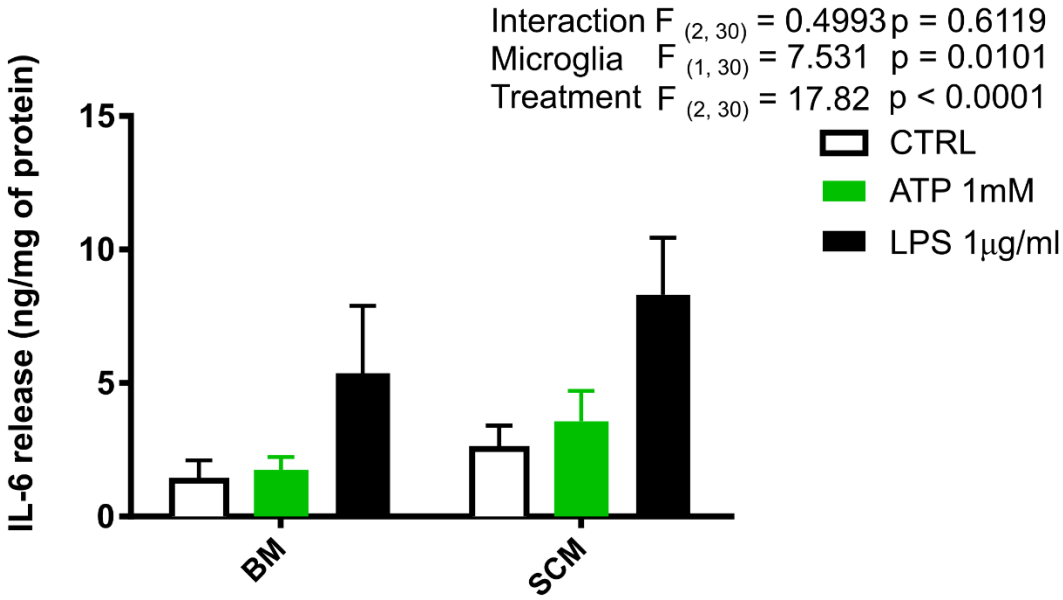

Interaction F (2, 30) = 0.4993 p = 0.6119
Microglia F (1, 30) = 7.531 p = 0.0101
Treatment F (2, 30) = 17.82 p < 0.0001
IL-6 release (ng/mg of protein)
0
5
10
15
BM
SCM
CTRL
ATP 1mM
LPS 1μg/ml

Figure 4-2 Secretion of the pro-inflammatory effectors TNF and IL6 by ATP activated BM and SCM.

A) Two-away ANOVA identified significant interaction between microglia and treatment TNF ($F_{(2,42)} = 20.00$, $p < 0.0001$). A, B) A significant main effect of microglia (TNF ($F_{(1,42)} = 23.20$, $p < 0.0001$) and IL6 ($F_{(1,30)} = 7.531$, $p < 0.010$) and treatment (TNF ($F_{(2,42)} = 78.35$, $p < 0.0001$) and IL6 ($F_{(2,30)} = 17.82$, $p < 0.001$) was also observed. However, ATP treatment did not induce a significant difference in the release of inflammatory factors TNF and IL6 between BM and SCM ATP treatments. A significant difference between BM LPS and SCM LPS was revealed by the Sidak *post-hoc* test, * represents $p < 0.05$, $n = 8$ for TNF and $n = 6$ for IL6 where n represents the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

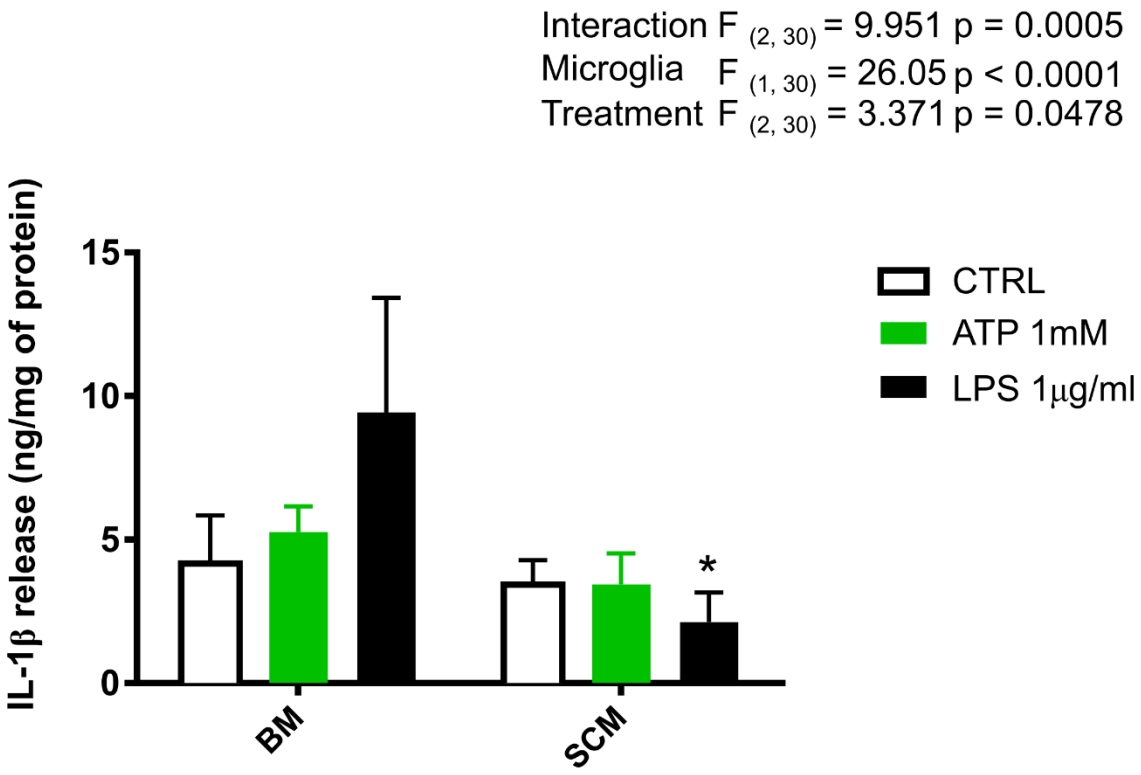


Figure 4-3 Secretion of the pro-inflammatory effector IL-β by ATP activated BM and SCM.

Two-away ANOVA identified significant interaction between microglia and treatment for IL-1β ($F_{(2,30)} = 9.951$, $p < 0.0005$), A significant main effect of microglia ($F_{(1,30)} = 26.05$, $p < 0.0001$) and treatment ($F_{(2,30)} = 3.372$, $p = 0.0478$) was also observed for IL-1β. However ATP treatment did not induce a significant difference in the release of inflammatory factor IL-β between BM and SCM after ATP treatments. A Sidak *post-hoc* test revealed a significant difference between BM LPS and SCM after LPS treatment. * represents $p < 0.05$ for comparison, $n = 6$ where n represent the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

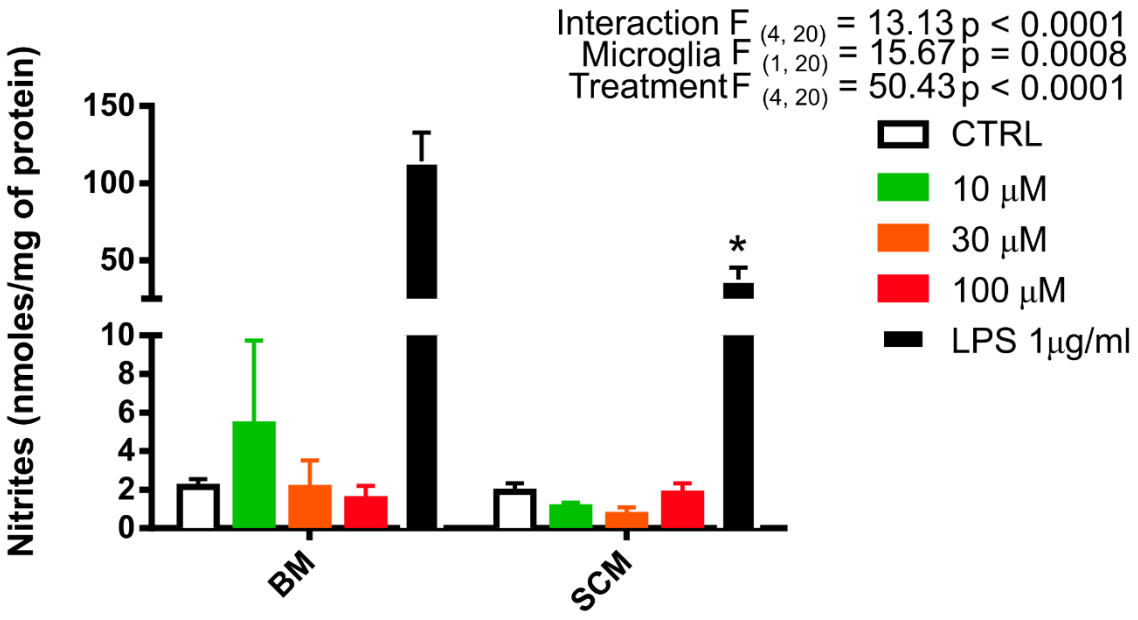
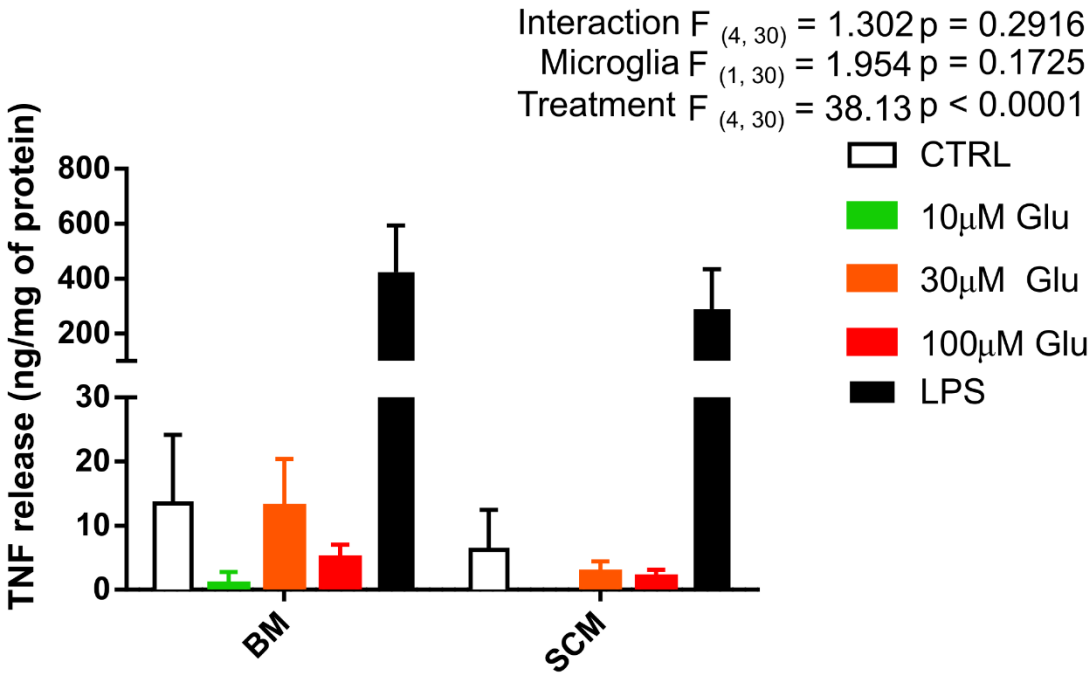


Figure 4-4 Release of the pro-inflammatory effector NO by BM and SCM exposed to glutamate.

Two-way ANOVA identified significant interaction between microglia and treatment for NO ($F_{(4,20)}$ =13.13, p <0.0001). There was also a significant main effect of microglia ($F_{(1,20)}$ =15.67, p = 0.0008) and treatment ($F_{(4,20)}$ =50.43, p < 0.0001), * represents significant difference between BM LPS and SCM LPS revealed by the Sidak *post-hoc* test p < 0.05. n = 3 where n represent the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

A


Interaction F (4, 30) = 1.302 p = 0.2916
Microglia F (1, 30) = 1.954 p = 0.1725
Treatment F (4, 30) = 38.13 p < 0.0001
CTRL
10μM Glu
30μM Glu
100μM Glu
LPS
TNF release (ng/mg of protein)
800
600
400
200
30
20
10
0
BM
SCM

B

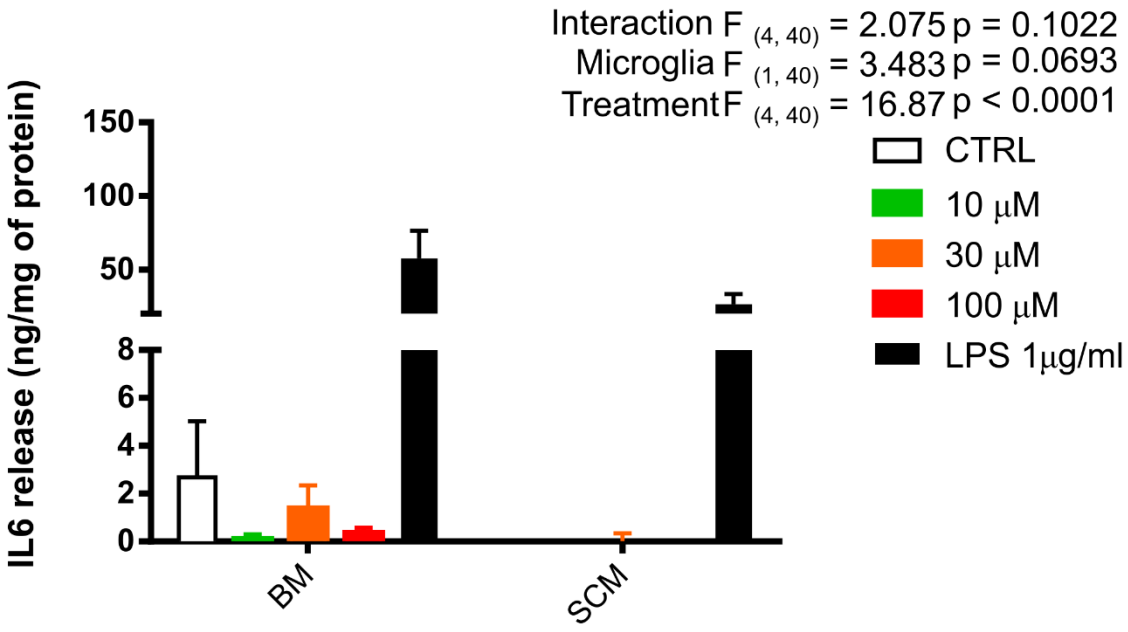

Interaction F (4, 40) = 2.075 p = 0.1022
Microglia F (1, 40) = 3.483 p = 0.0693
Treatment F (4, 40) = 16.87 p < 0.0001
CTRL
10 μM
30 μM
100 μM
LPS 1μg/ml
IL6 release (ng/mg of protein)
150
100
50
8
6
4
2
0
BM
SCM

Figure 4-5 Secretion of the pro-inflammatory effectors TNF and IL6 by BM and SCM exposed to glutamate.

A, B) Two-way ANOVA revealed a significant main effect for glutamate treatment for TNF ($F_{(4,30)} = 38.13$ $p < 0.0001$) and IL6 ($F_{(4,40)} = 16.87$, $p < 0.0001$). * represents a significant difference between respective LPS treatments revealed by the Sidak *post-hoc* test. * = $p < 0.05$. n= 4 for TNF and n = for IL6 where n represent the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

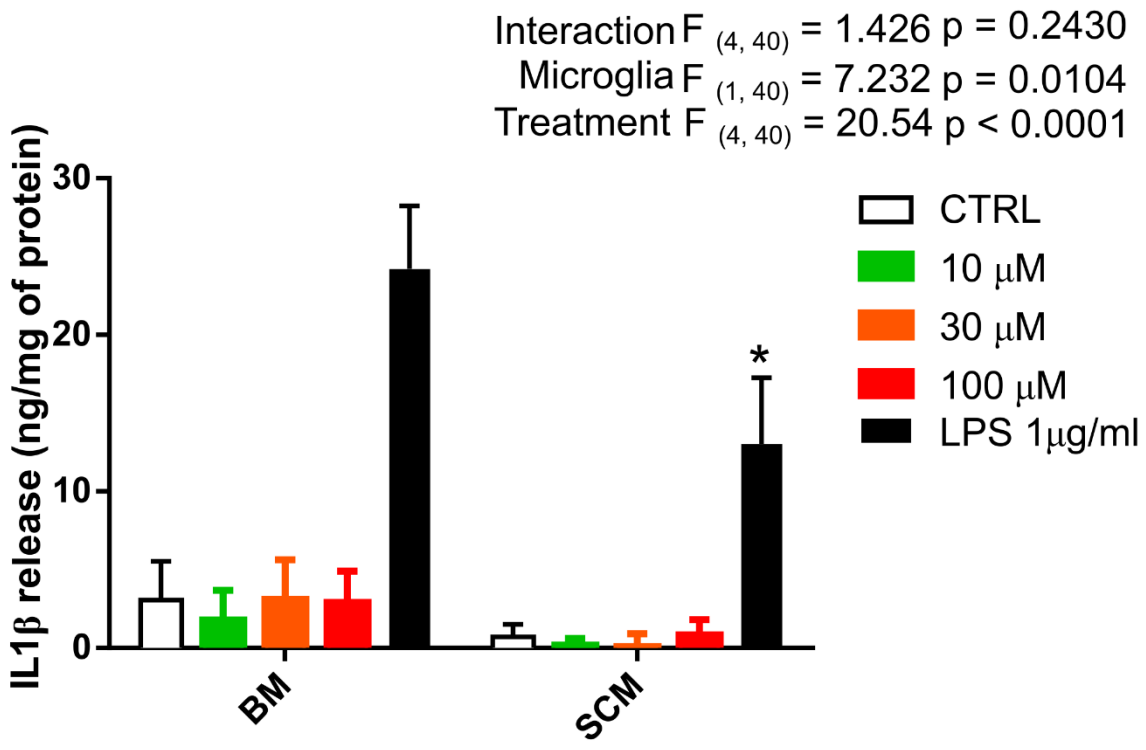


Figure 4-6 Secretion of the pro-inflammatory effector IL-1β by BM and SCM exposed to glutamate.

Two-way ANOVA revealed a significant main effect for microglia ($F_{(1,40)} = 7.232$ $p = 0.0104$) and glutamate treatment for IL-1β ($F_{(4,40)} = 20.54$ $p < 0.0001$). * represents significant difference between respective LPS treatment conditions revealed by the Sidak *post-hoc* test. * = $p < 0.05$. n= 5 where n represent the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars mean ± s.e.m

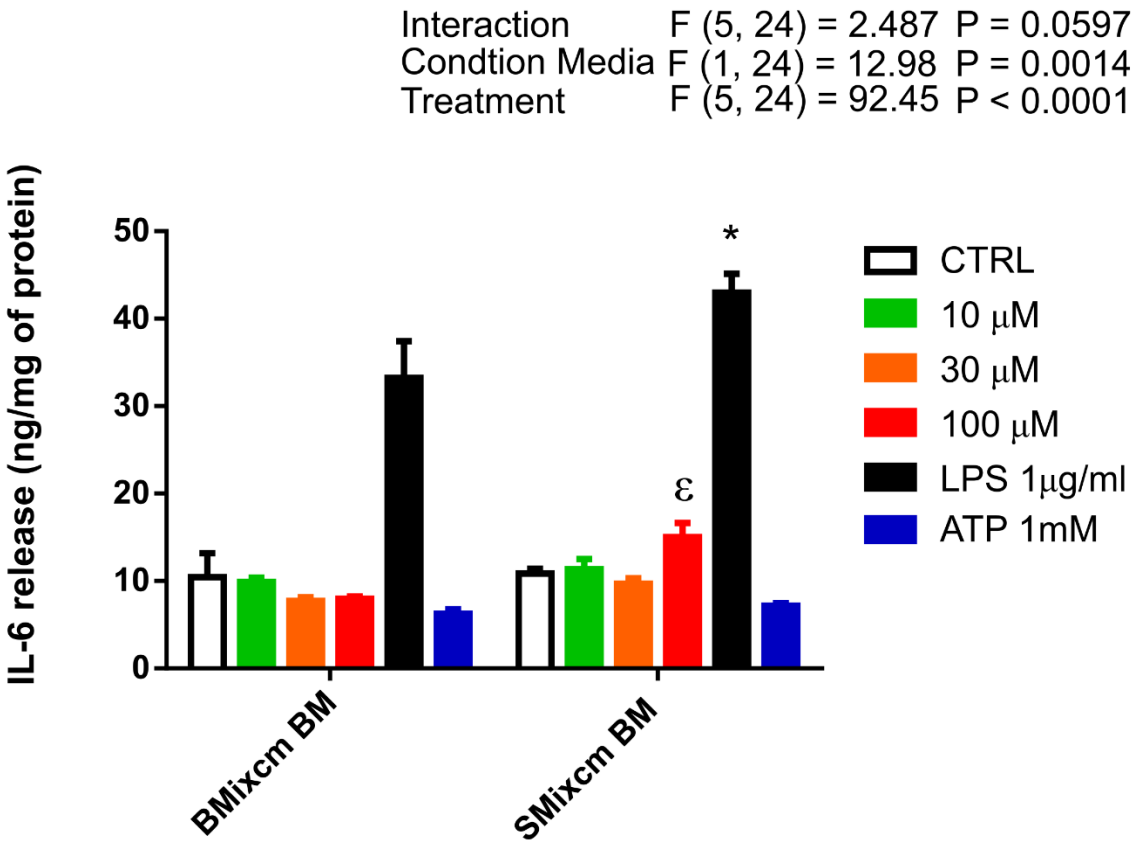

Interaction F (5, 24) = 2.487 P = 0.0597
Condtion Media F (1, 24) = 12.98 P = 0.0014
Treatment F (5, 24) = 92.45 P < 0.0001
IL-6 release (ng/mg of protein)
50
40
30
20
10
0
*
ε
CTRL
10 μM
30 μM
100 μM
LPS 1μg/ml
ATP 1mM
BMixcm BM
SMixcm BM

Figure 4-7 Secretion of the pro-inflammatory effector IL6 by BM exposed to BMix CM and SMix CM.

Two-way ANOVA revealed a trend towards interaction of conditioned media for IL6 release ($F_{(1, 24)} = 2.487$, $p = 0.058$). Two-way ANOVA also revealed a significant main effect of conditioned media ($F_{(1, 24)} = 12.98$, $p = 0.001$) and treatment $F_{(5, 24)} = 92.45$, $p < 0.0001$) for IL-6 release. ε represents significant difference between brain mixed glia conditioned media (BMixcm) 100 μM glutamate and spinal mixed glia conditioned media (SMixcm)100μM glutamate treatment and * significant difference between BMixcm LPS and SMixcm LPS revealed by the Sidak *post-hoc* test, ε = $p < 0.05$, * = $p < 0.05$. n = 3 where n represent the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

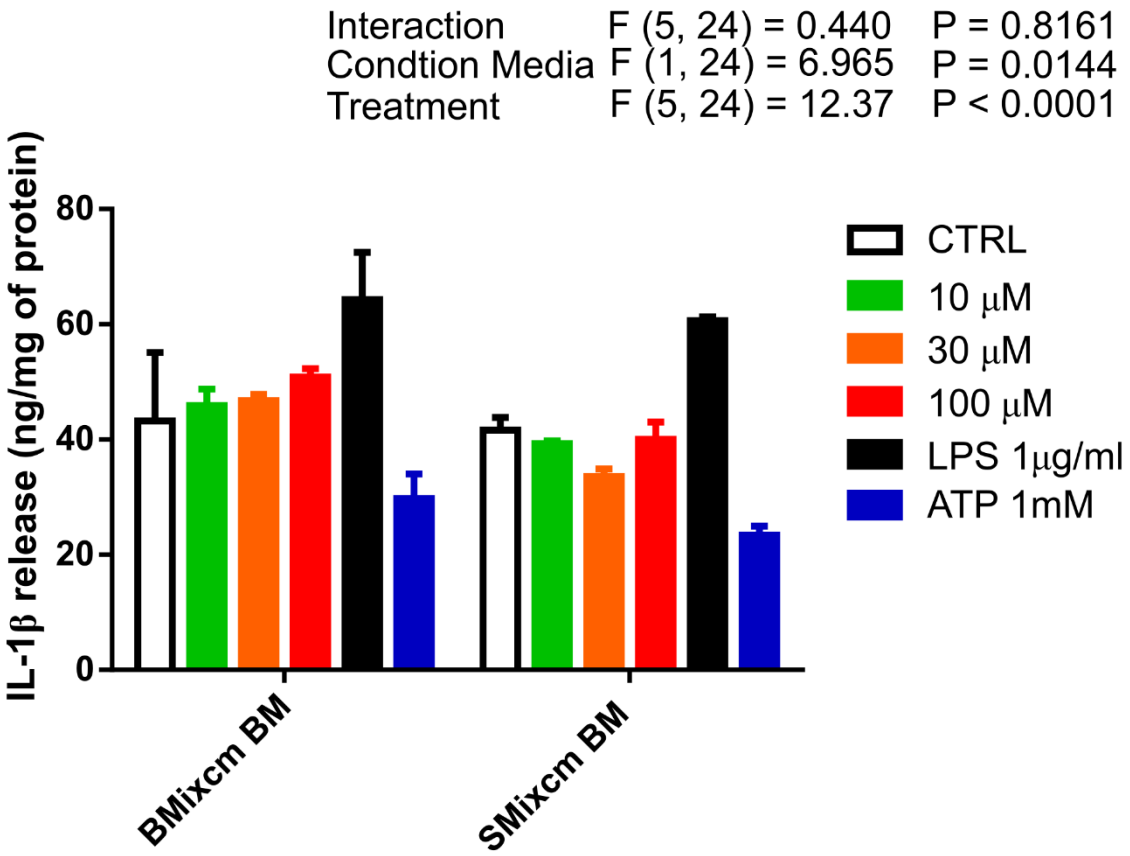


Figure 4-8 Secretion of the pro-inflammatory effector IL-1β by BM exposed to BMix CM and SMix CM.

Two-way ANOVA revealed a significant main effect of conditioned media ($F_{(1, 24)} = 6.965$, $p < 0.05$) and treatment ($F_{(5, 24)} = 12.37$, $p < 0.001$). However, the Sidak *post-hoc* test did not reveal any significant differences between BMix CM and SMix CM. n= 3 where n represents the number of independent experiments, where an independent experiment is a separate microglia preparation. Bars represent mean ± s.e.m

Chapter Five: Neuroprotective characterization of spinal microglia in an *in vitro* model of ischaemic injury

5.1 Introduction

The role of microglia in CNS homeostasis is dependent on factors such as region of origin, age, and sex (Ferrazzano et al., 2013; Grabert et al., 2016a; Lai et al., 2011; Lai and Todd, 2008; Sorge et al., 2011). This chapter focuses on the effects of region of origin of microglia in an *in vitro* model of ischaemic injury. Ischaemic injury in the CNS induces neuronal death and release of DAMPs (damage associated molecular patterns) (Kettenmann et al., 2011). Microglia are capable of sensing DAMPs and migrate to the site of injury where they contribute to both neurotoxic and neuroprotective processes (Giulian and Baker, 1986; Lai et al., 2011; Rogove and Tsirka, 1998; Vilhardt, 2005; Wake et al., 2009).

Several previous studies have demonstrated that activation of microglia can be neurotoxic or neurotrophic depending on factors including the severity of injury (Giulian and Baker, 1986; Rogove and Tsirka, 1998; Vilhardt, 2005; Wake et al., 2009). For example, microglia isolated from one day old neo-natal pups exposed to media from cultured neurons subjected to various degrees of hypoxic injury *in vitro* [mild (30 min), moderate (2 hrs) or severe (6hrs)] increase their release of brain-derived neurotropic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) in all conditions (Lai and Todd, 2008). However, only mild hypoxic injury increased release of IL-1β (interleukin-1beta), TNF (tumor necrosis factor-alpha), and NO (nitric oxide). Thus, microglia are a highly dynamic population of cells that react differentially to severity of hypoxic injury to neurons (Lai and Todd, 2008).

Previous studies have also shown that ablation of microglia can also exacerbate neuronal injury and that administration of microglia to the site of neuronal injury can improve neuronal viability after an ischaemic injury (Faustino et al., 2011; Hayashi et al., 2006; Imai et al., 2007;

Kitamura et al., 2004; Lalancette-Hébert et al., 2007; Neumann et al., 2006). This suggests that microglia can be protective during neuronal injury. Microglia are a primary source of both pro-inflammatory (NO, TNF, IL1-β , and IL6) and anti-inflammatory (IL-10) mediators during acute ischaemic injury (Clausen et al., 2008; Gregersen et al., 2000; Lai and Todd, 2008; Lambertsen et al., 2007; Sist et al., 2014). Surprisingly, acute block or inhibition of inflammatory factors such as TNF has been shown to be detrimental to the outcome of ischaemic injury, suggesting that inflammation is beneficial and needed for normal neuronal recovery (Bruce et al., 1996; Imai et al., 2007; Lai and Todd, 2008; Neumann et al., 2006). Studies *in vitro* suggest that BM (brain microglia) can rescue moderately, but not severely, injured cortical neurons, suggesting that there is a threshold for microglia mediated neuroprotection and recovery after injury (Lai and Todd, 2008). In Chapter 2, we demonstrated that the inflammatory profile of SCM (spinal cord microglia was reduced relative to BM after activation with LPS. However, it is not known whether SCM will provide more or less neuroprotection in a model of neuronal injury relative to BM. In this study, the neuroprotective effects of SCM on injured cortical neurons was tested against BM in a co-culture system (BM/SCM with cortical neurons). Neurons were subjected to moderate (30 min) or severe (4 hr) injury by OGD (oxygen-glucose deprivation, a model of ischaemic injury) and then co-cultured with BM or SCM. Viability assays and measurement of inflammatory markers suggest that SCM-cortical neuron co-culture was less viable than BM-cortical neuron co-culture after OGD injury.

5.2 Methods

Hanks Balanced Saline solutions (HBSS), Dulbecco's Modified Eagle Medium – Hams'F12 nutrient mixture (DMEM-F12), DMEM-F12 with HEPES (DMEM-F12/HEPES), Dulbecco's Modified Eagle Medium (DMEM), 0.25%trypsin-EDTA, fetal bovine serum (FBS), Neurobasal Medium, minus phenol red, Neurobasal Medium-A, B27, and Penicillin-Streptomycin (P/S) were from Gibco (Thermo-Fisher Scientific, Burlington, ON). Cytosine arabinoside (Ara-C) and sodium nitrite standard solution were from Sigma (Oakville, ON).

5.2.1 Primary mixed glia preparation

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. Brain and spinal cord for establishing primary mixed glial cultures were obtained from postnatal day one or two male SD rat pups as previously described (Churchward and Todd, 2014; Lai and Todd, 2008). The SD rat pups were euthanized; their brains (four) and spinal cords (twenty) were dissected and placed in dissection buffer (HBSS with 200 U/ml penicillin, 200 μg/ml streptomycin). Meninges and blood vessels were removed under a dissection microscope. Tissues were cut into small pieces and incubated in 0.25% trypsin-EDTA for 25 mins at 37°C, and collected by centrifugation (2000 x g, 2 mins). Trypsin was inactivated with maintenance media (DMEM/F12 supplemented with 10% FBS and 200 U/ml penicillin, 200 μg/ml streptomycin), tissues were dissociated by trituration in maintenance media and centrifuged at 2000 x g for 2 mins. The brain and spinal cord cell pellet was re-suspended in maintenance medium and seeded at equal density into cell culture treated T75 flasks coated with

poly-L-lysine. Cells were maintained in a 37°C, 5% CO_2 humidified incubator with maintenance media replaced twice weekly.

5.2.2 Primary cortical neuron culture

All animal protocols were conducted in accordance with Canadian Council on Animal Care Guidelines and approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta. A D18 pregnant SD rat was anesthetised and embryos were extracted. The embryos were decapitated on ice. The heads were placed on ice-cold HBSS, the brain was extracted, and meninges and blood vessels were removed under a dissection microscope. The cortices were isolated from the whole brain under a dissection microscope. The cortices are enzymatically dissociated in 0.25% Trypsin-EDTA for 20 mins at 37°C. The cortices were centrifuged at 2000 x g for 2 mins. Trypsin-EDTA was inactivated with DMEM supplemented with 10% FBS and 1% P/S. The cortices were centrifuged at 2000 x g for 2 mins and the cell pellet was re-suspended in neurobasal media supplemented with 2 % B27 and 1 % P/S. The cortical mixed cells were seeded at density of $5X10^5$ cells/ml in 12 well plates coated with poly-L-lysine. Three-day old mixed cortical cultures were treated with Ara-C at 3mM concentration for three days to deprive glial cells. The cortical neurons were then refreshed with fresh medium (Neurobasal media supplemented with 2 % B27 and 1 % P/S). The cortical neurons were maintained in a 37°C, 5% CO_2 humidified incubator with maintenance medium (1:1 mix of 0.22 µm filtered conditioned medium from cortical neurons and freshly made neurobasal medium supplemented with 2 % B27 and 1 % P/S) replaced twice weekly. All experiments were carried out on two-week old cortical neurons.

5.2.3 Cortical neurons OGD and establishment of cortical neuron -microglia co-culture

One day before OGD experiment two-week-old cortical neuron culture were refreshed with Neurobasal media supplemented with 2 % B27 and 1 % P/S (NB medium). On the day of OGD experiments the cells were refreshed once again with NB medium. The cortical neuron assigned for OGD were refreshed with NB media without glucose (NB^- media) and immediately placed in oxygen deprived chamber (chamber O^2 level < 1%) for 30 mins (moderate injury) and 4 hrs (severe injury). Control cortical neuron cultures were placed in normal oxygen conditions in a CO^2 incubator for the same duration as OGD exposed cortical neurons. After OGD, OGD-treated and control cortical neurons were allowed 1 hr to recover in NB media. Microglia were isolated from primary mixed glial cultures as described before in Chapter 3. BM and SCM were seeded on top of control and OGD injured cortical neurons at a density of $1X10^5$ cells/ml immediately after 1 hr recovery. The microglia were allowed to settle for 10 mins after which the co-culture was gently washed and fresh NB media were added and were allowed to recover overnight. At 24 hrs, the cortical neuron-microglia co-culture viability was assessed using an MTT assay and in parallel media and cell lysates were collected for measuring pro-and anti-inflammatory factors.

5.2.4 Viability assay

Viability of cortical neuron-microglia co-cultures was assessed via a colorimetric assay for metabolic activity (mitochondrial activity). MTT can be reduced to an insoluble formazan dye by NAD(P)H-dependent cellular oxidoreductase enzymes. The absorbance of light by the formazan dye in solution can be measured at 540 nm by an UV spectrometer. Control neurons

only, neurons + BM co-cultures (control and OGD) and neurons + SCM co-cultures (control and OGD) were incubated in 0.05mg/ml of MTT in PBS for 2 hrs in the CO_2 incubator. After 2 hrs, the MTT solution was aspirated and the cells were lysed in 200 microliters of DMSO. The absorbance of lysates was measured at 540 nm by an UV spectrometer.

5.2.5 Nitric Oxide (NO)

NO release was measured indirectly by quantifying the stable metabolite nitrite in culture media using a method described by Griess, (1879)). Media were collected 24 hours after OGD experiment and were added to 96-well plate in duplicates followed by 50 μL eac of 1% sulphanilamide (in 3N HCl) and 0.02% N-napthylethylenediamine per well. Absorbance was read at 540 nm and the amount of nitrite metabolite was interpolated from a set of standards measured in parallel.

5.2.6 Enzyme Linked Immunosorbent assays (ELISA)

A commercial ELISA kit was used to measure TNF, IL-1β, IL6 and IL-10 in media (DuoSet, R&D Systems Minneapolis, USA). ELISA procedures were carried out according to manufacturer protocols.

5.2.7 Statistics

Statistical analyses were carried out using two-way ANOVA followed by Sidak's method to test for significance between treatment groups except for MTT viability study where one-way ANOVA followed by Newman-Keul *post hoc* correction to test for significance between groups.

n represents the number of single independent experiments (i.e. an independent culture preparation) with a minimum of three technical replicates. Each technical replicate is a well in a 12, 24 or 48 well culture plate. All statistical analyses were done using Graphpad Prism version 6.

5.3 Results

5.3.1 Viability of OGD cortical neuron – microglia co-culture

In chapter 2, it was shown that the pro-inflammatory profile of SCM was reduced relative to BM in *in vitro* models mimicking pathogen-mediated injury (Baskar Jesudasan et al., 2014). Inflammation can affect the viability and recovery of injured neurons, and previous studies suggest that factors released by BM exposed to conditioned media from moderately injured cortical neurons (2hrs of oxygen deprivation, ~30% loss of viability) is neuroprotective (Lai and Todd, 2008). The protective effects of SCM has not been investigated. Here, BM and SCM were co-cultured with cortical neurons in an *in vitro* model of ischaemic injury (oxygen-glucose depravation of cortical neurons (OGD) for 30 mins (moderate) and 4 hrs (Figure 5-1). 30 mins was chosen to induce a moderate injury, based on previous data that suggest that severity of injury alters neuronal viability by altering release of microglial effectors (Lai and Todd, 2008). A 4 hrs condition served as a positive control for OGD injury where cortical neurons had acquired unrecoverable injury. In control (non-OGD) conditions, there was no effect on viability of the BM-cortical neuron or the SCM-cortical neurons co-culture systems ($F_{(2,12)} = 0.074$ $p = 0.9294$, and 4 hrs OGD ($F_{(2,12)} = 0.0581$ $p = 0.9438$) (Figure 5-1A, B). However, a main effect of culture conditions was observed with 30 min OGD ($F_{(2,12)} = 4.156$ $p = 0.0425$) (Figure 5.7.1B).

Newman-Keuls *post-hoc* test revealed that 30 mins GD injury conditions significantly reduced the metabolic activity in SCM-cortical neurons co-culture relative to BM-cortical neurons co-culture ($p < 0.05$). There was no effect of culture conditions on viability in the 4 hrs OGD condition (Figure 5-1C).

5.3.2 Inflammatory factors released by OGD cortical neuron – microglia co-culture

Release of pro-inflammatory (NO, TNF, IL6, IL1 β) and anti-inflammatory factors (IL10) were measured in the different co-culture and injury conditions. One-way ANOVA revealed no effect of culture conditions on NO, TNF, IL6, IL1-β or IL10 measured in BM and SCM-cortical co-cultures under control conditions (Figure 5.5.2, all $p > .05$). Similarly, there was no effect of culture conditions on release of NO, TNF, IL6, IL1-β or IL10 in co-culture systems in 30 mins or 4 hrs OGD conditions (Figure 5-3 and Figure 5-4, all $p > .05$). Thus, while metabolic activity of SCM-cortical neurons co-culture was significantly reduced relative to BM-cortical neurons co-cultures after 30 min OGD, there were no significant changes in the release of pro- or anti-inflammatory molecules in the co-culture systems.

5.4 Discussion

5.4.1 OGD cortical neurons – SCM co-culture exhibited reduced viability

In Chapter 2 it was demonstrated that the pro-inflammatory release and phagocytotic activity of SCM was reduced relative to BM after activation by LPS. As LPS is PAMP and a potent activator of microglia and inflammatory trigger, the current study investigated the

protective profile of SCM and BM in co-culture systems and models of moderate or severe ischaemic injury. Cortical neurons injured by OGD were then co-cultured with microglia to provide an *in vitro* paradigm to model ischaemic injury. We found that SCM-cortical neurons co-culture had reduced viability compared to BM-cortical neurons co-culture after moderate OGD injury (Figure 5-1B). However, the pro- and anti-inflammatory factors released by SCM-cortical neurons co-cultures was not significantly different from that of BM-cortical neurons co-cultures and neurons alone culture (Figure 5-2, Figure 5-3 and Figure 5-4). Numerous studies have demonstrated an increase in the pro-inflammatory factors within 24 hrs of neuronal injury (Choi and Rothman, 1990; Clausen et al., 2008; Lambertsen et al., 2012; Sist et al., 2014, 2012). However, this effect is not always present, and previous work from our group suggest that BM exposed to conditioned media from neurons with ischaemic injury do not release greater levels of pro-inflammatory factors (Lai and Todd, 2008). This could be due to greater proportion of apoptotic and necrotic neurons present *in vivo* compared to an *in vitro* model and a lack of additional inflammation from infiltrating peripheral immune cells in the culture models.

5.4.2 OGD cortical neurons-microglia co-culture in vitro mimic of penumbra

In vitro ischaemic models such as oxygen deprivation only or OGD have been extensively utilized to elucidate the molecular mechanisms that could be targeted for therapeutic intervention after an ischaemic injury (Kaushal and Schlichter, 2008; Lai and Todd, 2008; Meloni et al., 2011, 2001). In this study, OGD-injured cortical neurons and microglia co-culture was employed to study potential differences between BM- and SCM-cortical neurons co-cultures on viability and cytokine release. The *in vivo* OGD model mimics the border of the penumbra

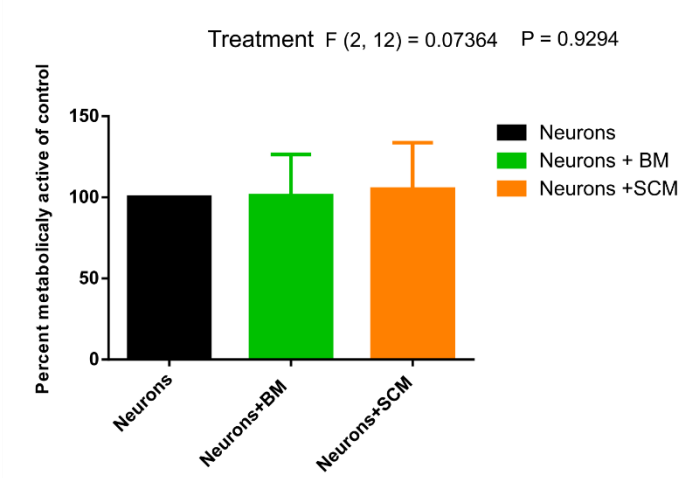
region of a stroke wherein the microglia themselves have not faced severe metabolic deficit but are exposed to the signals from the injured neurons. Our data suggest that BM and SCM respond comparably to neuronal injury, with a difference in viability after moderate neuronal injury.

5.4.3 Summary

Our results suggest that microglia alone do not trigger an inflammatory profile when exposed to moderately injured neurons *in vitro* in a contact culture. These data contradict previous data using non-contact microglia and OGD-injured neurons co-culture systems (Hu et al., 2012; Kaushal and Schlichter, 2008). This suggests a differential response of microglia that are in immediate contact with neurons versus those responding purely to chemical cues. However, while previous studies in this dissertation have highlighted differential response profiles in SCM vs. BM, these data suggest that the overall response to models of ischaemic injury between SCM and BM is comparable. While reduced viability was observed in SCM co-cultures relative to BM co-cultures in moderate injury conditions, this effect did not persist after 4 hrs of OGD and there were no significant differences in the release of inflammatory mediators. Thus, while our previous work suggests that SCM and BM are functionally unique subpopulations of cells, they exhibit a comparable response to models of ischaemic injury.

5.5 Figure

A



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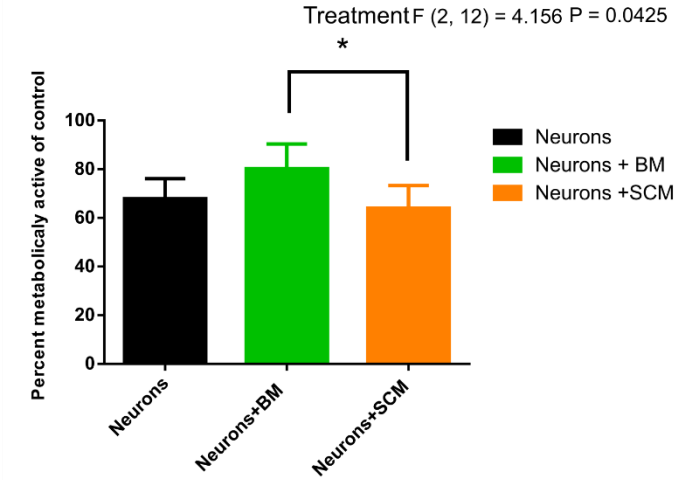


C

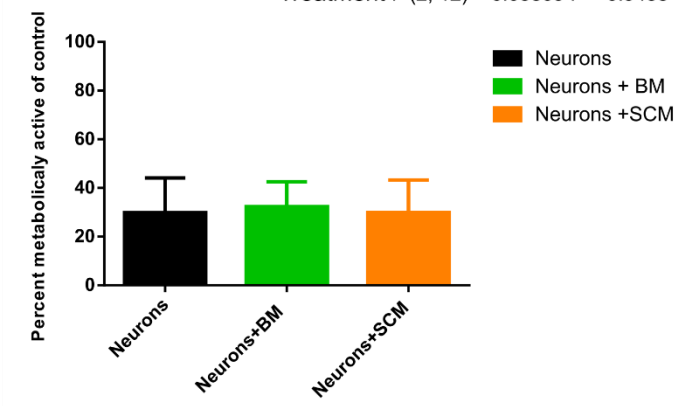
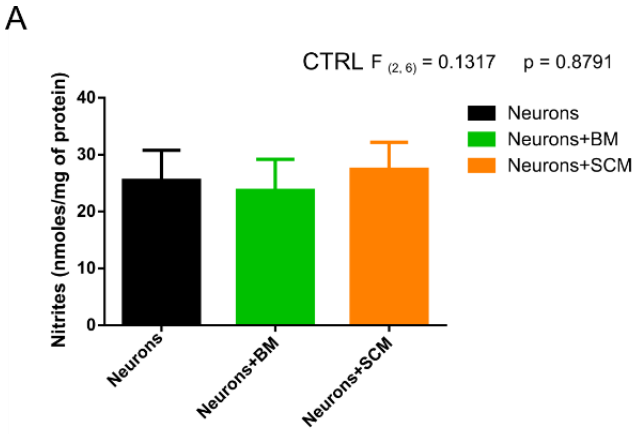
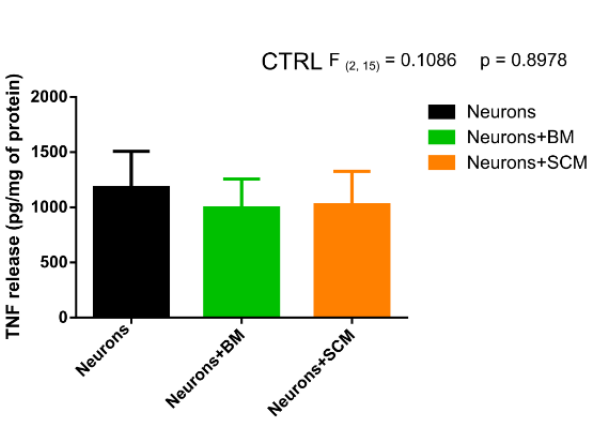
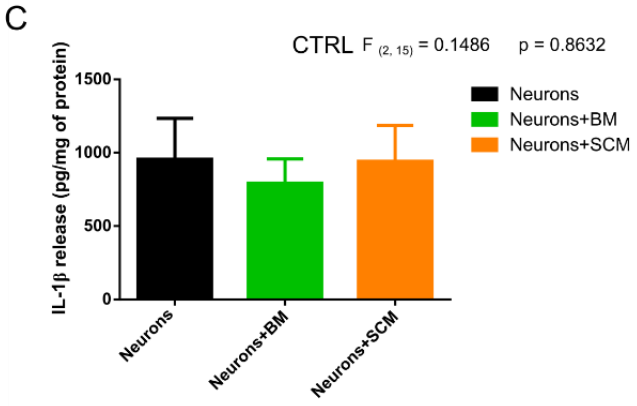


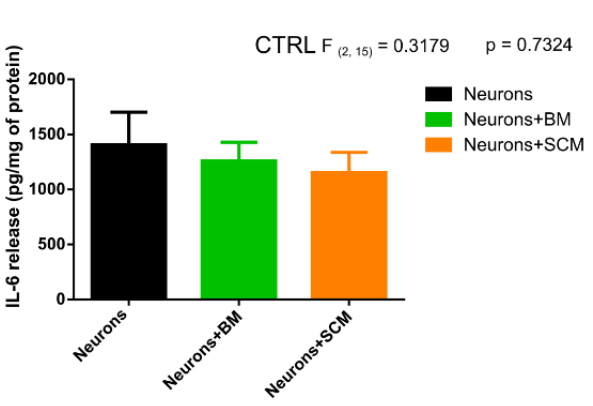
Figure 5-1 Metabolic activity of cortical neurons-microglia co-cultures after OGD.

A) One-way ANOVA did not reveal a main effect for viability of BM- and SCM-cortical neurons co-culture CTRL (CTRL $F_{(2,12)} = 0.074$ $p = 0.9294$). B) BM- and SCM-cortical neurons co-culture 30 min OGD had a main effect for viability of co-culture ($F_{(2,12)} = 4.156$ $p = 0.0425$). the Newman-Keuls *post-hoc* test revealed that 30 mins OGD injury conditions significantly reduced the metabolic activity of SCM-cortical neurons co-culture compared to BM-cortical neurons co-culture, $p < 0.05$. C) Cortical neurons only, BM- or SCM-cortical neurons co-culture 4 hrs OGD injury condition did not have significant main effect ($F_{(2,12)} = 0.0581$ $p = 0.9438$). n = 5 where n represents the number of independent experiments with a minimum of three replicates. Bars represent mean ± s.e.m


A
CTRL F (2, 6) = 0.1317 p = 0.8791
Nitrites (nmoles/mg of protein)
0
10
20
30
40
Neurons
Neurons+BM
Neurons+SCM


B
CTRL F (2, 15) = 0.1086 p = 0.8978
TNF release (pg/mg of protein)
0
500
1000
1500
2000
Neurons
Neurons+BM
Neurons+SCM


C
CTRL F (2, 15) = 0.1486 p = 0.8632
IL-1β release (pg/mg of protein)
0
500
1000
1500
Neurons
Neurons+BM
Neurons+SCM


D
CTRL F (2, 15) = 0.3179 p = 0.7324
IL-6 release (pg/mg of protein)
0
500
1000
1500
2000
Neurons
Neurons+BM
Neurons+SCM

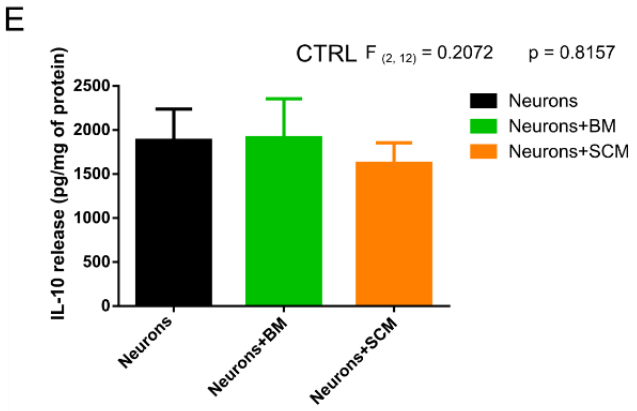

E
CTRL F (2, 12) = 0.2072 p = 0.8157
IL-10 release (pg/mg of protein)
0
500
1000
1500
2000
2500
Neurons
Neurons+BM
Neurons+SCM

Figure 5-2 Inflammatory factors released by CTRL cortical neurons-microglia co-cultures.

One-way ANOVA did not reveal a main effect for A) NO released in CTRL OGD cortical neurons-BM- and -SCM co-cultures ($F_{(2,6)} = 0.1317$ $p = 0.8791$), n = 3, B) TNF released in CTRL OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.1086$ $p = 0.8978$), n = 6, C) IL-1β released in CTRL OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.1486$ $p = 0.8632$), n= 6, D) IL-6 released in CTRL OGD cortical neurons-BM- and -SCM co-cultures ($F_{(2,12)} = 0.3179$ $p = 0.7324$), n = 6, E) IL-10 released in CTRL OGD cortical neurons-BM and -SCM co-cultures (CTRL $F_{(2,12)} = 0.2072$ $p = 0.8157$), n = 5. n represents the number of independent experiments with a minimum of three replicates. Bars represent mean ± s.e.m

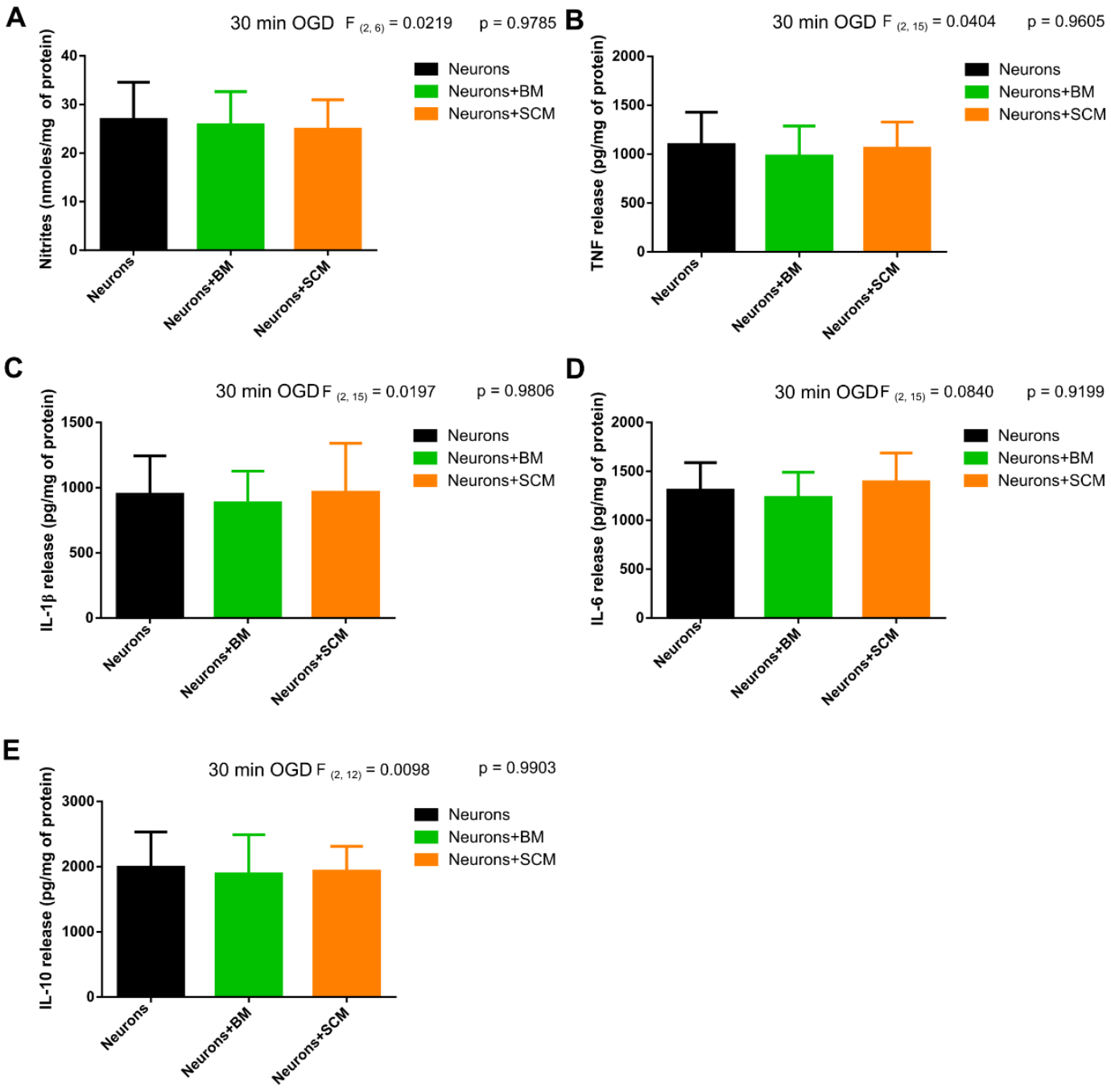

A
30 min OGD F (2, 6) = 0.0219 p = 0.9785
Nitrites (nmoles/mg of protein)
40
30
20
10
0
Neurons
Neurons+BM
Neurons+SCM
B
30 min OGD F (2, 15) = 0.0404 p = 0.9605
TNF release (pg/mg of protein)
2000
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
C
30 min OGD F (2, 15) = 0.0197 p = 0.9806
IL-1β release (pg/mg of protein)
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
D
30 min OGD F (2, 15) = 0.0840 p = 0.9199
IL-6 release (pg/mg of protein)
2000
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
E
30 min OGD F (2, 12) = 0.0098 p = 0.9903
IL-10 release (pg/mg of protein)
3000
2000
1000
0
Neurons
Neurons+BM
Neurons+SCM

Figure 5-3 Inflammatory factors released by 30 mins OGD cortical neurons-microglia co-cultures.

One-way ANOVA did not reveal a main effect for A) NO released in 30 mins OGD cortical neurons-BM and -SCM co-cultures (OGD $F_{(2,6)} = 0.0219$ $p = 0.9785$), n = 3,B) TNF released in 30 min OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.0404$ $p = 0.9605$), n =6,C) IL-1β released in 30 mins OGD cortical neurons-BM and -SCM co-cultures (OGD $F_{(2,12)} = 0.0197$ $p = 0.9806$), n =6, D) IL-6 released in 30 mins OGD cortical neurons-BM and -SCM co-cultures (OGD $F_{(2,12)} = 0.0840$ $p = 0.9199$), n = 6,E) IL-10 released in 30 mins OGD cortical neurons-BM and -SCM co-cultures (OGD $F_{(2,12)} = 0.0098$ $p = 0.8157$), n=5. n represents the number of independent experiments with a minimum of three replicates. Bars represent mean ± s.e.m

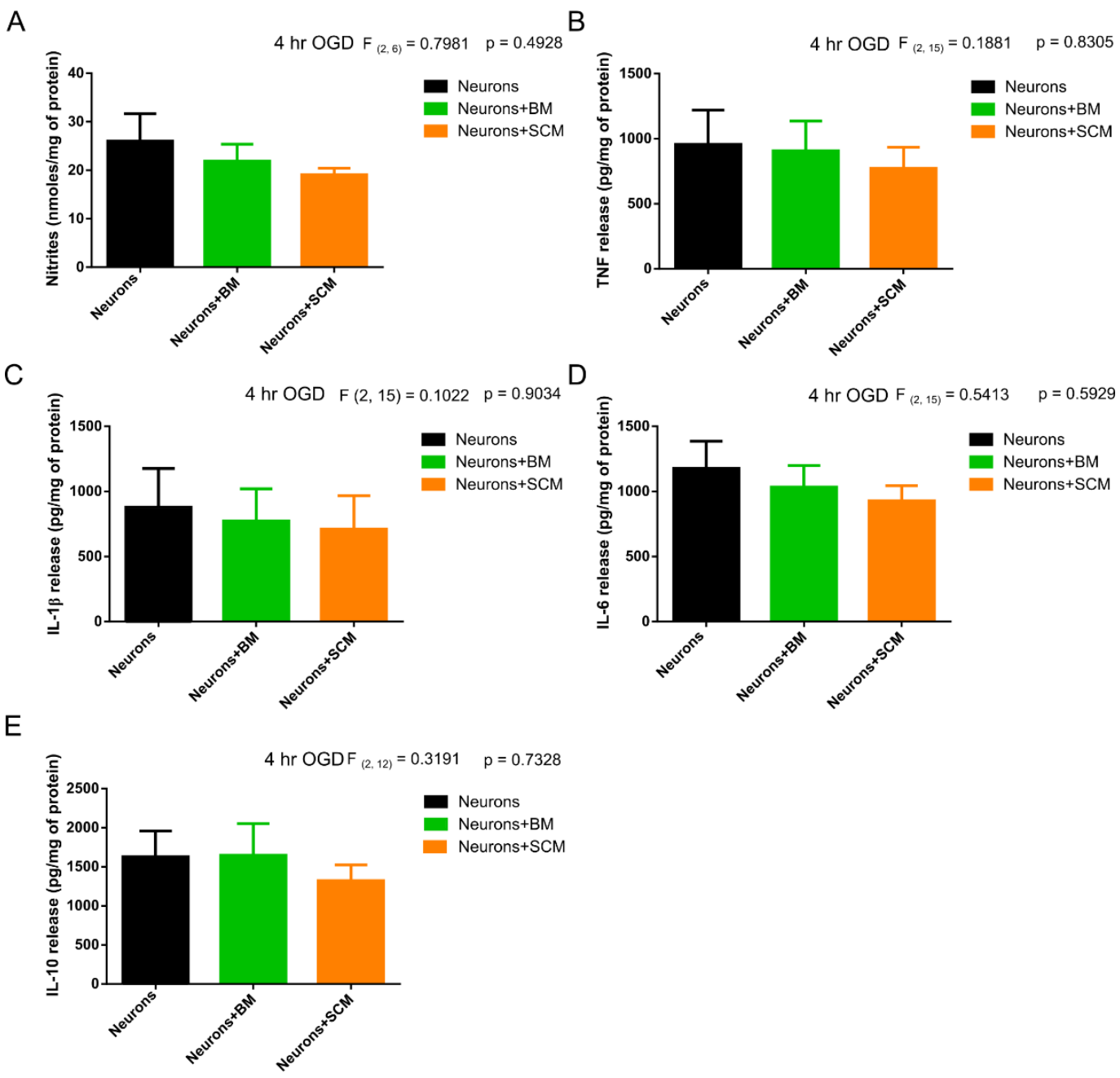

A
4 hr OGD F (2, 6) = 0.7981 p = 0.4928
Nitrites (nmoles/mg of protein)
40
30
20
10
0
Neurons
Neurons+BM
Neurons+SCM
B
4 hr OGD F (2, 15) = 0.1881 p = 0.8305
TNF release (pg/mg of protein)
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
C
4 hr OGD F (2, 15) = 0.1022 p = 0.9034
IL-1β release (pg/mg of protein)
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
D
4 hr OGD F (2, 15) = 0.5413 p = 0.5929
IL-6 release (pg/mg of protein)
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM
E
4 hr OGD F (2, 12) = 0.3191 p = 0.7328
IL-10 release (pg/mg of protein)
2500
2000
1500
1000
500
0
Neurons
Neurons+BM
Neurons+SCM

Figure 5-4 Inflammatory factors released by 4 hrs OGD cortical neurons-microglia co-cultures.

One-way ANOVA did not reveal a main effect for A) NO released in 4 hrs OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,6)} = 0.7981$ $p = 0.4928$), n = 3,B) TNF released in 4 hrs OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.1881$ $p = 0.8305$), n =6,C) IL-1β released in 4 hrs OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.1022$ $p = 0.9034$), n =6, D) IL-6 released in 4 hrs OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.5413$ $p = 0.5929$), n = 6,E) IL-10 released in 4 hrs OGD cortical neurons-BM and -SCM co-cultures ($F_{(2,12)} = 0.3191$ $p = 0.7328$), n=5. n represents the number of independent experiments with a minimum of three replicates. Bars represent mean ± s.e.m

Chapter Six: General Discussion

6.1 Summary of Thesis

SCM (spinal cord microglia) have not been characterized in the literature to the extent that BM (brain microglia) have been investigated. The main aim of this thesis was to characterize SCM and identify any functional differences between BM and SCM. To do so, a protocol to reliably culture SCM and to characterize and compare inflammatory factors released by SCM and BM was established. In Chapter 2, SCM and BM were activated with LPS (lipopolysaccharide, a TLR4 receptor agonist), with the results suggesting that inflammatory mediators (TNF, IL-1β) are released less by SCM relative to BM after LPS activation. There was no difference between BM and SCM in the release of anti-inflammatory factors or trophic factors after activation with LPS.

Mild trypsinization (as used in Chapter 2) is a viable isolation method for SCM. However, due to the differences in the composition of spinal mixed glia relative to mixed glia derived from the brain, trypsinization results in a higher propensity for contamination by fibroblast-like cells in the SCM culture relative to BM cultures (Figure 3-1and Figure 3-3). This contamination results in a loss of an unacceptably high percentage of isolations. Hence, in Chapter 3, a better method of isolation for SCM was developed and optimized. We found that mild shaking with lidocaine HCl provides higher purity cultures without cellular contaminants, suggesting that this is a better protocol than mild trypsinization for isolating SCM. Notably, microglia (BM and SCM) isolated by the lidocaine HCl method are functionally similar to cultures isolated by mild trypsinization (Figure 3-5).

Chapter 4 investigated whether BM and SCM exhibited a differential release of inflammatory factors (NO, TNF, IL6 and IL-1β) after physiological activation with mediators such as ATP and glutamate. DAMPs (damage associated molecular patterns) such as ATP and glutamate bind target receptors on microglia and have been associated with inflammatory processes. However, most studies that have investigated the function of these mediators used specific receptor agonists (rather than the endogenous compounds) to induce constitutive signalling of specific receptor subpopulations. This approach helps in understanding the signalling mechanisms of the target receptor but does not mimic physiological activation of the receptor (Dai et al., 2010; Vincent and Maiese, 2000). Furthermore, ATP and glutamate can act as cues to guide microglia to the site of injury rather than causing inflammation directly (Honda et al., 2001; Liu et al., 2009; Wu et al., 2007). Our data show that ATP and glutamate do not induce significant release of inflammatory factors by BM and SCM (Figure 4-1, Figure 4-2, Figure 4-3, Figure 4-4, Figure 4-5, and Figure 4-6) and that there was no clear difference between the inflammatory profiles of BM and SCM after treatment with ATP (Figure 4-1, Figure 4-2, and Figure 4-3). An overall reduction in NO and IL-1β release by SCM relative to BM was observed in glutamate experiments (Figure 4-4 and Figure 4-6). The results of this study support the postulate that the major action of ATP and glutamate at physiological concentrations on microglia is related to recruitment of microglia and other inflammatory cells to the site of injury or perturbation, rather than inducing release of cytokines or inflammatory mediators. The role of the immediate extracellular environment in dictating the phenotype of microglia was also investigated in Chapter 4. Our data confirm that microglia are plastic and sensitive to their extracellular environment, as conditioned media from spinal mixed glia cultures altered BM phenotype. Specifically, BM in conditioned media from spinal mixed glia cultured increased the

release of IL-6 and reduced release of IL-1β relative to release by BM in conditioned media from brain mixed glial cultures (Figure 4-7 and Figure 4-8). Thus, it is evident that microglia phenotype is not fixed and the immediate environment can selectively modify aspects of the microglial inflammatory phenotype.

Chapter 5 investigated the presence of functional differences between BM and SCM during OGD (oxygen glucose deprivation), a model of ischaemic injury. SCM and BM were cultured with cortical neurons in contact co-culture systems. The data suggest that OGD of cortical neuron-SCM co-cultures for 30 mins significantly reduced viability relative to neurons co-cultured with BM (Figure 5-1). However, this difference in cellular viability between SCM and BM co-cultures did not persist after 4 hrs of OGD (Figure 5-1). Release of inflammatory factors (TNF, IL6, IL-1β and IL10) was not significantly different between SCM and BM co-cultures, suggesting that overall functional response to OGD does not differ between microglia derived from brain vs spinal cord (Figure 5-2, Figure 5-3, and Figure 5-4). However, since an extensive temporal analysis of the release of pro- and anti-inflammatory factor release was not performed (only a single time point was sampled), these data provide only a limited temporal snapshot of these cells inflammatory profile after OGD injury.

6.2 Specific findings and significance

Regional heterogeneity of microglia has been observed both *in vitro* and *in vivo* in the CNS (central nervous system) (Hua et al., 2012; Lai et al., 2011; Yang et al., 2005).Furthermore, *in vitro* microglia from cortex were found to me more inflammatory compared to other regions of the brain (Lai et al., 2011). It was also found that microglia *in vitro* and *in vivo* respond to

injury in a severity-dependent fashion (Lai and Todd, 2008; Yang et al., 2005). This suggests that microglial phenotype is determined by region of origin and condition of the immediate environment. Interestingly, heterogeneity between BM and SCM has not been compared *in vitro*. Hence, the primary goal of Chapter 2 was to study differences in inflammatory factors released by brain and spinal cord microglia. Mild trypsinization was used as a reliable and quick method to isolate SCM. BM and SCM were activated by LPS and differences in morphology, release of inflammatory factors and phagocytic activity were measured. It was found that at basal conditions BM and SCM did not have measurable differences in morphology; however, LPS activated SCM exhibited a reduction in amoeboid morphology compared to BM (Figure 2-2). It was also found that phagocytic activity of SCM was reduced relative to BM, a finding that aligns well with the reduced amoeboid morphology (Figure 2-5). Similarly, LPS-induced release of NO, TNF and IL-1β was significantly reduced in SCM relative to BM, suggesting a reduced inflammatory profile overall (Figure 2-3).

The mild trypsinization method was an established method to isolate BM; however, only 78% of isolations for SCM were highly pure microglia (>95 purity). The contaminating cells had a fibroblast-like phenotype and were resilient to mild trypsinization. Due to the non-selectivity of this method of isolation for SCM, an alternative method using lidocaine HCl was developed to isolate SCM (Chapter 3). Notably, the lidocaine isolation method is quicker than other conventional methods such as vigorous shaking or gentle shaking over several hours (Gingras et al., 2007; Tamashiro et al., 2012). The lidocaine HCl isolation method also had the benefit of allowing for control of the seeding density of BM and SCM, a notable limitation of the mild trypsinization isolation method. BM and SCM isolated by this method activated with LPS do not exhibit differential release of NO (nitric oxide) relative to microglia isolated by mild

trypszinization (Figure 3-5). Thus, Chapter 3 provides a robust and improved method to isolate BM and SCM that will help in the accurate investigation of the role of microglia in *in vivo* models of spinal injury or spinal disorders.

Chapter 4 further investigated the functional differences and similarities between SCM and BM in isolated cultures. Chapter 2 suggests that the TLR4 signalling in SCM compared to BM is reduced, leading to less release of pro-inflammatory factors (NO, TNF and IL-1β) (Figure 2-3). Chapter 4 address the question of whether physiological signalling molecules such as ATP and glutamate can induce an inflammatory profile in SCM that is different than that of BM. Ischaemic stroke, traumatic brain injury and spinal cord injury result in large concentrations of ATP and glutamate being released in to the extracellular milieu (Kettenmann et al., 2011; Pocock and Kettenmann, 2007; Rodrigues et al., 2015; Ueda et al., 1992). In Chapter 4, microglia were treated with concentrations of ATP (1mM) and glutamate (10 μM, 30 μM, 100 μM) based on actual measured concentrations in brain parenchyma during normal physiology, transient ischemia/traumatic brain injury and excitotoxic injury (Dai et al., 2010; Hinzman et al., 2010; Marini and Paul, 1992, 1992; Ueda et al., 1992). BM and SCM were treated with the above-mentioned concentrations of ATP and glutamate and the release of NO, TNF, IL6 and IL-1β was measured. It was found that neither ATP nor glutamate significantly affect the release of, TNF, and IL6. However, an overall reduction in NO and IL-1β release by SCM was observed in glutamate experiments (Figure 4-4 and Figure 4-6). These data suggest that ATP and glutamate at physiologically relevant concentrations do not trigger the release of inflammatory factors. There is growing evidence that ATP and glutamate are chemo attractants involved in recruiting microglia to sites of injury or disease, and the lack of release of inflammatory mediators after treatment suggests this may be the major role for ATP and glutamate during injury (Honda et al.,

2001; Liu et al., 2009; Wu et al., 2007). Chapter 4 also investigated the role of the immediate extracellular environment in dictating the functional profile of microglia. Previously, it was shown that exposing thalamic microglia to conditioned media from the striatum resulted in the thalamic microglia adopting a striatum-like pattern of NO release (Lai, 2010). Here, BM were exposed to conditioned media from brain mixed glia (BMix CM) or spinal mixed glia (SMix CM) and treated with ATP, glutamate or LPS (Chapter 4). IL6, and IL-1β released by BMix BM and SMix BM were measured (Figure 4-7 and Figure 4-8). Notably, SMix-CM treated BM released significantly more IL6 compared to BMix BM after both 100 μM glutamate and LPS treatment (Figure 4-7). This IL6 release by BMix CM BM matches trends in IL6 release by SCM and BM in Chapter 2 (Figure 2-4). Similarly, an overall reduction in IL-1β release was observed in BM in SMix CM, providing further evidence that the phenotype of BM can be modulated by their immediate extracellular environment, a finding relevant to CNS injury or perturbation in different regions (Figure 4-8). Overall, Chapter 4 demonstrates that (unlike TLR4 activation by LPS), ATP and glutamate at physiological concentrations do not initiate an inflammatory response *in vitro*. However, an overall reduction in NO and IL-1β release by SCM was observed in glutamate experiments (Figure 4-4 and Figure 4-6).

In vitro ischaemic models such as oxygen deprivation or OGD are excellent functional tools to elucidate the molecular mechanisms as well determine neurotrophic as well neurotoxic functions of microglia (Kaushal and Schlichter, 2008; Lai and Todd, 2008; Meloni et al., 2011, 2001). Chapter 5 was designed to investigate whether any difference in the functional response profile of SCM relative to BM (i.e. differential response to TLR4 activation) would confer different neuroprotective effects in models of CNS injury. Interestingly, the data showed that microglia alone do not induce an inflammatory profile on exposure to moderately injured

neurons in an *in vitro* contact culture (Figure 5-2, Figure 5-3, and Figure 5-4). These data contradict previous studies that suggest that conditioned media from moderately injured neurons induced a significant increase in NO, TNF and IL-1β in microglia (Lai and Todd, 2008). We did not observe such microglial activation, perhaps suggesting differences in the microglia inflammatory response in contact cultures relative to microglia responding only to diffusible chemical cues. Notably, SCM and cortical neuron co-cultures after 30 min of OGD exhibited reduced viability compared to BM cortical neuron co-cultures; however, this loss of viability did not persist after 4 hrs of OGD (Figure 5-1). There were no differences between culture systems in release of inflammatory mediators after OGD (Figure 5-2, Figure 5-3, and Figure 5-4). Thus, Chapter 5 suggests that even though SCM are a heterogenous population compared to BM, with differences in response profile after specific activators (i.e. LPS), the functional response of BM and SCM to *in vitro* models of ischaemic injury is comparable.

6.3 Limitations of the approach

In vitro models of inflammation and ischaemic injury are great tools to reduce the complexity of *in vivo* model and tease apart mechanisms involved in injury or disease. Another benefit of an *in vitro* model is that the inflammatory factors released by BM and SCM can be quantified without interference from other cells types. Thus *in vitro* cell culture models are effective in studying receptor and intracellular signalling. However, cell culture models do have limitations. One major limitation is the typical use of neonatal microglia and embryonic neurons. A major concern with such young cells would be the lack of myelination and maturation in a neonate, leading to a different environment and that different response profiles relative to adult

microglia (Downes and Mullins, 2014). Our research and that of others have shown that microglia respond to their immediate environment, and it is likely that the environment of the CNS of a neonate has functional consequences for isolated cultures. Notably, recent studies have found increased expression of purinergic receptors involved in chemotaxis and phagocytosis in adult microglia relative to neonates (Butovsky et al., 2013; Haynes et al., 2006; Kurpius et al., 2007; Lai et al., 2013). Interestingly, the genes regulating factors such as cell adhesion, proliferation, migration, complement system activity, and transcription regulation also exhibit differential expression in adult vs. neonatal microglia (Butovsky et al., 2013). Thus, neonatal microglia may lack the exposure to various factors during developmental stages in different brain regions (Butovsky et al., 2013; Lai et al., 2013) and care should be taken when interpreting results from neonates and embryonic cells. For example, embryonic neurons and neonatal microglia might be more or less viable in models of injury/ischaemia compared to an adult cell. As injury severity alters functional profile, the magnitude of injury and factors released would likely be different than with adult cells. Isolated cell cultures also prevent crosstalk and feedback from other cells that might influence injury/disease in vivo or in mixed cell cultures. Thus, while neonatal microglia and embryonic neurons are effective tools for mechanistic studies, the translation of findings in these systems to adult cultures and to adults *in vivo* must be confirmed.

6.4 Conclusion and future directions

This thesis suggests that BM and SCM share many functional properties, but that the inflammatory profile of SCM after activation with the PAMP LPS is markedly different than BM. This finding is interesting and suggests that SCM response to infection or pathogens *in vivo*

might be different or slightly attenuated relative to microglia in the brain. The reduced inflammatory profile in non-sterile inflammation might result from increased inflammatory activity of peripheral cells in the spinal cord. Notably, SCM *in vivo* might have better potential to recruit peripheral macrophages since the BSCB (blood-spinal cord barrier) is more permeable to cytokines than the BBB (brain-blood barrier) (Bartanusz et al., 2011). Interestingly, in OGD contact co-culture experiments (a model of sterile injury), there were no significant differences in the inflammatory profile of BM and SCM.

Current developments in cell culture techniques have facilitated successful isolation and culture of adult brain and spinal cord microglia (Nikodemova and Watters, 2012; Yip et al., 2009). It would be interesting to test in future studies whether adult BM and SCM have similarities in inflammatory profiles on activation with LPS. Potential differences that the microglia might have acquired during development and adulthood in response to other activators could also be explored in this model. As microglia are an integral part of the CNS at all stages of development, understating their role in normal physiology, development, and during neuropathology is crucial for normal brain structure, function, and outcome during disease. Mechanistic studies in microglia cultures from different regions may therefore help in designing better therapeutics for CNS pathologies.

References

Abbracchio, M.P., 2006. International Union of Pharmacology LVIII: Update on the P2Y G Protein-Coupled Nucleotide Receptors: From Molecular Mechanisms and Pathophysiology to Therapy. Pharmacol. Rev. 58, 281–341. doi:10.1124/pr.58.3.3

Abbracchio, M.P., Burnstock, G., Verkhratsky, A., Zimmermann, H., 2009. Purinergic signalling in the nervous system: an overview. Trends Neurosci. 32, 19–29. doi:10.1016/j.tins.2008.10.001

Aderka, D., Le, J.M., Vilcek, J., 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J. Immunol. Baltim. Md 1950 143, 3517–3523.

Akdis, M., Aab, A., Altunbulakli, C., Azkur, K., Costa, R.A., Crameri, R., Duan, S., Eiwegger, T., Eljaszewicz, A., Ferstl, R., Frei, R., Garbani, M., Globinska, A., Hess, L., Huitema, C., Kubo, T., Komlosi, Z., Konieczna, P., Kovacs, N., Kucuksezer, U.C., Meyer, N., Morita, H., Olzhausen, J., O'Mahony, L., Pezer, M., Prati, M., Rebane, A., Rhyner, C., Rinaldi, A., Sokolowska, M., Stanic, B., Sugita, K., Treis, A., van de Veen, W., Wanke, K., Wawrzyniak, M., Wawrzyniak, P., Wirz, O.F., Zakzuk, J.S., Akdis, C.A., 2016. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. J. Allergy Clin. Immunol. 138, 984–1010. doi:10.1016/j.jaci.2016.06.033

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499–511. doi:10.1038/nri1391

Alliot, F., Godin, I., Pessac, B., 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Brain Res. Dev. Brain Res. 117, 145–152.

Aloisi, F., 2001. Immune function of microglia. Glia 36, 165–179.

Araki, E., Forster, C., Dubinsky, J.M., Ross, M.E., Iadecola, C., 2001. Cyclooxygenase-2 Inhibitor NS-398 Protects Neuronal Cultures From Lipopolysaccharide-Induced Neurotoxicity. Stroke 32, 2370–2375. doi:10.1161/hs1001.096057

Armstrong, L., Jordan, N., Millar, A., 1996. Interleukin 10 (IL-10) regulation of tumour necrosis factor alpha (TNF-alpha) from human alveolar macrophages and peripheral blood monocytes. Thorax 51, 143–149.

Asensio, V.C., Campbell, I.L., 1999. Chemokines in the CNS: plurifunctional mediators in diverse states. Trends Neurosci. 22, 504–512.

Astrup, J., Siesjö, B.K., Symon, L., 1981. Thresholds in cerebral ischemia - the ischemic penumbra. Stroke J. Cereb. Circ. 12, 723–725.

Baggiolini, M., Dewald, B., Moser, B., 1997. Human chemokines: an update. Annu. Rev. Immunol. 15, 675–705. doi:10.1146/annurev.immunol.15.1.675

Bartanusz, V., Jezova, D., Alajajian, B., Digicaylioglu, M., 2011. The blood-spinal cord barrier: morphology and clinical implications. Ann. Neurol. 70, 194–206. doi:10.1002/ana.22421

Basiri, M., Doucette, R., 2010. Sensorimotor cortex aspiration: a model for studying Wallerian degeneration-induced glial reactivity along the entire length of a single CNS axonal pathway. Brain Res. Bull. 81, 43–52. doi:10.1016/j.brainresbull.2009.11.002

Baskar Jesudasan, S.J., Todd, K.G., Winship, I.R., 2014. Reduced Inflammatory Phenotype in Microglia Derived from Neonatal Rat Spinal Cord versus Brain. PLoS ONE 9, e99443. doi:10.1371/journal.pone.0099443

Bethea, J.R., Nagashima, H., Acosta, M.C., Briceno, C., Gomez, F., Marcillo, A.E., Loor, K., Green, J., Dietrich, W.D., 1999. Systemically Administered Interleukin-10 Reduces

Tumor Necrosis Factor-Alpha Production and Significantly Improves Functional Recovery Following Traumatic Spinal Cord Injury in Rats. J. Neurotrauma 16, 851–863. doi:10.1089/neu.1999.16.851

Bianco, F., Pravettoni, E., Colombo, A., Schenk, U., Moller, T., Matteoli, M., Verderio, C., 2005. Astrocyte-Derived ATP Induces Vesicle Shedding and IL-1 Release from Microglia. J. Immunol. 174, 7268–7277. doi:10.4049/jimmunol.174.11.7268

Biber, K., Vinet, J., Boddeke, H.W.G.M., 2008. Neuron-microglia signaling: chemokines as versatile messengers. J. Neuroimmunol. 198, 69–74. doi:10.1016/j.jneuroim.2008.04.012

Blinzinger, K., Kreutzberg, G., 1968. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. Z. Für Zellforsch. Mikrosk. Anat. Vienna Austria 1948 85, 145–157.

Block, M.L., Zecca, L., Hong, J.-S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat. Rev. Neurosci. 8, 57–69. doi:10.1038/nrn2038

Boraschi, D., Tagliabue, A., 2013. The interleukin-1 receptor family. Semin. Immunol. 25, 394–407. doi:10.1016/j.smim.2013.10.023

Boucsein, C., Zacharias, R., Färber, K., Pavlovic, S., Hanisch, U.-K., Kettenmann, H., 2003. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. Eur. J. Neurosci. 17, 2267–2276.

Broderick, C., Duncan, L., Taylor, N., Dick, A.D., 2000. IFN-gamma and LPS-mediated IL-10-dependent suppression of retinal microglial activation. Invest. Ophthalmol. Vis. Sci. 41, 2613–2622.

Bruce, A.J., Boling, W., Kindy, M.S., Peschon, J., Kraemer, P.J., Carpenter, M.K., Holtsberg, F.W., Mattson, M.P., 1996. Altered neuronal and microglial responses to excitotoxic and

ischemic brain injury in mice lacking TNF receptors. Nat. Med. 2, 788–794. doi:10.1038/nm0796-788

Burnstock, G., 2007. Purine and pyrimidine receptors. Cell. Mol. Life Sci. CMLS 64, 1471–1483. doi:10.1007/s00018-007-6497-0

Burnstock, G., 2006. Historical review: ATP as a neurotransmitter. Trends Pharmacol. Sci. 27, 166–176. doi:10.1016/j.tips.2006.01.005

Burnstock, G., Verkhratsky, A., 2010. Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. Cell Death Dis. 1, e9. doi:10.1038/cddis.2009.11

Burnstock, G., Verkhratsky, A., 2009. Evolutionary origins of the purinergic signalling system. Acta Physiol. 195, 415–447. doi:10.1111/j.1748-1716.2009.01957.x

Butovsky, O., Jedrychowski, M.P., Moore, C.S., Cialic, R., Lanser, A.J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P.M., Doykan, C.E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J.D., Ransohoff, R.M., Gygi, S.P., Antel, J.P., Weiner, H.L., 2013. Identification of a unique TGF-β–dependent molecular and functional signature in microglia. Nat. Neurosci. 17, 131–143. doi:10.1038/nn.3599

Byrnes, K.R., Stoica, B., Loane, D.J., Riccio, A., Davis, M.I., Faden, A.I., 2009. Metabotropic glutamate receptor 5 activation inhibits microglial associated inflammation and neurotoxicity. Glia 57, 550–560. doi:10.1002/glia.20783

Cafferty, W.B.J., 2004. Conditioning Injury-Induced Spinal Axon Regeneration Fails in Interleukin-6 Knock-Out Mice. J. Neurosci. 24, 4432–4443. doi:10.1523/JNEUROSCI.2245-02.2004

Carmichael, S.T., Archibeque, I., Luke, L., Nolan, T., Momiy, J., Li, S., 2005. Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. Exp. Neurol. 193, 291–311. doi:10.1016/j.expneurol.2005.01.004

Castillo, J., Rama, R., Dávalos, A., 2000. Nitric oxide-related brain damage in acute ischemic stroke. Stroke 31, 852–857.

Centonze, D., Muzio, L., Rossi, S., Cavasinni, F., De Chiara, V., Bergami, A., Musella, A., D'Amelio, M., Cavallucci, V., Martorana, A., Bergamaschi, A., Cencioni, M.T., Diamantini, A., Butti, E., Comi, G., Bernardi, G., Cecconi, F., Battistini, L., Furlan, R., Martino, G., 2009. Inflammation Triggers Synaptic Alteration and Degeneration in Experimental Autoimmune Encephalomyelitis. J. Neurosci. 29, 3442–3452. doi:10.1523/JNEUROSCI.5804-08.2009

Chaboub, L.S., Deneen, B., 2012. Developmental Origins of Astrocyte Heterogeneity: The Final Frontier of CNS Development. Dev. Neurosci. 34, 379–388. doi:10.1159/000343723

Choi, D.W., Rothman, S.M., 1990. The Role of Glutamate Neurotoxicity in Hypoxic-Ischemic Neuronal Death. Annu. Rev. Neurosci. 13, 171–182. doi:10.1146/annurev.ne.13.030190.001131

Choi, G.B., Yim, Y.S., Wong, H., Kim, S., Kim, H., Kim, S.V., Hoeffer, C.A., Littman, D.R., Huh, J.R., 2016. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. Science 351, 933–939. doi:10.1126/science.aad0314

Churchward, M.A., Todd, K.G., 2014. Statin treatment affects cytokine release and phagocytic activity in primary cultured microglia through two separable mechanisms. Mol. Brain 7, 85. doi:10.1186/s13041-014-0085-7

Clausen, B.H., Lambertsen, K.L., Babcock, A.A., Holm, T.H., Dagnaes-Hansen, F., Finsen, B., 2008. Interleukin-1beta and tumor necrosis factor-alpha are expressed by different subsets of microglia and macrophages after ischemic stroke in mice. J. Neuroinflammation 5, 46. doi:10.1186/1742-2094-5-46

Clausen, B.H., Lambertsen, K.L., Meldgaard, M., Finsen, B., 2005. A quantitative in situ hybridization and polymerase chain reaction study of microglial-macrophage expression of interleukin-1beta mRNA following permanent middle cerebral artery occlusion in mice. Neuroscience 132, 879–892. doi:10.1016/j.neuroscience.2005.01.031

Colton, C.A., Gilbert, D.L., 1987. Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett. 223, 284–288.

Dai, S.-S., Zhou, Y.-G., Li, W., An, J.-H., Li, P., Yang, N., Chen, X.-Y., Xiong, R.-P., Liu, P., Zhao, Y., Shen, H.-Y., Zhu, P.-F., Chen, J.-F., 2010. Local glutamate level dictates adenosine A2A receptor regulation of neuroinflammation and traumatic brain injury. J. Neurosci. Off. J. Soc. Neurosci. 30, 5802–5810. doi:10.1523/JNEUROSCI.0268-10.2010

Daniele, S.G., Edwards, A.A., Maguire-Zeiss, K.A., 2014. Isolation of cortical microglia with preserved immunophenotype and functionality from murine neonates. J. Vis. Exp. JoVE e51005. doi:10.3791/51005

Danton, G.H., Dietrich, W.D., 2003. Inflammatory mechanisms after ischemia and stroke. J. Neuropathol. Exp. Neurol. 62, 127–136.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., Gan, W.-B., 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. 8, 752–758. doi:10.1038/nn1472

David, S., Kroner, A., 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nat. Rev. Neurosci. 12, 388–399. doi:10.1038/nrn3053

Dawson, D.A., Martin, D., Hallenbeck, J.M., 1996. Inhibition of tumor necrosis factor-alpha reduces focal cerebral ischemic injury in the spontaneously hypertensive rat. Neurosci. Lett. 218, 41–44.

de Haas, A.H., Boddeke, H.W.G.M., Biber, K., 2008. Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. Glia 56, 888–894. doi:10.1002/glia.20663

de Haas, A.H., van Weering, H.R.J., de Jong, E.K., Boddeke, H.W.G.M., Biber, K.P.H., 2007. Neuronal chemokines: versatile messengers in central nervous system cell interaction. Mol. Neurobiol. 36, 137–151. doi:10.1007/s12035-007-0036-8

Del Rio Hortega, P., 1932. Microglia, in: Cytology and Cellular Pathology of the Nervous System. Paul B. Hoeber, New York, pp. 481–534.

Dhandapani, K.M., Brann, D.W., 2003. Transforming growth factor-beta: a neuroprotective factor in cerebral ischemia. Cell Biochem. Biophys. 39, 13–22. doi:10.1385/CBB:39:1:13

Diab, A., Zhu, J., Xiao, B.G., Mustafa, M., Link, H., 1997. High IL-6 and Low IL-10 in the Central Nervous System Are Associated with Protracted Relapsing EAE in DA Rats: J. Neuropathol. Exp. Neurol. 56, 641–650. doi:10.1097/00005072-199756060-00002

Dibaj, P., Nadrigny, F., Steffens, H., Scheller, A., Hirrlinger, J., Schomburg, E.D., Neusch, C., Kirchhoff, F., 2010. NO mediates microglial response to acute spinal cord injury under ATP control in vivo. Glia 58, 1133–1144. doi:10.1002/glia.20993

Dinarello, C.A., 2009. Immunological and Inflammatory Functions of the Interleukin-1 Family. Annu. Rev. Immunol. 27, 519–550. doi:10.1146/annurev.immunol.021908.132612

Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 22, 391–397.

Domercq, M., Vázquez-Villoldo, N., Matute, C., 2013. Neurotransmitter signaling in the pathophysiology of microglia. Front. Cell. Neurosci. 7. doi:10.3389/fncel.2013.00049

Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 209, 378–388. doi:10.1016/j.expneurol.2007.06.009

Downes, N., Mullins, P., 2014. The Development of Myelin in the Brain of the Juvenile Rat. Toxicol. Pathol. 42, 913–922. doi:10.1177/0192623313503518

Duan, Y., Sahley, C.L., Muller, K.J., 2009. ATP and NO dually control migration of microglia to nerve lesions. Dev. Neurobiol. 69, 60–72. doi:10.1002/dneu.20689

Dumont, R.J., Okonkwo, D.O., Verma, S., Hurlbert, R.J., Boulos, P.T., Ellegala, D.B., Dumont, A.S., 2001. Acute spinal cord injury, part I: pathophysiologic mechanisms. Clin. Neuropharmacol. 24, 254–264.

Durafourt, B.A., Moore, C.S., Zammit, D.A., Johnson, T.A., Zaguia, F., Guiot, M.-C., Bar-Or, A., Antel, J.P., 2012. Comparison of polarization properties of human adult microglia and blood-derived macrophages. Glia 60, 717–727. doi:10.1002/glia.22298

Ehrlich, Y.H., Lenox, R.H., Kornecki, E., Berry, W.O. (Eds.), 1987. Molecular Mechanisms of Neuronal Responsiveness, Advances in Experimental Medicine and Biology. Springer US, Boston, MA.

Erta, M., Quintana, A., Hidalgo, J., 2012. Interleukin-6, a Major Cytokine in the Central Nervous System. Int. J. Biol. Sci. 8, 1254–1266. doi:10.7150/ijbs.4679

Esiri, M.M., al Izzi, M.S., Reading, M.C., 1991. Macrophages, microglial cells, and HLA-DR antigens in fetal and infant brain. J. Clin. Pathol. 44, 102–106.

Färber, K., Kettenmann, H., 2006. Purinergic signaling and microglia. Pflugers Arch. 452, 615–621. doi:10.1007/s00424-006-0064-7

Farso, M.C., O'Shea, R.D., Beart, P.M., 2009. Evidence group I mGluR drugs modulate the activation profile of lipopolysaccharide-exposed microglia in culture. Neurochem. Res. 34, 1721–1728. doi:10.1007/s11064-009-9999-3

Faustino, J.V., Wang, X., Johnson, C.E., Klibanov, A., Derugin, N., Wendland, M.F., Vexler, Z.S., 2011. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. J. Neurosci. Off. J. Soc. Neurosci. 31, 12992–13001. doi:10.1523/JNEUROSCI.2102-11.2011

Ferguson, A.R., Christensen, R.N., Gensel, J.C., Miller, B.A., Sun, F., Beattie, E.C., Bresnahan, J.C., Beattie, M.S., 2008. Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J. Neurosci. Off. J. Soc. Neurosci. 28, 11391–11400. doi:10.1523/JNEUROSCI.3708-08.2008

Fernandez, E.J., Lolis, E., 2002. Structure, function, and inhibition of chemokines. Annu. Rev. Pharmacol. Toxicol. 42, 469–499. doi:10.1146/annurev.pharmtox.42.091901.115838

Ferrari, D., Chiozzi, P., Falzoni, S., Hanau, S., Di Virgilio, F., 1997. Purinergic Modulation of Interleukin-1β Release from Microglial Cells Stimulated with Bacterial Endotoxin. J. Exp. Med. 185, 579–582. doi:10.1084/jem.185.3.579

Ferrazzano, P., Chanana, V., Uluc, K., Fidan, E., Akture, E., Kintner, D.B., Cengiz, P., Sun, D., 2013. Age-dependent microglial activation in immature brains after hypoxia- ischemia. CNS Neurol. Disord. Drug Targets 12, 338–349.

Fiebich, B.L., Biber, K., Lieb, K., van Calker, D., Berger, M., Bauer, J., Gebicke-Haerter, P.J., 1996. Cyclooxygenase-2 expression in rat microglia is induced by adenosine A2a-receptors. Glia 18, 152–160. doi:10.1002/(SICI)1098-1136(199610)18:2<152::AID-GLIA7>3.0.CO;2-2

Fischer, W., Krügel, U., 2007. P2Y receptors: focus on structural, pharmacological and functional aspects in the brain. Curr. Med. Chem. 14, 2429–2455.

Floden, A.M., Combs, C.K., 2011. Microglia demonstrate age-dependent interaction with amyloid-β fibrils. J. Alzheimers Dis. JAD 25, 279–293. doi:10.3233/JAD-2011-101014

Fredholm, B.B., Chern, Y., Franco, R., Sitkovsky, M., 2007. Aspects of the general biology of adenosine A2A signaling. Prog. Neurobiol. 83, 263–276. doi:10.1016/j.pneurobio.2007.07.005

Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Klotz, K.-N., Linden, J., 2001. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. Pharmacol. Rev. 53, 527.

Fujita, R., Ma, Y., Ueda, H., 2008. Lysophosphatidic acid-induced membrane ruffling and brain-derived neurotrophic factor gene expression are mediated by ATP release in primary microglia. J. Neurochem. 107, 152–160. doi:10.1111/j.1471-4159.2008.05599.x

Gadani, S.P., Walsh, J.T., Lukens, J.R., Kipnis, J., 2015. Dealing with Danger in the CNS: The Response of the Immune System to Injury. Neuron 87, 47–62. doi:10.1016/j.neuron.2015.05.019

Garcia, J.A., Cardona, S.M., Cardona, A.E., 2014. Isolation and Analysis of Mouse Microglial Cells: Isolation and Analysis of Mouse Microglial Cells, in: Coligan, J.E., Bierer, B.E., Margulies, D.H., Shevach, E.M., Strober, W. (Eds.), Current Protocols in Immunology. John Wiley & Sons, Inc., Hoboken, NJ, USA, p. 14.35.1-14.35.15.

Gelderblom, M., Leypoldt, F., Steinbach, K., Behrens, D., Choe, C.-U., Siler, D.A., Arumugam, T.V., Orthey, E., Gerloff, C., Tolosa, E., Magnus, T., 2009. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke J. Cereb. Circ. 40, 1849–1857. doi:10.1161/STROKEAHA.108.534503

Gingras, M., Gagnon, V., Minotti, S., Durham, H.D., Berthod, F., 2007. Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. J. Neurosci. Methods 163, 111–118. doi:10.1016/j.jneumeth.2007.02.024

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., Samokhvalov, I.M., Merad, M., 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330, 841–845. doi:10.1126/science.1194637

Ginhoux, F., Prinz, M., 2015. Origin of microglia: current concepts and past controversies. Cold Spring Harb. Perspect. Biol. 7, a020537. doi:10.1101/cshperspect.a020537

Giulian, D., Baker, T.J., 1986. Characterization of ameboid microglia isolated from developing mammalian brain. J. Neurosci. Off. J. Soc. Neurosci. 6, 2163–2178.

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D.I., Gonçalves, N., Cunha, R.A., 2013. Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner:

A2A receptor blockade prevents BDNF release and proliferation of microglia. J. Neuroinflammation 10. doi:10.1186/1742-2094-10-16

Grabert, K., Michoel, T., Karavolos, M.H., Clohisey, S., Baillie, J.K., Stevens, M.P., Freeman, T.C., Summers, K.M., McColl, B.W., 2016a. Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat. Neurosci. 19, 504–516. doi:10.1038/nn.4222

Grabert, K., Michoel, T., Karavolos, M.H., Clohisey, S., Baillie, J.K., Stevens, M.P., Freeman, T.C., Summers, K.M., McColl, B.W., 2016b. Microglial brain region−dependent diversity and selective regional sensitivities to aging. Nat. Neurosci. 19, 504–516. doi:10.1038/nn.4222

Green, H.F., Nolan, Y.M., 2012. GSK-3 mediates the release of IL-1β, TNF-α and IL-10 from cortical glia. Neurochem. Int. 61, 666–671. doi:10.1016/j.neuint.2012.07.003

Greenhalgh, A.D., David, S., 2014. Differences in the Phagocytic Response of Microglia and Peripheral Macrophages after Spinal Cord Injury and Its Effects on Cell Death. J. Neurosci. 34, 6316–6322. doi:10.1523/JNEUROSCI.4912-13.2014

Gregersen, R., Lambertsen, K., Finsen, B., 2000. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 20, 53–65. doi:10.1097/00004647-200001000-00009

Griess, P., 1879. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt „Ueber einige Azoverbindungen". Berichte Dtsch. Chem. Ges. 12, 426–428. doi:10.1002/cber.187901201117

Guo, L.-H., Trautmann, K., Schluesener, H.J., 2005. Expression of P2X4 receptor by lesional activated microglia during formalin-induced inflammatory pain. J. Neuroimmunol. 163, 120–127. doi:10.1016/j.jneuroim.2005.03.007

Ha, Y., Kim, Y.S., Cho, J.M., Yoon, S.H., Park, S.R., Yoon, D.H., Kim, E.Y., Park, H.C., 2005. Role of granulocyte-macrophage colony-stimulating factor in preventing apoptosis and improving functional outcome in experimental spinal cord contusion injury. J. Neurosurg. Spine 2, 55–61. doi:10.3171/spi.2005.2.1.0055

Hagino, Y., Kariura, Y., Manago, Y., Amano, T., Wang, B., Sekiguchi, M., Nishikawa, K., Aoki, S., Wada, K., Noda, M., 2004. Heterogeneity and potentiation of AMPA type of glutamate receptors in rat cultured microglia. Glia 47, 68–77. doi:10.1002/glia.20034

Hanisch, U.-K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10, 1387–1394. doi:10.1038/nn1997

Harada, K., Hide, I., Seki, T., Tanaka, S., Nakata, Y., Sakai, N., 2011. Extracellular ATP differentially modulates Toll-like receptor 4-mediated cell survival and death of microglia: LPS/ATP-mediated survival and death of microglia. J. Neurochem. 116, 1138–1147. doi:10.1111/j.1471-4159.2011.07170.x

Haselkorn, M.L., Shellington, D.K., Jackson, E.K., Vagni, V.A., Janesko-Feldman, K., Dubey, R.K., Gillespie, D.G., Cheng, D., Bell, M.J., Jenkins, L.W., Homanics, G.E., Schnermann, J., Kochanek, P.M., 2010. Adenosine A1 receptor activation as a brake on the microglial response after experimental traumatic brain injury in mice. J. Neurotrauma 27, 901–910. doi:10.1089/neu.2009.1075

Hayashi, Y., Kawaji, K., Sun, L., Zhang, X., Koyano, K., Yokoyama, T., Kohsaka, S., Inoue, K., Nakanishi, H., 2011. Microglial Ca(2+)-activated K(+) channels are possible molecular

targets for the analgesic effects of S-ketamine on neuropathic pain. J. Neurosci. Off. J. Soc. Neurosci. 31, 17370–17382. doi:10.1523/JNEUROSCI.4152-11.2011

Hayashi, Y., Tomimatsu, Y., Suzuki, H., Yamada, J., Wu, Z., Yao, H., Kagamiishi, Y., Tateishi, N., Sawada, M., Nakanishi, H., 2006. The intra-arterial injection of microglia protects hippocampal CA1 neurons against global ischemia-induced functional deficits in rats. Neuroscience 142, 87–96. doi:10.1016/j.neuroscience.2006.06.003

Haynes, S.E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M.E., Gan, W.-B., Julius, D., 2006. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat. Neurosci. 9, 1512–1519. doi:10.1038/nn1805

Heese, K., Fiebich, B.L., Bauer, J., Otten, U., 1997. Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A2a-receptors. Neurosci. Lett. 231, 83–86.

Hill, J.K., Gunion-Rinker, L., Kulhanek, D., Lessov, N., Kim, S., Clark, W.M., Dixon, M.P., Nishi, R., Stenzel-Poore, M.P., Eckenstein, F.P., 1999. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. Brain Res. 820, 45–54.

Hinzman, J.M., Thomas, T.C., Burmeister, J.J., Quintero, J.E., Huettl, P., Pomerleau, F., Gerhardt, G.A., Lifshitz, J., 2010. Diffuse Brain Injury Elevates Tonic Glutamate Levels and Potassium-Evoked Glutamate Release in Discrete Brain Regions at Two Days Post-Injury: An Enzyme-Based Microelectrode Array Study. J. Neurotrauma 27, 889–899. doi:10.1089/neu.2009.1238

Honda, S., Sasaki, Y., Ohsawa, K., Imai, Y., Nakamura, Y., Inoue, K., Kohsaka, S., 2001. Extracellular ATP or ADP Induce Chemotaxis of Cultured Microglia through $G_{i/o}$-Coupled P2Y Receptors. J. Neurosci. 21, 1975.

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R.K., Chen, S., Gao, Y., Chen, J., 2012. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 43, 3063–3070. doi:10.1161/STROKEAHA.112.659656

Hua, K., Schindler, M.K., McQuail, J.A., Forbes, M.E., Riddle, D.R., 2012. Regionally distinct responses of microglia and glial progenitor cells to whole brain irradiation in adult and aging rats. PloS One 7, e52728. doi:10.1371/journal.pone.0052728

Huang, Y.-H., Tsai, P.-S., Kai, Y.-F., Yang, C.-H., Huang, C.-J., 2006. Lidocaine inhibition of inducible nitric oxide synthase and cationic amino acid transporter-2 transcription in activated murine macrophages may involve voltage-sensitive Na+ channel. Anesth. Analg. 102, 1739–1744. doi:10.1213/01.ANE.0000219593.15109.DB

Huang, Y.-J., Lee, K.H., Grau, J.W., 2016. Complete spinal cord injury (SCI) transforms how brain derived neurotrophic factor (BDNF) affects nociceptive sensitization. Exp. Neurol. 288, 38–50. doi:10.1016/j.expneurol.2016.11.001

Huang, Z., Huang, P., Panahian, N., Dalkara, T., Fishman, M., Moskowitz, M., 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265, 1883–1885. doi:10.1126/science.7522345

Hynds, D.L., Rangappa, N., Ter Beest, J., Snow, D.M., Rabchevsky, A.G., 2004. Microglia enhance dorsal root ganglion outgrowth in Schwann cell cultures. Glia 46, 218–223. doi:10.1002/glia.10353

Idriss, H.T., Naismith, J.H., 2000. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microsc. Res. Tech. 50, 184–195. doi:10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H

Imai, F., Suzuki, H., Oda, J., Ninomiya, T., Ono, K., Sano, H., Sawada, M., 2007. Neuroprotective effect of exogenous microglia in global brain ischemia. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 27, 488–500. doi:10.1038/sj.jcbfm.9600362

Inoue, K., 2006a. ATP receptors of microglia involved in pain. Novartis Found. Symp. 276, 263-272-281.

Inoue, K., 2006b. The function of microglia through purinergic receptors: neuropathic pain and cytokine release. Pharmacol. Ther. 109, 210–226. doi:10.1016/j.pharmthera.2005.07.001

Inoue, K., Tsuda, M., 2009. Microglia and neuropathic pain. Glia 57, 1469–1479. doi:10.1002/glia.20871

Inoue, K., Tsuda, M., Koizumi, S., 2004. Chronic pain and microglia: the role of ATP. Novartis Found. Symp. 261, 55-64-67, 149–154.

Inoue, K., Tsuda, M., Tozaki-Saitoh, H., 2007. Modification of neuropathic pain sensation through microglial ATP receptors. Purinergic Signal. 3, 311–316. doi:10.1007/s11302-007-9071-1

Jiang, S.X., Slinn, J., Aylsworth, A., Hou, S.T., 2012. Vimentin participates in microglia activation and neurotoxicity in cerebral ischemia. J. Neurochem. 122, 764–774. doi:10.1111/j.1471-4159.2012.07823.x

Jin, R., Yang, G., Li, G., 2010. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J. Leukoc. Biol. 87, 779–789. doi:10.1189/jlb.1109766

Kader, A., Frazzini, V.I., Solomon, R.A., Trifiletti, R.R., 1993. Nitric oxide production during focal cerebral ischemia in rats. Stroke 24, 1709–1716.

Kaindl, A.M., Degos, V., Peineau, S., Gouadon, E., Chhor, V., Loron, G., Le Charpentier, T., Josserand, J., Ali, C., Vivien, D., Collingridge, G.L., Lombet, A., Issa, L., Rene, F., Loeffler, J.-P., Kavelaars, A., Verney, C., Mantz, J., Gressens, P., 2012. Activation of microglial N-methyl-D-aspartate receptors triggers inflammation and neuronal cell death in the developing and mature brain. Ann. Neurol. 72, 536–549. doi:10.1002/ana.23626

Kaushal, V., Schlichter, L.C., 2008. Mechanisms of Microglia-Mediated Neurotoxicity in a New Model of the Stroke Penumbra. J. Neurosci. 28, 2221–2230. doi:10.1523/JNEUROSCI.5643-07.2008

Kawamura, M., 2004. Direct Excitation of Inhibitory Interneurons by Extracellular ATP Mediated by P2Y1 Receptors in the Hippocampal Slice. J. Neurosci. 24, 10835–10845. doi:10.1523/JNEUROSCI.3028-04.2004

Kerstetter, A.E., Miller, R.H., 2012. Isolation and Culture of Spinal Cord Astrocytes, in: Milner, R. (Ed.), Astrocytes. Humana Press, Totowa, NJ, pp. 93–104.

Kettenmann, H., Hanisch, U.-K., Noda, M., Verkhratsky, A., 2011. Physiology of Microglia. Physiol. Rev. 91, 461–553. doi:10.1152/physrev.00011.2010

Khakh, B.S., Burnstock, G., Kennedy, C., King, B.F., North, R.A., Séguéla, P., Voigt, M., Humphrey, P.P.A., 2001. International Union of Pharmacology. XXIV. Current Status of the Nomenclature and Properties of P2X Receptors and Their Subunits. Pharmacol. Rev. 53, 107.

Kielian, T., Esen, N., Bearden, E.D., 2005. Toll-like receptor 2 (TLR2) is pivotal for recognition of S. aureus peptidoglycan but not intact bacteria by microglia. Glia 49, 567–576. doi:10.1002/glia.20144

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E.G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., Müller, D.N., Luckow, B., Brocker, T., Debowski, K., Fritz, G., Opdenakker, G., Diefenbach, A., Biber, K., Heikenwalder, M., Geissmann, F., Rosenbauer, F., Prinz, M., 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat. Neurosci. 16, 273–280. doi:10.1038/nn.3318

Kigerl, K.A., de Rivero Vaccari, J.P., Dietrich, W.D., Popovich, P.G., Keane, R.W., 2014. Pattern recognition receptors and central nervous system repair. Exp. Neurol. 258, 5–16. doi:10.1016/j.expneurol.2014.01.001

Kim, D., Kim, M.A., Cho, I.-H., Kim, M.S., Lee, S., Jo, E.-K., Choi, S.-Y., Park, K., Kim, J.S., Akira, S., Na, H.S., Oh, S.B., Lee, S.J., 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J. Biol. Chem. 282, 14975–14983. doi:10.1074/jbc.M607277200

Kim, E., Cho, S., 2016. Microglia and Monocyte-Derived Macrophages in Stroke. Neurother. J. Am. Soc. Exp. Neurother. 13, 702–718. doi:10.1007/s13311-016-0463-1

Kitamura, Y., Takata, K., Inden, M., Tsuchiya, D., Yanagisawa, D., Nakata, J., Taniguchi, T., 2004. Intracerebroventricular injection of microglia protects against focal brain ischemia. J. Pharmacol. Sci. 94, 203–206.

Knoblach, S.M., Faden, A.I., 1998. Interleukin-10 Improves Outcome and Alters Proinflammatory Cytokine Expression after Experimental Traumatic Brain Injury. Exp. Neurol. 153, 143–151. doi:10.1006/exnr.1998.6877

Kobayashi, K., Yamanaka, H., Fukuoka, T., Dai, Y., Obata, K., Noguchi, K., 2008. P2Y12 Receptor Upregulation in Activated Microglia Is a Gateway of p38 Signaling and Neuropathic Pain. J. Neurosci. 28, 2892–2902. doi:10.1523/JNEUROSCI.5589-07.2008

Kohta, M., Kohmura, E., Yamashita, T., 2009. Inhibition of TGF-beta1 promotes functional recovery after spinal cord injury. Neurosci. Res. 65, 393–401. doi:10.1016/j.neures.2009.08.017

Kong, T., Choi, J.-K., Park, H., Choi, B.H., Snyder, B.J., Bukhari, S., Kim, N.-K., Huang, X., Park, S.R., Park, H.C., Ha, Y., 2009. Reduction in programmed cell death and improvement in functional outcome of transient focal cerebral ischemia after administration of granulocyte-macrophage colony-stimulating factor in rats. Laboratory investigation. J. Neurosurg. 111, 155–163. doi:10.3171/2008.12.JNS08172

Kreutzberg, G.W., 1995. Microglia, the first line of defence in brain pathologies. Arzneimittelforschung. 45, 357–360.

Kulhara, P., Gupta, S., 2010. What is schizophrenia: A neurodevelopmental or neurodegenerative disorder or a combination of both? A critical analysis. Indian J. Psychiatry 52, 21. doi:10.4103/0019-5545.58891

Kurpius, D., Nolley, E.P., Dailey, M.E., 2007. Purines induce directed migration and rapid homing of microglia to injured pyramidal neurons in developing hippocampus. Glia 55, 873–884. doi:10.1002/glia.20509

Lafon, M., Megret, F., Lafage, M., Prehaud, C., 2006. The Innate Immune Facet of Brain: Human Neurons Express TLR-3 and Sense Viral dsRNA. J. Mol. Neurosci. 29, 185–194. doi:10.1385/JMN:29:3:185

Lai, A., 2010. Determining factors in the differential activation of microglia. University of Alberta, Edmonton, Alberta. Canada.

Lai, A.Y., Dhami, K.S., Dibal, C.D., Todd, K.G., 2011. Neonatal rat microglia derived from different brain regions have distinct activation responses. Neuron Glia Biol. 7, 5–16. doi:10.1017/S1740925X12000154

Lai, A.Y., Dibal, C.D., Armitage, G.A., Winship, I.R., Todd, K.G., 2013. Distinct activation profiles in microglia of different ages: a systematic study in isolated embryonic to aged microglial cultures. Neuroscience 254, 185–195. doi:10.1016/j.neuroscience.2013.09.010

Lai, A.Y., Todd, K.G., 2008. Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury. Glia 56, 259–270. doi:10.1002/glia.20610

Lai, A.Y., Todd, K.G., 2006a. Hypoxia-activated microglial mediators of neuronal survival are differentially regulated by tetracyclines. Glia 53, 809–816. doi:10.1002/glia.20335

Lai, A.Y., Todd, K.G., 2006b. Microglia in cerebral ischemia: molecular actions and interactions. Can. J. Physiol. Pharmacol. 84, 49–59. doi:10.1139/Y05-143

Laing, K.J., Secombes, C.J., 2004. Chemokines. Dev. Comp. Immunol. 28, 443–460. doi:10.1016/j.dci.2003.09.006

Lalancette-Hébert, M., Gowing, G., Simard, A., Weng, Y.C., Kriz, J., 2007. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J. Neurosci. Off. J. Soc. Neurosci. 27, 2596–2605. doi:10.1523/JNEUROSCI.5360-06.2007

Lambertsen, K.L., Biber, K., Finsen, B., 2012. Inflammatory cytokines in experimental and human stroke. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 32, 1677–1698. doi:10.1038/jcbfm.2012.88

Lambertsen, K.L., Clausen, B.H., Babcock, A.A., Gregersen, R., Fenger, C., Nielsen, H.H., Haugaard, L.S., Wirenfeldt, M., Nielsen, M., Dagnaes-Hansen, F., Bluethmann, H., Faergeman, N.J., Meldgaard, M., Deierborg, T., Finsen, B., 2009. Microglia Protect Neurons against Ischemia by Synthesis of Tumor Necrosis Factor. J. Neurosci. 29, 1319–1330. doi:10.1523/JNEUROSCI.5505-08.2009

Lambertsen, K.L., Clausen, B.H., Fenger, C., Wulf, H., Owens, T., Dagnaes-Hansen, F., Meldgaard, M., Finsen, B., 2007. Microglia and macrophages express tumor necrosis factor receptor p75 following middle cerebral artery occlusion in mice. Neuroscience 144, 934–949. doi:10.1016/j.neuroscience.2006.10.046

Ledeboer, A., Brevé, J.J.P., Wierinckx, A., van der Jagt, S., Bristow, A.F., Leysen, J.E., Tilders, F.J.H., Van Dam, A.-M., 2002. Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. Eur. J. Neurosci. 16, 1175–1185.

Lee, P.-Y., Tsai, P.-S., Huang, Y.-H., Huang, C.-J., 2008. INHIBITION OF TOLL-LIKE RECEPTOR-4, NUCLEAR FACTOR-κB AND MITOGEN-ACTIVATED PROTEIN KINASE BY LIGNOCAINE MAY INVOLVE VOLTAGE-SENSITIVE SODIUM CHANNELS. Clin. Exp. Pharmacol. Physiol. 35, 1052–1058. doi:10.1111/j.1440-1681.2008.04962.x

Leibinger, M., Müller, A., Gobrecht, P., Diekmann, H., Andreadaki, A., Fischer, D., 2013. Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell Death Dis. 4, e609. doi:10.1038/cddis.2013.126

Leow-Dyke, S., Allen, C., Denes, A., Nilsson, O., Maysami, S., Bowie, A.G., Rothwell, N.J., Pinteaux, E., 2012. Neuronal toll-like receptor 4 signaling induces brain endothelial

activation and neutrophil transmigration in vitro. J. Neuroinflammation 9. doi:10.1186/1742-2094-9-230

Li, Z., Li, W., Li, Q., Tang, M., 2013. Extracellular nucleotides and adenosine regulate microglial motility and their role in cerebral ischemia. Acta Pharm. Sin. B 3, 205–212. doi:10.1016/j.apsb.2013.06.003

Liu, G.J., Nagarajah, R., Banati, R.B., Bennett, M.R., 2009. Glutamate induces directed chemotaxis of microglia. Eur. J. Neurosci. 29, 1108–1118. doi:10.1111/j.1460-9568.2009.06659.x

Ma, T.-C., Zhu, X.-Z., 2011. Neurotoxic Effects of lnterleukin-6 and Sodium Nitroprusside on Cultured Rat Hippocampal Neurons. Arzneimittelforschung 50, 512–514. doi:10.1055/s-0031-1300239

MacEwan, D.J., 2002. TNF ligands and receptors--a matter of life and death. Br. J. Pharmacol. 135, 855–875. doi:10.1038/sj.bjp.0704549

Marini, A.M., Paul, S.M., 1992. N-methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis. Proc. Natl. Acad. Sci. U. S. A. 89, 6555–6559.

Matsui, T., Svensson, C.I., Hirata, Y., Mizobata, K., Hua, X.-Y., Yaksh, T.L., 2010. Release of prostaglandin E(2) and nitric oxide from spinal microglia is dependent on activation of p38 mitogen-activated protein kinase. Anesth. Analg. 111, 554–560. doi:10.1213/ANE.0b013e3181e3a2a2

McKimmie, C.S., Fazakerley, J.K., 2005. In response to pathogens, glial cells dynamically and differentially regulate Toll-like receptor gene expression. J. Neuroimmunol. 169, 116–125. doi:10.1016/j.jneuroim.2005.08.006

Mead, E.L., Mosley, A., Eaton, S., Dobson, L., Heales, S.J., Pocock, J.M., 2012. Microglial neurotransmitter receptors trigger superoxide production in microglia; consequences for microglial-neuronal interactions. J. Neurochem. 121, 287–301. doi:10.1111/j.1471-4159.2012.07659.x

Mele, T., Madrenas, J., 2010. TLR2 signalling: At the crossroads of commensalism, invasive infections and toxic shock syndrome by Staphylococcus aureus. Int. J. Biochem. Cell Biol. 42, 1066–1071. doi:10.1016/j.biocel.2010.03.021

Meloni, B.P., Majda, B.T., Knuckey, N.W., 2001. Establishment of neuronal in vitro models of ischemia in 96-well microtiter strip-plates that result in acute, progressive and delayed neuronal death. Neuroscience 108, 17–26.

Meloni, B.P., Meade, A.J., Kitikomolsuk, D., Knuckey, N.W., 2011. Characterisation of neuronal cell death in acute and delayed in vitro ischemia (oxygen-glucose deprivation) models. J. Neurosci. Methods 195, 67–74. doi:10.1016/j.jneumeth.2010.11.023

Mihara, M., Hashizume, M., Yoshida, H., Suzuki, M., Shiina, M., 2012. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. Clin. Sci. Lond. Engl. 1979 122, 143–159. doi:10.1042/CS20110340

Mildner, A., Schmidt, H., Nitsche, M., Merkler, D., Hanisch, U.-K., Mack, M., Heikenwalder, M., Brück, W., Priller, J., Prinz, M., 2007. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. Nat. Neurosci. 10, 1544–1553. doi:10.1038/nn2015

Milligan, C.E., Cunningham, T.J., Levitt, P., 1991. Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain. J. Comp. Neurol. 314, 125–135. doi:10.1002/cne.903140112

Mingam, R., De Smedt, V., Amédée, T., Bluthé, R.-M., Kelley, K.W., Dantzer, R., Layé, S., 2008. In vitro and in vivo evidence for a role of the P2X7 receptor in the release of IL-1 beta in the murine brain. Brain. Behav. Immun. 22, 234–244. doi:10.1016/j.bbi.2007.08.007

Mitrasinovic, O.M., Grattan, A., Robinson, C.C., Lapustea, N.B., Poon, C., Ryan, H., Phong, C., Murphy, G.M., 2005. Microglia overexpressing the macrophage colony-stimulating factor receptor are neuroprotective in a microglial-hippocampal organotypic coculture system. J. Neurosci. Off. J. Soc. Neurosci. 25, 4442–4451. doi:10.1523/JNEUROSCI.0514-05.2005

Miwa, T., Furukawa, S., Nakajima, K., Furukawa, Y., Kohsaka, S., 1997. Lipopolysaccharide enhances synthesis of brain-derived neurotrophic factor in cultured rat microglia. J. Neurosci. Res. 50, 1023–1029. doi:10.1002/(SICI)1097-4547(19971215)50:6<1023::AID-JNR13>3.0.CO;2-5

Mizoguchi, Y., Kato, T.A., Seki, Y., Ohgidani, M., Sagata, N., Horikawa, H., Yamauchi, Y., Sato-Kasai, M., Hayakawa, K., Inoue, R., Kanba, S., Monji, A., 2014. Brain-derived neurotrophic factor (BDNF) induces sustained intracellular Ca2+ elevation through the up-regulation of surface transient receptor potential 3 (TRPC3) channels in rodent microglia. J. Biol. Chem. 289, 18549–18555. doi:10.1074/jbc.M114.555334

Mizutani, M., Pino, P.A., Saederup, N., Charo, I.F., Ransohoff, R.M., Cardona, A.E., 2012. The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. J. Immunol. Baltim. Md 1950 188, 29–36. doi:10.4049/jimmunol.1100421

Molyneaux, B.J., Arlotta, P., Menezes, J.R.L., Macklis, J.D., 2007. Neuronal subtype specification in the cerebral cortex. Nat. Rev. Neurosci. 8, 427–437. doi:10.1038/nrn2151

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765. doi:10.1146/annurev.immunol.19.1.683

Morsch, M., Radford, R., Lee, A., Don, E.K., Badrock, A.P., Hall, T.E., Cole, N.J., Chung, R., 2015. In vivo characterization of microglial engulfment of dying neurons in the zebrafish spinal cord. Front. Cell. Neurosci. 9, 321. doi:10.3389/fncel.2015.00321

Mukaino, M., Nakamura, M., Yamada, O., Okada, S., Morikawa, S., Renault-Mihara, F., Iwanami, A., Ikegami, T., Ohsugi, Y., Tsuji, O., Katoh, H., Matsuzaki, Y., Toyama, Y., Liu, M., Okano, H., 2010. Anti-IL-6-receptor antibody promotes repair of spinal cord injury by inducing microglia-dominant inflammation. Exp. Neurol. 224, 403–414. doi:10.1016/j.expneurol.2010.04.020

Murugan, M., Ling, E.-A., Kaur, C., 2013. Glutamate receptors in microglia. CNS Neurol. Disord. Drug Targets 12, 773–784.

Nagamoto-Combs, K., McNeal, D.W., Morecraft, R.J., Combs, C.K., 2007. Prolonged microgliosis in the rhesus monkey central nervous system after traumatic brain injury. J. Neurotrauma 24, 1719–1742. doi:10.1089/neu.2007.0377

Nakamura, Y., Si, Q.S., Kataoka, K., 1999. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci. Res. 35, 95–100.

Nayak, D., Roth, T.L., McGavern, D.B., 2014. Microglia Development and Function*. Annu. Rev. Immunol. 32, 367–402. doi:10.1146/annurev-immunol-032713-120240

Nesic, O., Xu, G.Y., McAdoo, D., High, K.W., Hulsebosch, C., Perez-Pol, R., 2001. IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. J. Neurotrauma 18, 947–956. doi:10.1089/089771501750451857

Neumann, J., Gunzer, M., Gutzeit, H.O., Ullrich, O., Reymann, K.G., Dinkel, K., 2006. Microglia provide neuroprotection after ischemia. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 20, 714–716. doi:10.1096/fj.05-4882fje

Nicke, A., Bäumert, H.G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E., Schmalzing, G., 1998. P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels. EMBO J. 17, 3016–3028. doi:10.1093/emboj/17.11.3016

Nikodemova, M., Watters, J.J., 2012. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. J. Neuroinflammation 9. doi:10.1186/1742-2094-9-147

Nimmerjahn, A., 2005. Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. Science 308, 1314–1318. doi:10.1126/science.1110647

Niswender, C.M., Conn, P.J., 2010. Metabotropic Glutamate Receptors: Physiology, Pharmacology, and Disease. Annu. Rev. Pharmacol. Toxicol. 50, 295–322. doi:10.1146/annurev.pharmtox.011008.145533

Noda, M., Nakanishi, H., Nabekura, J., Akaike, N., 2000. AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia. J. Neurosci. Off. J. Soc. Neurosci. 20, 251–258.

North, R.A., 2002. Molecular Physiology of P2X Receptors. Physiol. Rev. 82, 1013–1067. doi:10.1152/physrev.00015.2002

North, R.A., Verkhratsky, A., 2006. Purinergic transmission in the central nervous system. Pflugers Arch. 452, 479–485. doi:10.1007/s00424-006-0060-y

Ogata, T., Chuai, M., Morino, T., Yamamoto, H., Nakamura, Y., Schubert, P., 2003. Adenosine triphosphate inhibits cytokine release from lipopolysaccharide-activated microglia via P2y receptors. Brain Res. 981, 174–183.

Okada, S., Nakamura, M., Mikami, Y., Shimazaki, T., Mihara, M., Ohsugi, Y., Iwamoto, Y., Yoshizaki, K., Kishimoto, T., Toyama, Y., Okano, H., 2004. Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. J. Neurosci. Res. 76, 265–276. doi:10.1002/jnr.20044

Olson, J.K., 2010. Immune response by microglia in the spinal cord: Microglia in the spinal cord. Ann. N. Y. Acad. Sci. 1198, 271–278. doi:10.1111/j.1749-6632.2010.05536.x

Olson, J.K., Miller, S.D., 2004. Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses through Multiple TLRs. J. Immunol. 173, 3916–3924. doi:10.4049/jimmunol.173.6.3916

Oshima, T., Lee, S., Sato, A., Oda, S., Hirasawa, H., Yamashita, T., 2009. TNF-α contributes to axonal sprouting and functional recovery following traumatic brain injury. Brain Res. 1290, 102–110. doi:10.1016/j.brainres.2009.07.022

Oyinbo, C.A., 2011. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta Neurobiol. Exp. (Warsz.) 71, 281–299.

Pankratov, Y., Lalo, U., Verkhratsky, A., North, R.A., 2007. Quantal Release of ATP in Mouse Cortex. J. Gen. Physiol. 129, 257–265. doi:10.1085/jgp.200609693

Pankratov, Y., Lalo, U., Verkhratsky, A., North, R.A., 2006. Vesicular release of ATP at central synapses. Pflugers Arch. 452, 589–597. doi:10.1007/s00424-006-0061-x

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T., 2011. Synaptic

Pruning by Microglia Is Necessary for Normal Brain Development. Science 333, 1456–1458. doi:10.1126/science.1202529

Pineau, I., Lacroix, S., 2007. Proinflammatory cytokine synthesis in the injured mouse spinal cord: Multiphasic expression pattern and identification of the cell types involved. J. Comp. Neurol. 500, 267–285. doi:10.1002/cne.21149

Pocock, J.M., Kettenmann, H., 2007. Neurotransmitter receptors on microglia. Trends Neurosci. 30, 527–535. doi:10.1016/j.tins.2007.07.007

Prinz, M., Priller, J., 2010. Tickets to the brain: role of CCR2 and CX3CR1 in myeloid cell entry in the CNS. J. Neuroimmunol. 224, 80–84. doi:10.1016/j.jneuroim.2010.05.015

Prüss, H., Kopp, M.A., Brommer, B., Gatzemeier, N., Laginha, I., Dirnagl, U., Schwab, J.M., 2011. Non-Resolving Aspects of Acute Inflammation after Spinal Cord Injury (SCI): Indices and Resolution Plateau: Non-Resolving Inflammation. Brain Pathol. 21, 652–660. doi:10.1111/j.1750-3639.2011.00488.x

Qureshi, O.S., Paramasivam, A., Yu, J.C.H., Murrell-Lagnado, R.D., 2007. Regulation of P2X4 receptors by lysosomal targeting, glycan protection and exocytosis. J. Cell Sci. 120, 3838–3849. doi:10.1242/jcs.010348

Ren, Y., Zhou, X., He, X., 2014. Function of microglia and macrophages in secondary damage after spinal cord injury. Neural Regen. Res. 9, 1787. doi:10.4103/1673-5374.143423

Rezaie, P., Male, D., 1999. Colonisation of the developing human brain and spinal cord by microglia: a review. Microsc. Res. Tech. 45, 359–382. doi:10.1002/(SICI)1097-0029(19990615)45:6<359::AID-JEMT4>3.0.CO;2-D

Roberts, J.A., Vial, C., Digby, H.R., Agboh, K.C., Wen, H., Atterbury-Thomas, A., Evans, R.J., 2006. Molecular properties of P2X receptors. Pflugers Arch. 452, 486–500. doi:10.1007/s00424-006-0073-6

Rodrigues, R.J., Tomé, A.R., Cunha, R.A., 2015. ATP as a multi-target danger signal in the brain. Front. Neurosci. 9, 148. doi:10.3389/fnins.2015.00148

Rogove, A.D., Tsirka, S.E., 1998. Neurotoxic responses by microglia elicited by excitotoxic injury in the mouse hippocampus. Curr. Biol. CB 8, 19–25.

Saijo, K., Glass, C.K., 2011. Microglial cell origin and phenotypes in health and disease. Nat. Rev. Immunol. 11, 775–787. doi:10.1038/nri3086

Sallusto, F., Baggiolini, M., 2008. Chemokines and leukocyte traffic. Nat. Immunol. 9, 949–952. doi:10.1038/ni.f.214

Sanz, J.M., Virgilio, F.D., 2000. Kinetics and Mechanism of ATP-Dependent IL-1 Release from Microglial Cells. J. Immunol. 164, 4893–4898. doi:10.4049/jimmunol.164.9.4893

Sato, A., Ohtaki, H., Tsumuraya, T., Song, D., Ohara, K., Asano, M., Iwakura, Y., Atsumi, T., Shioda, S., 2012. Interleukin-1 participates in the classical and alternative activation of microglia/macrophages after spinal cord injury. J. Neuroinflammation 9. doi:10.1186/1742-2094-9-65

Saura, J., Tusell, J.M., Serratosa, J., 2003. High-yield isolation of murine microglia by mild trypsinization. Glia 44, 183–189. doi:10.1002/glia.10274

Sawada, M., Suzumura, A., Marunouchi, T., 1995. Induction of functional interleukin-2 receptor in mouse microglia. J. Neurochem. 64, 1973–1979.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., Stevens, B., 2012. Microglia Sculpt

Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. Neuron 74, 691–705. doi:10.1016/j.neuron.2012.03.026

Schafer, D.P., Lehrman, E.K., Stevens, B., 2013. The “quad-partite” synapse: microglia-synapse interactions in the developing and mature CNS. Glia 61, 24–36. doi:10.1002/glia.22389

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W., Pollard, J.W., Frampton, J., Liu, K.J., Geissmann, F., 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science 336, 86–90. doi:10.1126/science.1219179

Seeland, S., Kettiger, H., Murphy, M., Treiber, A., Giller, J., Kiss, A., Sube, R., Krähenbühl, S., Hafner, M., Huwyler, J., 2015. ATP-induced cellular stress and mitochondrial toxicity in cells expressing purinergic P2X7 receptor. Pharmacol. Res. Perspect. 3, e00123. doi:10.1002/prp2.123

Siao, C.-J., Tsirka, S.E., 2002. Tissue plasminogen activator mediates microglial activation via its finger domain through annexin II. J. Neurosci. Off. J. Soc. Neurosci. 22, 3352–3358. doi:20026281

Sierra, A., de Castro, F., Del Río-Hortega, J., Rafael Iglesias-Rozas, J., Garrosa, M., Kettenmann, H., 2016. The “Big-Bang” for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia. Glia 64, 1801–1840. doi:10.1002/glia.23046

Simon, C.M., Sharif, S., Tan, R.P., LaPlaca, M.C., 2009. Spinal cord contusion causes acute plasma membrane damage. J. Neurotrauma 26, 563–574. doi:10.1089/neu.2008.0523

Sims, J.E., Smith, D.E., 2010. The IL-1 family: regulators of immunity. Nat. Rev. Immunol. 10, 89–102. doi:10.1038/nri2691

Sist, B., Baskar Jesudasan, S.J., R., I., 2012. Diaschisis, Degeneration, and Adaptive Plasticity After Focal Ischemic Stroke, in: Garcia Rodriguez, J.C. (Ed.), Acute Ischemic Stroke. InTech.

Sist, B., Fouad, K., Winship, I.R., 2014. Plasticity beyond peri-infarct cortex: spinal up regulation of structural plasticity, neurotrophins, and inflammatory cytokines during recovery from cortical stroke. Exp. Neurol. 252, 47–56. doi:10.1016/j.expneurol.2013.11.019

Smith, P.D., Puskas, F., Meng, X., Lee, J.H., Cleveland, J.C., Weyant, M.J., Fullerton, D.A., Reece, T.B., 2012. The evolution of chemokine release supports a bimodal mechanism of spinal cord ischemia and reperfusion injury. Circulation 126, S110-117. doi:10.1161/CIRCULATIONAHA.111.080275

Sokolowski, J.D., Chabanon-Hicks, C.N., Han, C.Z., Heffron, D.S., Mandell, J.W., 2014. Fractalkine is a “find-me” signal released by neurons undergoing ethanol-induced apoptosis. Front. Cell. Neurosci. 8, 360. doi:10.3389/fncel.2014.00360

Sorge, R.E., LaCroix-Fralish, M.L., Tuttle, A.H., Sotocinal, S.G., Austin, J.-S., Ritchie, J., Chanda, M.L., Graham, A.C., Topham, L., Beggs, S., Salter, M.W., Mogil, J.S., 2011. Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male But Not Female Mice. J. Neurosci. 31, 15450–15454. doi:10.1523/JNEUROSCI.3859-11.2011

Sperlágh, B., Illes, P., 2007. Purinergic modulation of microglial cell activation. Purinergic Signal. 3, 117–127. doi:10.1007/s11302-006-9043-x

Sriram, K., 2002. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: Implications for Parkinson’s disease. FASEB J. doi:10.1096/fj.02-0216fje

Sroga, J.M., Jones, T.B., Kigerl, K.A., McGaughy, V.M., Popovich, P.G., 2003. Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. J. Comp. Neurol. 462, 223–240. doi:10.1002/cne.10736

Surprenant, A., North, R.A., 2009. Signaling at purinergic P2X receptors. Annu. Rev. Physiol. 71, 333–359. doi:10.1146/annurev.physiol.70.113006.100630

Takeda, K., Akira, S., 2004. TLR signaling pathways. Semin. Immunol. 16, 3–9.

Takeuchi, H., Jin, S., Wang, J., Zhang, G., Kawanokuchi, J., Kuno, R., Sonobe, Y., Mizuno, T., Suzumura, A., 2006. Tumor Necrosis Factor- Induces Neurotoxicity via Glutamate Release from Hemichannels of Activated Microglia in an Autocrine Manner. J. Biol. Chem. 281, 21362–21368. doi:10.1074/jbc.M600504200

Tamashiro, T.T., Dalgard, C.L., Byrnes, K.R., 2012. Primary Microglia Isolation from Mixed Glial Cell Cultures of Neonatal Rat Brain Tissue. J. Vis. Exp. doi:10.3791/3814

Tang, Z., Gan, Y., Liu, Q., Yin, J.-X., Liu, Q., Shi, J., Shi, F.-D., 2014. CX3CR1 deficiency suppresses activation and neurotoxicity of microglia/macrophage in experimental ischemic stroke. J. Neuroinflammation 11, 26. doi:10.1186/1742-2094-11-26

Tanga, F.Y., Nutile-McMenemy, N., DeLeo, J.A., 2005. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc. Natl. Acad. Sci. U. S. A. 102, 5856–5861. doi:10.1073/pnas.0501634102

Taoufik, E., Valable, S., Muller, G.J., Roberts, M.L., Divoux, D., Tinel, A., Voulgari-Kokota, A., Tseveleki, V., Altruda, F., Lassmann, H., Petit, E., Probert, L., 2007. FLIPL Protects Neurons against In Vivo Ischemia and In Vitro Glucose Deprivation-Induced Cell Death. J. Neurosci. 27, 6633–6646. doi:10.1523/JNEUROSCI.1091-07.2007

Tarkowski, E., Rosengren, L., Blomstrand, C., Wikkelsö, C., Jensen, C., Ekholm, S., Tarkowski, A., 1995. Early intrathecal production of interleukin-6 predicts the size of brain lesion in stroke. Stroke J. Cereb. Circ. 26, 1393–1398.

Taylor, D.L., 2005. Stimulation of Microglial Metabotropic Glutamate Receptor mGlu2 Triggers Tumor Necrosis Factor -Induced Neurotoxicity in Concert with Microglial-Derived Fas Ligand. J. Neurosci. 25, 2952–2964. doi:10.1523/JNEUROSCI.4456-04.2005

Taylor, D.L., Diemel, L.T., Cuzner, M.L., Pocock, J.M., 2002. Activation of group II metabotropic glutamate receptors underlies microglial reactivity and neurotoxicity following stimulation with chromogranin A, a peptide up-regulated in Alzheimer's disease. J. Neurochem. 82, 1179–1191.

Taylor, D.L., Diemel, L.T., Pocock, J.M., 2003. Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. J. Neurosci. Off. J. Soc. Neurosci. 23, 2150–2160.

Temple, S., 2001. The development of neural stem cells. Nature 414, 112–117.

Tian, L., Ma, L., Kaarela, T., Li, Z., 2012. Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases. J. Neuroinflammation 9. doi:10.1186/1742-2094-9-155

Tikka, T.M., Koistinaho, J.E., 2001. Minocycline Provides Neuroprotection Against N-Methyl-D-aspartate Neurotoxicity by Inhibiting Microglia. J. Immunol. 166, 7527–7533. doi:10.4049/jimmunol.166.12.7527

Tozaki-Saitoh, H., Tsuda, M., Miyata, H., Ueda, K., Kohsaka, S., Inoue, K., 2008. P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve

injury. J. Neurosci. Off. J. Soc. Neurosci. 28, 4949–4956. doi:10.1523/JNEUROSCI.0323-08.2008

Trang, T., Beggs, S., Salter, M.W., 2006. Purinoceptors in microglia and neuropathic pain. Pflugers Arch. 452, 645–652. doi:10.1007/s00424-006-0074-5

Trang, T., Beggs, S., Wan, X., Salter, M.W., 2009. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. J. Neurosci. Off. J. Soc. Neurosci. 29, 3518–3528. doi:10.1523/JNEUROSCI.5714-08.2009

Tremblay, M.-È., Lowery, R.L., Majewska, A.K., 2010. Microglial Interactions with Synapses Are Modulated by Visual Experience. PLoS Biol. 8, e1000527. doi:10.1371/journal.pbio.1000527

Tremblay, M.-E., Stevens, B., Sierra, A., Wake, H., Bessis, A., Nimmerjahn, A., 2011. The Role of Microglia in the Healthy Brain. J. Neurosci. 31, 16064–16069. doi:10.1523/JNEUROSCI.4158-11.2011

Tsuda, M., Inoue, K., Salter, M.W., 2005. Neuropathic pain and spinal microglia: a big problem from molecules in “small” glia. Trends Neurosci. 28, 101–107. doi:10.1016/j.tins.2004.12.002

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., Inoue, K., 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424, 778–783. doi:10.1038/nature01786

Twohig, J.P., Cuff, S.M., Yong, A.A., Wang, E.C.Y., 2011. The role of tumor necrosis factor receptor superfamily members in mammalian brain development, function and homeostasis. Rev. Neurosci. 22. doi:10.1515/RNS.2011.041

Ueda, Y., Obrenovitch, T.P., Lok, S.Y., Sarna, G.S., Symon, L., 1992. Changes in extracellular glutamate concentration produced in the rat striatum by repeated ischemia. Stroke J. Cereb. Circ. 23, 1125–1130; discussion 1131.

Upender, M.B., Naegele, J.R., 1999. Activation of microglia during developmentally regulated cell death in the cerebral cortex. Dev. Neurosci. 21, 491–505. doi:17416

Verderio, C., Matteoli, M., 2001. ATP Mediates Calcium Signaling Between Astrocytes and Microglial Cells: Modulation by IFN-. J. Immunol. 166, 6383–6391. doi:10.4049/jimmunol.166.10.6383

Vilhardt, F., 2005. Microglia: phagocyte and glia cell. Int. J. Biochem. Cell Biol. 37, 17–21. doi:10.1016/j.biocel.2004.06.010

Vincent, A.M., Maiese, K., 2000. The metabotropic glutamate system promotes neuronal survival through distinct pathways of programmed cell death. Exp. Neurol. 166, 65–82. doi:10.1006/exnr.2000.7487

von Zahn, J., Möller, T., Kettenmann, H., Nolte, C., 1997. Microglial phagocytosis is modulated by pro- and anti-inflammatory cytokines. Neuroreport 8, 3851–3856.

Wake, H., Moorhouse, A.J., Jinno, S., Kohsaka, S., Nabekura, J., 2009. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J. Neurosci. Off. J. Soc. Neurosci. 29, 3974–3980. doi:10.1523/JNEUROSCI.4363-08.2009

Wake, H., Tremblay, M.-E., Sierra, A. (Eds.), 2015. Never-resting microglia: physiological roles in the healthy brain and pathological implications, Frontiers Research Topics. Frontiers SA Media.

Wang, X., 2004. Inhibition of Tumor Necrosis Factor- -Converting Enzyme by a Selective Antagonist Protects Brain from Focal Ischemic Injury in Rats. Mol. Pharmacol. 65, 890–896. doi:10.1124/mol.65.4.890

Ward-Kavanagh, L.K., Lin, W.W., Šedý, J.R., Ware, C.F., 2016. The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses. Immunity 44, 1005–1019. doi:10.1016/j.immuni.2016.04.019

Weishaupt, N., Silasi, G., Colbourne, F., Fouad, K., 2010. Secondary damage in the spinal cord after motor cortex injury in rats. J. Neurotrauma 27, 1387–1397. doi:10.1089/neu.2010.1346

Werry, E.L., Liu, G.J., Lovelace, M.D., Nagarajah, R., Hickie, I.B., Bennett, M.R., 2011. Lipopolysaccharide-stimulated interleukin-10 release from neonatal spinal cord microglia is potentiated by glutamate. Neuroscience 175, 93–103. doi:10.1016/j.neuroscience.2010.10.080

Wolf, Y., Yona, S., Kim, K.-W., Jung, S., 2013. Microglia, seen from the CX3CR1 angle. Front. Cell. Neurosci. 7. doi:10.3389/fncel.2013.00026

Wollmer, M.A., Lucius, R., Wilms, H., Held-Feindt, J., Sievers, J., Mentlein, R., 2001. ATP and adenosine induce ramification of microglia in vitro. J. Neuroimmunol. 115, 19–27.

Woodroofe, M.N., Sarna, G.S., Wadhwa, M., Hayes, G.M., Loughlin, A.J., Tinker, A., Cuzner, M.L., 1991. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J. Neuroimmunol. 33, 227–236. doi:10.1016/0165-5728(91)90110-S

Woolf, C.J., Mannion, R.J., 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet Lond. Engl. 353, 1959–1964. doi:10.1016/S0140-6736(99)01307-0

Wu, L.-J., Vadakkan, K.I., Zhuo, M., 2007. ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents. Glia 55, 810–821. doi:10.1002/glia.20500

Yanagisawa, D., Kitamura, Y., Takata, K., Hide, I., Nakata, Y., Taniguchi, T., 2008. Possible involvement of P2X7 receptor activation in microglial neuroprotection against focal cerebral ischemia in rats. Biol. Pharm. Bull. 31, 1121–1130.

Yang, L., Jones, N.R., Blumbergs, P.C., Van Den Heuvel, C., Moore, E.J., Manavis, J., Sarvestani, G.T., Ghabriel, M.N., 2005. Severity-dependent expression of pro-inflammatory cytokines in traumatic spinal cord injury in the rat. J. Clin. Neurosci. Off. J. Neurosurg. Soc. Australas. 12, 276–284. doi:10.1016/j.jocn.2004.06.011

Yenari, M.A., Kauppinen, T.M., Swanson, R.A., 2010. Microglial activation in stroke: therapeutic targets. Neurother. J. Am. Soc. Exp. Neurother. 7, 378–391. doi:10.1016/j.nurt.2010.07.005

Yip, P.K., Kaan, T.K.Y., Fenesan, D., Malcangio, M., 2009. Rapid isolation and culture of primary microglia from adult mouse spinal cord. J. Neurosci. Methods 183, 223–237. doi:10.1016/j.jneumeth.2009.07.002

Yu, T., Cheng, Y., Zhao, P., Kou, D., Sun, K., Chen, B., Wang, A., 2009. Immune therapy with cultured microglia grafting into the injured spinal cord promoting the recovery of rat's hind limb motor function. Chin. J. Traumatol. Zhonghua Chuang Shang Za Zhi Chin. Med. Assoc. 12, 291–295.

Zhang, Z., Chen, G., Zhou, W., Song, A., Xu, T., Luo, Q., Wang, W., Gu, X., Duan, S., 2007. Regulated ATP release from astrocytes through lysosome exocytosis. Nat. Cell Biol. 9, 945–953. doi:10.1038/ncb1620

Zhou, L.-J., Yang, T., Wei, X., Liu, Y., Xin, W.-J., Chen, Y., Pang, R.-P., Zang, Y., Li, Y.-Y., Liu, X.-G., 2011. Brain-derived neurotrophic factor contributes to spinal long-term potentiation and mechanical hypersensitivity by activation of spinal microglia in rat. Brain. Behav. Immun. 25, 322–334. doi:10.1016/j.bbi.2010.09.025

Zimmermann, H., 2006. Ectonucleotidases in the nervous system. Novartis Found. Symp. 276, 113-128-130, 233–237, 275–281.