IDENTIFICATION OF NOVEL ATP AND INTERLEUKIN-10 SOURCES IN THE SPINAL CORD

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A thesis submitted to the **University of Sydney**
in fulfilment of the requirements for the degree of
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DECLARATION

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy. The work presented in this thesis was undertaken by myself in the Bosch Institute and Brain and Mind Research Institute, University of Sydney apart from the electrophysiology experiments in section 3.3.1 and 3.3.4 which were carried out by Dr Guo Jun Liu. To the best of my knowledge the work presented in this thesis has not been published by another person, except where due reference is made in the text of the thesis. Parts of this thesis have been published in abstract form and in peer-reviewed journal form and they are listed on page vii.

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"Of making many books there is no end and much study wearies the body. Now all has been heard here is the conclusion of the matter: Fear God and keep his commandments, for this is the whole duty of man."

Ecclesiastes 12:12b-13.

SUMMARY

Chronic pain has a large individual and societal impact. Although effective treatments for acute pain exist, a specific and effective treatment for chronic pain is still to be discovered. Recent use of genetic techniques has revealed that spinal ATP plays a role in the generation of allodynia and hyperalgesia, two symptoms of chronic pain. Furthermore, it has been shown that the symptoms of chronic pain can be alleviated by increasing spinal levels of the anti-inflammatory cytokine interleukin-10 (IL-10). These observations suggest that decreasing the spinal release of ATP and increasing the spinal release of IL-10 may be novel approaches to chronic pain treatment. Yet knowledge of the endogenous sources of spinal ATP and IL-10 is limited. Therefore, the aim of this thesis was to identify novel sources of ATP and IL-10 in the spinal cord.

The first series of experiments (Chapter 3) investigated whether spinal cord astrocytes could be a source of ATP in response to the nociceptive neurotransmitters glutamate and substance P. Glutamate stimulated ATP release from cultured spinal cord astrocytes and this release was greatly potentiated by substance P, even though substance P alone did not elicit ATP release. Substance P also potentiated glutamate-induced inward currents without alone causing such currents. Glutamate, when applied on its own, acted exclusively through AMPA receptors to stimulate Ca^{2+} influx-dependent ATP release. However when substance P was co-applied with glutamate, ATP release could be elicited by activation of NMDA and metabotropic glutamate receptors. ATP release resulting from AMPA and kainate receptor stimulation was not affected by substance P. Activation of neurokinin receptor

subtypes, protein kinase C and phospholipase A_2, C and D were required for substance P to bring about its effects.

The second series of experiments (Chapter 4) explored whether spinal cord astrocytes could be a source of IL-10 in response to glutamate and stimulation of the Toll-like receptor 4 (TLR4), which is known to occur in chronic pain. TLR4 stimulation increased IL-10 release from cultured spinal cord astrocytes. TLR4-stimulated IL-10 release was enhanced in the presence of glutamate. Glutamate potentiated TLR4-stimulated IL-10 release by upregulating IL-10 mRNA and worked synergistically through metabotropic glutamate receptor groups I, II and possibly III.

The third series of experiments (Chapter 5) examined whether spinal cord microglia could be a source of IL-10 in response to glutamate and stimulation of the TLR4. TLR4 stimulation enhanced both IL-10 transcription and translation, resulting in an increase in IL-10 release from cultured spinal cord microglia. Glutamate significantly increased TLR4-stimulated IL-10 release from microglia by binding NMDA, AMPA, metabotropic glutamate receptors and possibly kainate receptors and enhancing TLR4-induced IL-10 mRNA expression.

The results of this thesis suggest that astrocytes may be a major source of ATP in the spinal cord on activation of nerve fibres that co-release substance P and glutamate. The results of this thesis also suggest that when glutamate and TLR4 agonists are released into the spinal cord after noxious stimulation, astrocytes and microglia may be a major source of IL-10. These results implicate spinal cord glia in both pro-nociceptive and anti-nociceptive responses to pain-related substances.

CONTENTS

PUBLICATIONS

Refereed journal articles related to this thesis

Werry EL, Liu GJ and Bennett MR (2006) Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P. *Journal of Neurochemistry* 99:924-36.

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Werry EL, Liu GJ, Nagarajah R, Lovelace MD, Hickie IB and Bennett MR (2008) Microglial interleukin-10 release on activation of Toll-like receptor 4 is potentiated by glutamate *Submitted.*

Other refereed journal articles

Liu GJ, Kalous A, **Werry EL** and Bennett MR (2006) Purine release from spinal cord microglia after elevation of calcium by glutamate *Molecular Pharmacology* 70:851-59.

Liu GJ, **Werry EL** and Bennett MR (2005) Secretion of ATP from Schwann cells in response to uridine triphosphate *European Journal of Neuroscience* 21:151-60.

Abdipronoto A, Liu GJ, **Werry EL** and Bennett MR (2003) Mechanisms of secretion of ATP from cortical astrocytes triggered by uridine triphosphate *Neuroreport* 14:2177-81.

Conference proceedings

Werry EL, Liu GJ and Bennett MR (2004) Substance P modulates glutamate-stimulated adenosine triphosphate release from spinal cord astrocytes *Proceedings of the Australian Health and Medical Research Congress* 2:119.

Werry EL, Liu GJ and Bennett MR (2005) Substance P allows glutamate to stimulate ATP release through NMDA and metabotropic glutamate receptors on spinal cord astrocytes *Proceedings of the Australian Neuroscience Society* 16:42.

Werry EL, Liu GJ and Bennett MR (2005) Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P *Journal of Neurochemistry* 94:101.

Werry EL, Liu GJ and Bennett MR (2006) Substance P enhances glutamate-stimulated ATP release from cultured rat spinal cord astrocytes *Society for Neuroscience* 733.10/H14.

Werry EL, Liu GJ and Bennett MR (2007) Glutamate enhances anti-inflammatory interleukin-10 release from spinal cord microglia and astrocytes after Toll-like receptor 4 stimulation *Proceedings of the World Congress of Neuroscience* 7:163.

Werry EL, Liu GJ and Bennett MR (2007) Interleukin-10 release from spinal cord microglia and astrocytes after Toll-like receptor 4 stimulation is enhanced by glutamate *Proceedings of the 25th International Australasian Winter Conference on Brain Research* 25:In press.

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ABBREVIATIONS

AC,	Adenylyl cyclase
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
AP-1	activation protein-1
AP5	D(−)-2-Amino-5-phosphonopentanoic acid
APDC	(2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate
ARL 67156	6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5-triphosphate
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD14	cluster determinant 14
cDNA	complementary deoxyribonucleic acid
c/EBP	CCAAT/enhancer binding protein
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPCCOEt,	7-Hydroxyiminocyclopropan[b]chromen-1α-carboxylic acid ethyl ester
DAG	diacyl glycerol
DAPI	4',6-diamidino-2-phenylindole
DDA	2'5'dideoxyadenosine
DHPG	(*S*)-3,5-Dihydroxyphenylglycine hydrate
DMEM	Dulbecco's modified Eagle's medium
DMEM+	supplemented Dulbecco's modified Eagle's medium
dNTP	2'-deoxynucleoside 5'-triphosphate
dsRNA	double stranded ribonucleic acid
EGLU	(2S)-α-Ethylglutamic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-related kinase
FAD^{2+}	flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GAMS	γ-D-glutamylamino methylsulfonic acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate and aspartate transporter
GLT	glutamate transporter
Glu	glutamate
GYKI52466	1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride
HBSS	Hank's balanced salt solution
IkB	inhibitor of nuclear factor-kB
IKK	inhibitor of nuclear factor-kB kinase
IL-6	interleukin-6

IL-10	interleukin-10
IL-10R	interleukin-10 receptor
IL-1β	interleukin-1β
IP_3	inositol triphosphate
IRAK	IL-1 receptor associated kinase
IRF3	interferon regulatory factor-3
ISRE	interferon-stimulated response element
JAK1	Janus Kinase 1
JNK	JUN N-terminal kinase
KA	kainate
L703606	cis-2-(Diphenylmethyl)-N-[(2iodophenyl)methyl]-1azabicyclo[2.2.2]octan-3-amine oxalate salt
L-AP4	L-(+)-2-Amino-4-phosphonobutyric acid
LBP	lipopolysaccharide binding protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAL	MyD88-adaptor-like
MAPK	mitogen activated protein kinase
MCPG	(+)□-α-methyl-4-carboxyphenylglycine
MD2	myeloid differentiation Ag
mGlu	metabotropic glutamate
mGluR	metabotropic glutamate receptor
MK-801	(+)-MK-801 hydrogen maleate
MKK	mitogen-activated protein kinase kinase
MPEP	6-Methyl-2-(phenylethynyl)pyridine
MSPG	(±)-α-Methyl-(4-sulfonophenyl)glycine
MyD88	myeloid differention 88
NAD^+	ñicotinamide adenine dinucleotide
NBMPR	*S*-(4-Nitrobenzyl)-6-thioinosine
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline
NF-kB	nuclear factor-kB
NK1	neurokinin-1
NK2	neurokinin-2
NK3	neurokinin-3
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
REST	relative expression software tool
R-LPS	rough lipopolysaccharide
RT-PCR	reverse transcription-polymerase chain reaction
siRNA	small interfering ribonucleic acid
S-LPS	smooth lipopolysaccharide

STAT	Signal Transducer and Activator of Transcription
Sp1	Simian virus 40 promoter factor 1
SP	substance P
tACPD	*trans*-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid
TAB2	transforming growth factor-β-activated kinase 1-binding protein 2
TAK1	transforming growth factor-β-activated kinase 1
TBOA	threo-β-hydroxyaspartic acid
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TRAF	tumor-necrosis-factor-receptor-associated factor
TRAM -1	TIR-containing adaptor molecule-1
TRPV1	transient receptor potential channel, vanilloid receptor 1
t-SNARE	target-soluble N-ethylmaleimide-sensitive factor attachment receptor
Tyk2	tyrosine kinase 2
UBC13	ubiquitin-conjugating enzyme 13
UEV1A	ubiquitin-conjugating enzyme E2 variant 1;
UTP	uridine triphosphate;
v-SNARE	vesicle-soluble N-ethylmaleimide-sensitive factor attachment receptor

CHAPTER 1

LITERATURE REVIEW AND AIMS

Contents

1.1 AN INTRODUCTION TO THE CONCEPT OF PAIN

1.1.1 Acute pain

The International Association for the Study of Pain (1994) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain can be acute where the area of sensation is the same as the area of noxious stimulation. Acute pain is reversible, that is, it goes away (Borsook, 2003; Holden and Pizzi, 2003). Acute pain is elicited by noxious stimuli such as mechanical force, thermal energy or chemicals like acid. The receptors that respond to these stimuli are called nociceptors and they are found in the skin, muscles, joint capsules and a range of internal organs such as the heart and gut. Nociceptors are the peripheral endings of Aδ and C fibres, which are intermediate (12–30 m/s) and slow (0.5–2 m/s) conducting peripheral afferent fibres respectively. When these nociceptors are activated to a degree whereby their fibres are brought to threshold, action potentials travel down Aδ and C fibres and synapse in the spinal cord dorsal horn. The cell body-containing grey matter of the spinal cord is divided up into 10 layers or laminae (I – X). C fibres synapse in laminae I, II, VI and X whilst Aδ fibres synapse mostly in laminae I, II and to a lesser extent IV. At these synapses, through release of neurotransmitters such as substance P (SP) and glutamate, action potentials are elicited in post-synaptic dorsal horn neurons (for a review see Millan (1999)). Some of these dorsal horn neurons are interneurons, which synapse on ventral horn neurons. The result of this is the propagation of action potentials toward muscles in the originally stimulated region and consequent reflex

withdrawal from the noxious stimulus. Other dorsal horn neurons send axons up ascending tracts to the limbic system, thalamus and areas of the cortex, such as the somatosensory cortex, resulting in perceptions of pain (Andersen et al., 2004; Holden and Pizzi, 2003; M illan, 1999). These pathways are summarized in Figure 1.1. Acute pain is useful, providing a warning of impending damage to tissues (Millan, 1999).

1.1.2 Chronic pain

Chronic pain is pain which persists beyond the normal time of healing and which is present everyday for three or more months (Access Economics Pty Ltd, 2007). Allodynia is pain perceived on application of a normally non-painful stimulus, such as pain felt when clothes brush on the skin (tactile allodynia), and hyperalgesia is a heightened response to a noxious stimulus (Merskey and Bogduk, 1994). Primary hyperalgesia occurs in the area of initial injury, whilst secondary hyperalgesia occurs in tissue surrounding the initial injury. Pain can also be spontaneous both in the area of injury and the surrounding tissues. Allodynia, hyperalgesia and spontaneous pain are the three key symptoms of chronic pain, although secondary hyperalgesia and allodynia are seen in severe acute pain and are classed as chronic pain symptoms when they persist longer than would be expected for the acute injury to heal (Holden and Pizzi, 2003). The enduring pain is referred to as neuropathic pain if it results from nerve damage and inflammatory pain if it results from inflammatory mediators. Conditions involving chronic pain include arthritis, chronic migraine and peripheral nerve damage (Borsook, 2003).

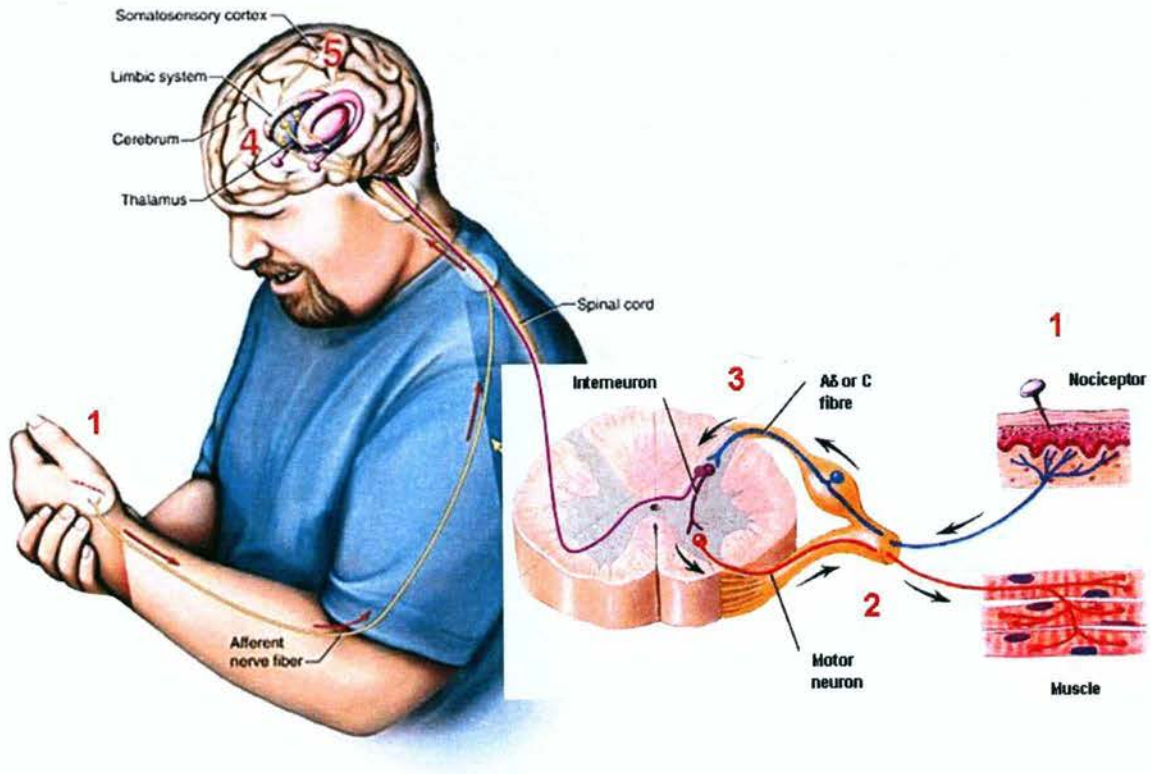


Figure 1.1 Neural pathways involved in acute pain. 1) A nociceptive stimulus activates nociceptors which project action potentials down A^{δ} or C peripheral afferent fibres into the dorsal horn of the spinal cord. 2) These neurons synapse on an interneuron then a motor neuron, leading to reflex withdrawal. 3) Peripheral afferent fibres also synapse on dorsal horn neurons which project to higher areas of the central nervous system such as the thalamus and limbic system (4) and the somatosensory cortex (5). Adapted from McAlexander (2007).

Chronic pain is a widespread condition with an estimated 3.2 million Australians having experienced it in 2007 (Access Economics Pty Ltd, 2007). In contrast to acute pain, chronic pain interferes with the everyday life and wellbeing of sufferers, with many sufferers experiencing reduced work performance and employment outcomes (Access Economics Pty Ltd, 2007). Additionally, a high correlation with psychopathology such as depressive disorders has been reported, although the nature of the relationship between chronic pain and psychopathology is yet to be fully elucidated (Dersh et al., 2002). Given the individual effects of chronic pain, it has a large economic impact on Australian society. In 2007, chronic pain cost Australians an estimated $34.3 billion (Access Economics Pty Ltd, 2007).

The large individual and societal impact of chronic pain means that an effective treatment is much sought after. Although treatments such as non-steroidal anti-inflammatories, opiods and anaesthetics allow effective management of acute pain, these treatments do not effectively diminish chronic pain (Nitu et al., 2003). Moreover, a specific and effective treatment for chronic pain is yet to be found. A deeper understanding of the cellular and molecular mechanisms of chronic pain is needed to develop treatments that specifically target the dysfunctioning pathways.

1.2 A BRIEF INTRODUCTION TO THE CELL TYPES IMPLICATED IN CHRONIC PAIN

Many cell types are present in the central nervous system (CNS). The most numerous group of cells are glial cells, which is a cell group comprised of microglia, astrocytes, oligodendrocytes and ependymoglia. Glial cells outnumber the other major class of cell, neurons, ten to one (Stevens, 2003). In addition to glia and neurons, the CNS also contains endothelial cells, which line CNS blood vessels, and cells such as leptomeninges, which comprise the pia mater, and arachnoid lining, which covers the CNS. Of all these cell types, evidence is strongest for the involvement of neurons, microglia and astrocytes in chronic pain.

1.2.1 Neurons

An increased activity of nociceptive peripheral afferent and dorsal horn neurons has been suggested as the causative factor behind hyperalgesia and allodynia. Supporting this hypothesis are observations that nociceptive peripheral afferent neurons can develop spontaneous action potential firing (Liu et al., 2000) and become hyperexcitable (Campbell and Meyer, 2006; Millan, 1999) when injured or inflamed. In part, this is thought to be the cellular basis of hyperalgesia. Additionally, in such circumstances neurons that would normally fire action potentials to non-noxious tactile stimuli start to express mRNA for transmitters released by pain neurons (Neumann et al., 1996; Woolf and Salter, 2000). This phenomenon is thought to underlie allodynia. Recently, evidence has emerged that the hyperalgesia and allodynia which characterise chronic pain may not be due

solely to dysfunction of neurons but that glial cells, such as microglia and astrocytes, may contribute to the aforementioned maladaptive neuronal changes.

1.2.2 Microglia and astrocytes

Microglia comprise 5–10 % of the total amount of glial cells in the CNS (Vaughan and Peters, 1974). They are the immune surveillance cells of the CNS, recognising foreign, infected or dead cells and proteins. When such cells and proteins are detected, microglia become "activated" and destroy and phagocytose the foreign, infected or dead material. This function restricts the spread of disease and infection in the CNS (Streit, 1995). Due to this T-cell-like responsibility of microglia, their morphology varies with their functional state as demonstrated in Figure 1.2A. In a non-pathological environment, microglia are described as "resting" and have thin, spiny processes radiating from a central compartment. "Activated" microglia, which have been exposed to pathological tissue but have not become phagocytic, have thicker processes than resting microglia. Phagocytic microglia are spherical (Streit, 1995). When activated, microglia exhibit an upregulation of cell surface markers such as OX-42 and endothelin-1 (Taylor et al., 2003; Tsuda et al., 2003).

Astrocytes comprise the majority (60–65 %) of glial cells in the CNS (Peinado et al., 1998). Astrocytes have processes that surround both blood vessels and synapses (Peters et al., 1991; Ventura and Harris, 1999) and were originally thought to function only as homeostatic regulators, uptaking neurotransmitter, H^+ and K^+ ions to generate perfect conditions for neuronal action potential propagation

(Ballanyani, 1995; Deitmer, 1995). Over the last ten years, evidence has accumulated that astrocytes can also release transmitters, termed gliotransmitters, which can actively modulate neuronal transmission (Koizumi et al., 2003), can elicit waves of intracellular calcium increases in surrounding astrocytes (Cornell-Bell et al., 1990; Koizumi et al., 2002) and can cause release of substances from microglia (Bianco et al., 2005). Figure 1.3A shows that two basic types of astrocytes can be observed *in vivo*, based on morphological characteristics. Protoplasmic astrocytes are found in grey matter and have thin processes with extensive process branching, whilst fibrillary astrocytes are found in white matter and have thicker, less branched processes and a stellate shape (Duffy, 1983). Categorisation into protoplasmic and fibrillary astrocytes may be too simplistic since white matter astrocytes that resemble protoplasmic astrocytes have been described, as have astrocytes that have features of both protoplasmic and fibrillary types (Levison and Goldman, 1993). Similarly, *in vitro*, there are several types of astrocytes. For example, in 10-day-old spinal cord astrocyte cultures, there are three major types of astrocyte present - type A, which are flat, polygonal astrocytes; type G, which have a small cell body with lots of small processes; and type H, which are flat, disk shaped astrocytes (Black et al., 1993). These can be seen in Figure 1.3C.

The role of glia in chronic pain is evidenced with careful use of fluorocitrate. Fluorocitrate acts in the Krebs cycle to decrease the activity of aconitase, preventing the conversion of citrate to isocitrate. As neurons do not use extracellular citrate in the Krebs cycle, the direct effect of fluorocitrate is to selectively and reversibly

inhibit glial metabolism, effectively inactivating glia. The need for care in use comes at high doses and on prolonged exposure to fluorocitrate where it leads to irreversible glial changes which indirectly affect neuronal health (Milligan et al., 2003; Paulsen et al., 1987; Wieseler-Frank et al., 2005). Hyperalgesia is blocked when fluorocitrate is added at an appropriate dose and incubation time to selectively effect glia prior to the induction of inflammatory pain (Meller et al., 1994; Watkins et al., 1997). Also, when minocycline, a microglial activation inhibitor, is applied before the induction of neuropathic and inflammatory pain, the degree of hyperalgesia and allodynia is decreased (Ledeboer et al., 2005; Raghavendra et al., 2003).

It should be noted that in the majority of experiments, application of glial inactivators does not completely stop pain. Given the wealth of evidence for the involvement of neuronal dysfunction in hyperalgesia and allodynia, it is obvious that a variety of spinal cord cell types interact to bring about hyperalgesia and allodynia and that one type of cell cannot be completely responsible for the changes. It is not completely understood how glia and neurons interact to result in the changes seen in chronic pain. However, part of this process has recently been revealed with emerging evidence of some key molecular pathways involved in both the genesis and alleviation of chronic pain.

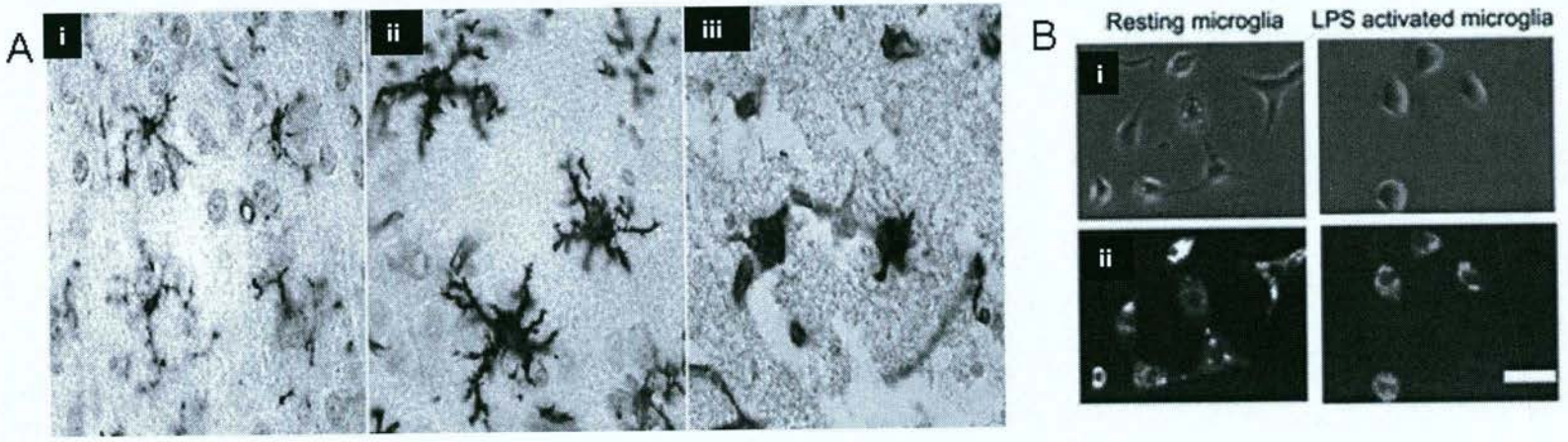


Figure 1.2 The morphology of microglia . A) *In situ* morphology of i) resting, ii) activated and iii) phagocytic cortical microglia stained by tomato lectin immunohistochemistry (see section 2.5 for details on immunohistochemistry). From Basu et al (2005). No scale was reported. B) *In vitro* morphology of resting and lipopolysaccharide (LPS) -activated spinal cord microglia as viewed by i) light microscopy and ii) immunohistochemical staining for cell marker isolectin B_4. The scale bar represents 20$^{\mu}$ m. Adapted from Liu et al (2006).

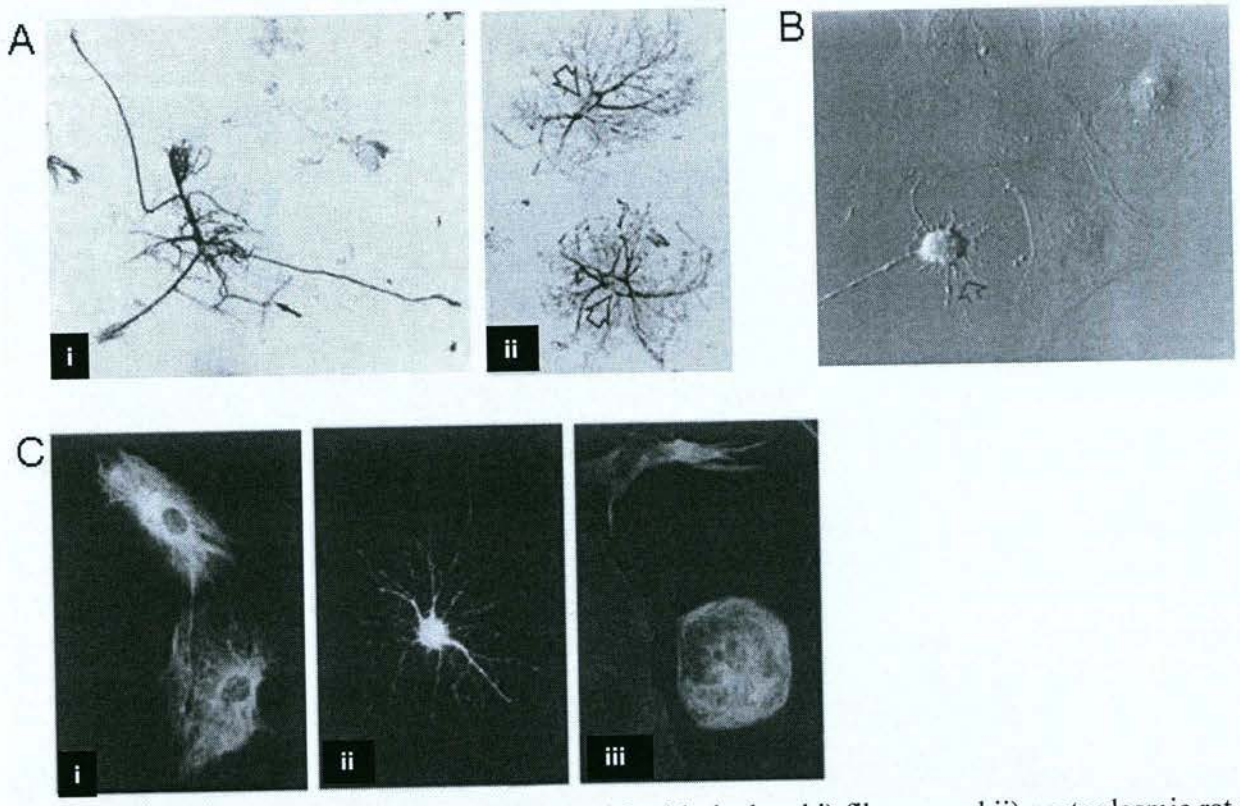


Figure 1. 3 The morphology of astrocytes. A) Morp hology of freshly isolated i) fibrous and ii) protoplasmic rat cortical astrocytes stained for glial fibrillary acidic protein (GFAP) by immunohistochemistry. The arrow in i) indicates the endfoot of an astrocytic process while the arrows in ii) indicate cell bodies (magnification is x1000). From Connor and Berkowitz (1985). B) *In vitro* morphology of rat spinal cord astrocytes as viewed by light microscopy. The arrow indicates a cultured astrocyte resembling an *in situ* fibrous astrocyte, while all other astrocytes resemble protoplasmic astrocytes. No scale was indicated. Taken from Rosewater and Sontheimer (1994)(Everett and Senogles, 2004; 1994). C) *In vitro* morphology of spinal cord astrocytes as viewed by immunocytochem ical staining for GFAP. Three types of cultured spinal cord astrocytes can be identified by immunocytochemical staining 10 days after initial culturing. These are type A (i), type G (ii) and type H (iii). x400. Taken from Black et al (1993).

1.3 MOLECULES IMPLICATED IN CHRONIC PAIN

Recent use of genetic techniques has shed light on some of the key molecules which may be involved in both generating and alleviating the symptoms of chronic pain like hyperalgesia and allodynia. Techniques such as receptor knock-down and knock-out have revealed a role for adenosine triphosphate (ATP) in the pathogenesis of neuropathic allodynia, neuropathic and inflammatory hyperalgesia, and gene therapy have revealed a role for interleukin-10 (IL-10) in alleviating the symptoms of chronic pain.

1.3.1 ATP

1.3.1.1 An introduction to ATP

ATP is a ubiquitous molecule and as illustrated in Figure 1.4A it consists of a ribose sugar with the purine base adenine attached to the 1' ribose carbon (the adenosine portion) and three phosphate groups attached to the 5' ribose carbon (the triphosphate portion). ATP acts on a large family of ionotropic and metabotropic purinergic receptors. Ionotropic purinergic receptors are referred to as P2X receptors and they are formed from a combination of 3 subunits. When a receptor is comprised of three subunits, it is termed a trimer. Each subunit of the P2X trimer has an intracellular N- and C-terminus connected to 2 hydrophobic transmembrane domains which are linked by a large extracellular loop as shown in Figure 1.4B. There are seven different receptor subunit proteins termed $P2X_{1-7}$ found throughout the CNS (Burnstock, 2007b). Apart from $P2X_6$, all can form homotrimer receptors in which all the subunits of the receptor are identical, for example a

$P2X_1$ homotrimer receptor is comprised of only $P2X_1$ subunits. Additionally, heteromeric forms of P2X receptors exist, where the receptor is comprised of several different subunits such as with $P2X_{2/3}$ receptors (Burnstock, 2007b). All P2X receptors are activated by ATP, leading to a flow of ions through the receptors. In addition to this when $P2X_7$ receptors are repeatedly activated, a pore forms allowing the flow of larger molecules such as a 629 Da nucleic acid stain (Surprenant et al., 1996).

Metabotropic receptors which respond to ATP are referred to as P2Y receptors and each receptor consists of just one subunit. Each subunit contains an extracellular N-terminus, seven transmembrane domains and an intracellular C-terminus as exemplified in Figure 1.4C. There are 8 types ($P2Y_{1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13,\ 14}$) (Fields and Burnstock, 2006). ATP effectively activates $P2Y_2$ and $P2Y_4$, while metabolites of ATP are more effective activators of other P2Y receptors. The first step in ATP metabolism is the removal of a phosphate group resulting in the nucleotide adenosine diphosphate (ADP) which activates $P2Y_{1,\ 12}$ and $P2Y_{13}$ receptors. Another nucleotide, uridine diphosphate, activates $P2Y_6$ and uridine diphosphate glucose activates $P2Y_{14}$ receptors (Burnstock, 2007b). P2Y receptors are coupled to intracellular signaling pathways, for example $P2Y_2$ and $P2Y_4$ receptors can activate phospholipase C (PLC)-β (see section 1.4.1.2.1 and Figure 1.8 for the PLC pathway).

Although within a cell ATP plays a key role in oxidative metabolism and without it cells would die, it is also released as an extracellular modulator by some cells. In the spinal cord, ATP has a number of roles as an extracellular modulator. The exercise pressor reflex is an increase in blood pressure and heart rate that occurs after static contraction of muscles. The first synapse in this reflex circuit is in the dorsal horn and ATP release from muscle afferent nerves at this synapse plays a key role in bringing about the exercise pressor reflex (Gao et al., 2005). ATP can also excite spinal motor neurons and elicit an initial increase then a delayed decrease in activity of the diaphragm-innervating phrenic motor nerves (Deng and Fyffe, 2004; Miles et al., 2002).

Additionally, ATP has been shown to excite neurons and glia in the first central pain synapse in the dorsal horn, suggesting a role for ATP in nociceptive pain transmission (Burnstock, 2007a; Fam et al., 2000). For example, ATP increases glutamate release from peripheral nociceptive afferents onto post-synaptic dorsal horn neurons, consequently increasing evoked excitatory post-synaptic currents in these dorsal horn neurons (Nakatsuka and Gu, 2001). Moreover, a role in not just acute pain transmission but chronic pain is suggested by studies showing application of 30 nmol ATP to the spinal cord elicits allodynia (Nakagawa et al., 2007). Additionally, one common method of administration of substances into the spinal cord is to inject them into the subarachnoid space. This is also referred to as an intrathecal injection. Intrathecal administration of a P2X agonist elicits hyperalgesia (Tsuda et al., 1999). Further evidence for the role of ATP in allodynia

and hyperalgesia has come with genetic and pharmacological targeting of the $P2X_4$, $P2X_7$ and $P2X_3$ subtypes of purinergic receptor.

1.3.1.2 The role of the $P2X_4$ receptor in neuropathic allodynia

The binding of ATP to the $P2X_4$ receptor on microglia appears to play a key role in the maintenance of allodynia after peripheral nerve injury. Allodynia can be completely reversed using an antagonist that blocks the $P2X_4$ receptor if intrathecally administered 7 days after injury, whereas allodynia isn't relieved with a broad spectrum P2X antagonist that doesn't block the $P2X_4$ receptor (Tsuda et al., 2003). Additionally, allodynia can be decreased by knockdown of the $P2X_4$ receptor through intraspinal administration of an antisense oligodeoxynucleotide (see Figure 1.5A). Antisense oligodeoxynucleotide is a small sequence of DNA complementary to a sequence of the $P2X_4$ mRNA, which when bound to the $P2X_4$ mRNA results in the inability of the $P2X_4$ subunit protein to be synthesized from its mRNA. Furthermore, the $P2X_4$ receptor is upregulated on dorsal horn microglia after injury but was not detected on neurons or astrocytes after injury (Tsuda et al., 2003). Although expression on oligodendrocytes was not examined, almost all $P2X_4$ staining was on OX-42 positive cells. As OX-42 is a cellular marker for microglia, this suggests that the site of action of ATP on $P2X_4$ receptors is microglia. Further supporting this is the observation that injection of $P2X_4$-stimulated microglia into the spinal cord results in allodynia (Tsuda et al., 2003).

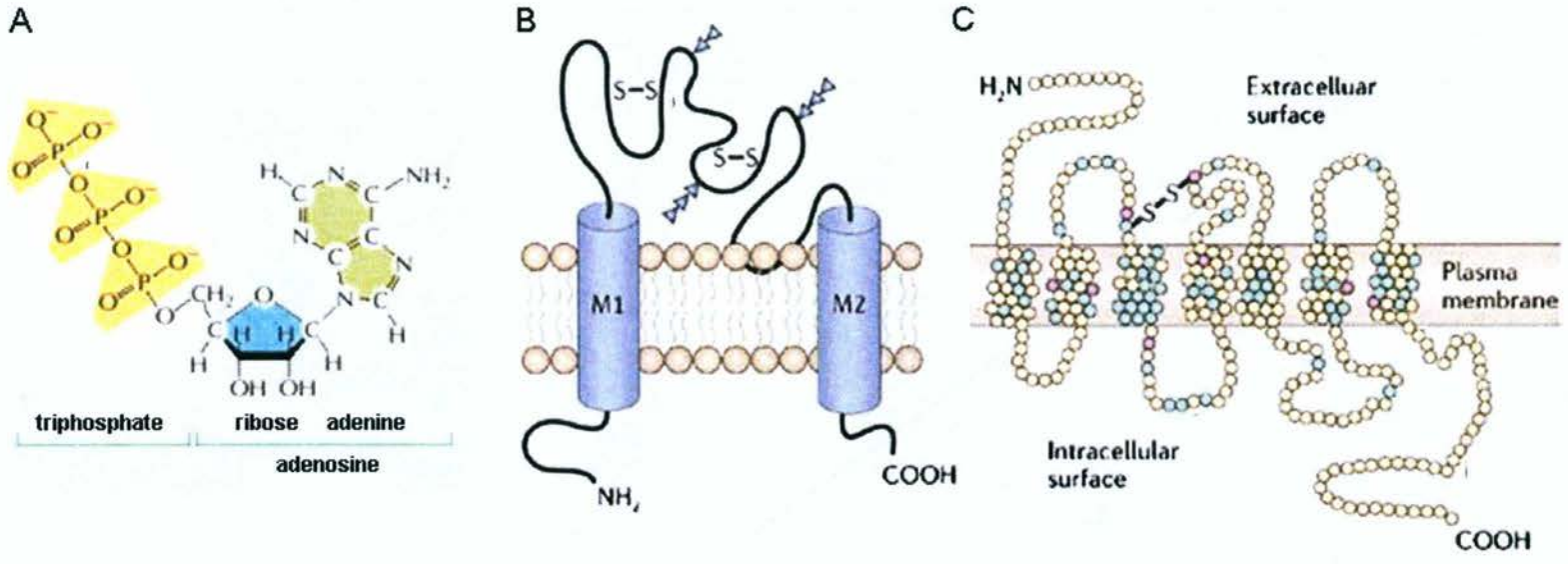


Figure 1.4 ATP and its receptors. A) The chemical structure of ATP made up of triphosphate, ribose and adenine. From Alberts et al (2002). B) A representation of the general structure of a P2X subunit. M1 and M2 indicate the two transmembrane segments. C) A representation of the general structure of a P2Y subunit. Adapted from Fields & Burnstock (2006).

One mechanism whereby binding of ATP to microglial $P2X_4$ receptors maintains allodynia is through production of brain-derived neurotrophic factor (BDNF). ATP-stimulated microglia release BDNF, and BDNF application to the spinal cord causes the neurotransmitter γ-aminobutyric acid (GABA) to produce depolarizing currents in lamina I neurons. This is in stark contrast to the usual hyperpolarizing effect of GABA on these neurons. This change in direction of the effect of GABA is commonly seen after nerve injury and may contribute to the hyperexcitability of nociceptive neurons after injury. This change is also accompanied by allodynia (Coull et al., 2005). When $P2X_4$-stimulated microglia are injected into the spinal cord and microglia are subjected to small interfering RNA (siRNA) specific for BDNF which interferes with BDNF production, allodynia and the change in polarization of GABA-induced currents is reversed. Additionally, allodynia resulting from neuropathy and $P2X_4$-stimulated microglial injection is prevented by application of an antibody to a receptor of BDNF (Coull et al., 2005).

The involvement of the $P2X_4$ receptor in chronic inflammatory pain and neuropathic hyperalgesia is unclear. Although one study has reported an increase in microglial expression of the $P2X_4$ receptor after formalin injection, one method of inducing inflammatory pain (Guo et al., 2005), results of other studies indicate the involvement of the $P2X_4$ receptor seems to be specific to neuropathic allodynia as no changes in $P2X_4$ expression are seen after injection of complete Freund's adjuvant, a diminished mycobacteria which induces inflammatory pain (Tsuda et al., 2003). Also, both inflammatory and neuropathic hyperalgesia can be

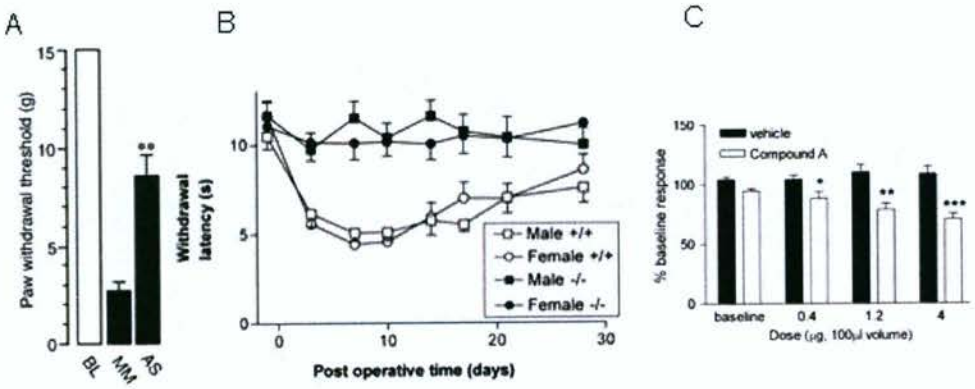
completely blocked by a $P2X_7$ gene deletion as discussed below (Chessell et al., 2005).

1.3.1.3 The role of the $P2X_7$ receptor in inflammatory and neuropathic hyperalgesia

It is unlikely that the $P2X_4$ receptor is the only purinergic receptor that ATP works on to contribute to chronic pain. Suramin is a poor antagonist at the $P2X_4$ receptor but a strong antagonist at most other P2X and P2Y receptors (excluding the $P2X_6$, $P2Y_4$ and $P2Y_6$ receptor) (Bo et al., 1995; Collo et al., 1996; Garcia-Guzman et al., 1997; Ralevic and Burnstock, 1998; Soto et al., 1996). Intrathecal application of suramin one day before induction of inflammatory hyperalgesia led to a decrease in hyperalgesia which lasted 4 weeks after cessation of suramin injection (Wu et al., 2004). Suramin is known to have inhibitory effects at non-purinergic receptors (Ralevic and Burnstock, 1998; Wu et al., 2004) and a portion of the observed inhibitory effect on hyperalgesia may be due to these non-purinergic actions, however a portion of the effects of suramin is likely due to effects on purinergic receptors since PPADS, an antagonist at all P2X receptors apart from $P2X_4$ and $P2X_6$ (Tsuda et al., 2003) had a similar effect on inflammatory hyperalgesia to suramin (Tsuda et al., 1999).

One non-$P2X_4$ microglial receptor that ATP may be working through in generating hyperalgesia is the $P2X_7$ receptor. Systemic and intraperitoneal application of specific $P2X_7$ receptor antagonists decreases the degree of tactile allodynia in

neuropathic pain (Honore et al., 2006; McGaraughty et al., 2007). Inflammatory and neuropathic hyperalgesia are compeletely removed in mice with a disrupted $P2X_7$ receptor gene, as shown in Figure 1.5B (Chessell et al., 2005). Peripherally, the $P2X_7$ receptor is found on cells such as monocytes, macrophages, lymphocytes, eosinophils, basophils, neutrophils, satellite cells and Schwann cells (Burnstock and Knight, 2004; Chessell et al., 2005). In the CNS, there is solid immunohistochemical evidence for the presence of microglial $P2X_7$ receptors (Sim et al., 2004). The presence of functional $P2X_7$ receptors on neurons and astrocytes, however, is controversial (Anderson and Nedergaard, 2006; Sperlagh et al., 2006). Antibodies used in previous immunohistochemical studies which identified $P2X_7$ receptors on neurons and astrocytes have been shown to be non-specific, positively staining these cells in $P2X_7$ knockout mice (Sim et al., 2004). By and large, pharmacological efforts to demonstrate functional $P2X_7$ receptors have been hampered by a lack of availability of specifc agonists and antagonists for the $P2X_7$, as well as the potential of agonists to be metabolized to adenosine (Anderson and Nedergaard, 2006). These factors make it difficult to identify which cell types contribute to the effect of $P2X_7$ gene disruption on allodynia and hyperalgesia and further work will be needed to accurately identify which cells have pain-related $P2X_7$ receptors. Although it is likely that peripheral cells play a role (Martucci et al., 2007), there is evidence for at least a partial role for the $P2X_7$ receptor on spinal microglia in the reported effects of gene deletion. After neuropathic hyperalgesia induction, PPADS administration in the spinal cord decreased levels of the chronic pain-inducing cytokine interleukin-1β (IL-1β; see


A
B
C
Paw withdrawal threshold (g)
15
10
5
0
BL
MM
AS
**
Withdrawal latency (s)
10
5
0
0
10
20
30
Post operative time (days)
Male +/+
Female +/+
Male -/-
Female -/-
% baseline response
150
100
50
0
vehicle
Compound A
*
**
***
baseline
0.4
1.2
4
Dose (µg, 100µl volume)

D

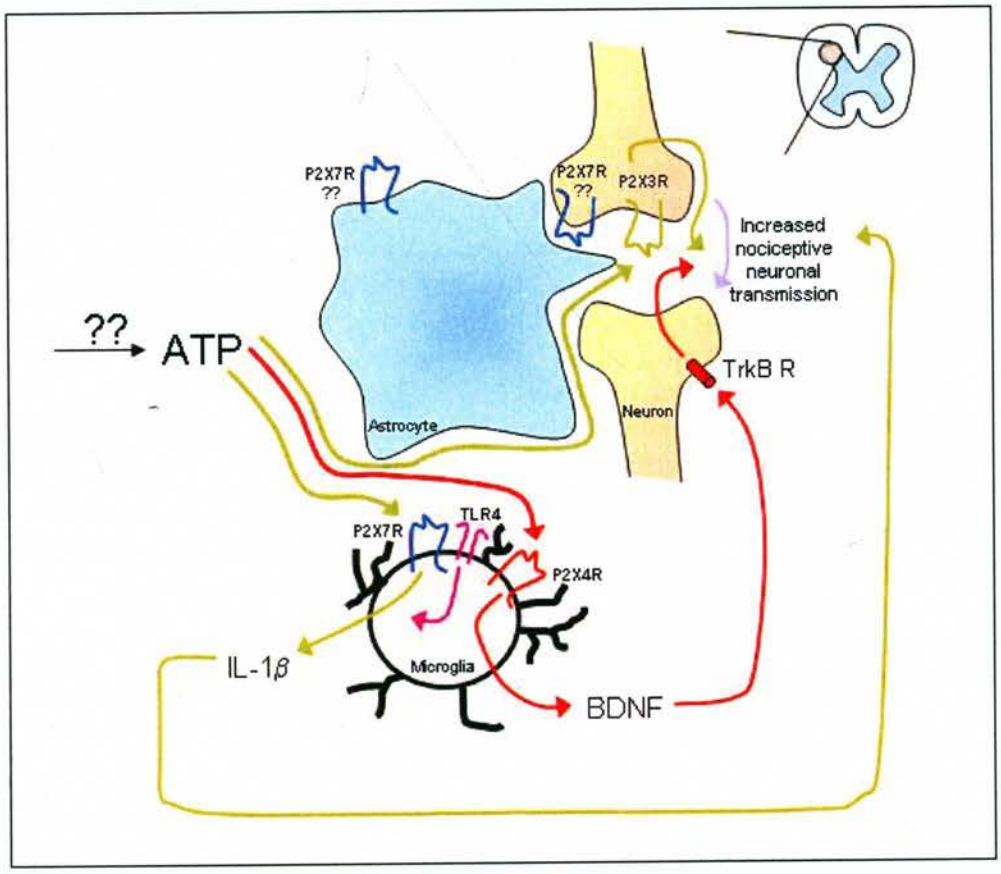

P2X7R
??
P2X7R
??
P2X3R
Increased nociceptive neuronal transmission
??
ATP
TrkB R
Astrocyte
Neuron
TLR4
P2X7R
P2X4R
IL-1β
Microglia
BDNF

Figure 1.5 Pathways of involvement of spinal ATP in chronic pain. A) A mouse with allodynia will withdraw its paw when subjected to a smaller amount of mechanical force (measured in g) than it would when in a non-allodynic state. The amount of force required to cause paw withdrawal before nerve injury (BL) was greater than that required after nerve injury (MM). When mice were treated with anti-sense oligodeoxynucleotide to the $P2X_4$ receptor (AS), nerve-injury induced allodynia was partially reversed. *$p<0.05$. Taken from Tsuda et al (2003). B) A mouse with hyperalgesia will remove its paw from a heat source faster than when in a non-hyperalgesic state. When mice were placed on a hotplate set at 50 – 55°C, male and female mice with functional $P2X_7$ receptors (+/+) displayed hyperalgesia after an operation that induced neuropathic damage. Male and female mice without $P2X_7$ receptors did not display this hyperalgesia when subjected to neuropathic damage. Initial recordings marked as post-operative day 0 were taken before neuropathy-inducing surgery. Taken from Coull et al (2005). C) Action potentials were electrically induced in Aδ fibres and the frequency of subsequent post-synaptic potentials was calculated. Responses of rats not subjected to further treatment (baseline) were set as 100%. A subpopulation of rats had either a vehicle liquid or a novel $P2X_3$ antagonist, compound A, applied to the spinal cord and subsequent frequency of electrically induced action potentials were given as a ratio of baseline values. It can be seen compound A significantly reduced the activity of Aδ fibres. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Taken from Sharp et al (2006). D) In the dorsal horn of the spinal cord, ATP from an unknown source can activate microglial $P2X_4$ receptors resulting in release of brain-derived neurotrophic factor (BDNF) which can activate its receptor TrkB. ATP may also activate microglial $P2X_7$ receptors (and possibly astrocytic and neuronal $P2X_7$ receptors). Activation of microglial $P2X_7$ receptors in conjunction with stimulation of microglial Toll-like receptor 4 (TLR4) results in interleukin-1β (IL-1β) release. ATP also activates neuronal $P2X_3$ receptors. The net result of this is increased nociceptive neuronal transmission. Pathways linked to neuropathic allodynia are indicated with red arrows and pathways linked to inflammatory and neuropathic hyperalgesia are indicated with green arrows.

section 1.3.2.1) and subsequently the degree of hyperalgesia displayed (Martucci et al., 2007). In neuropathic pain conditions, the Toll-like receptor 4 (TLR4, detailed in section 1.4.2.2.1) is activated (Tanga et al., 2005). To date, the only recorded spinal source of IL-1β in response to ATP is by $P2X_7$ stimulation of microglia that have had their TLR4s activated (Chessell et al., 2005; Sanz and Di Virgilio, 2000). This suggests IL-1β release from $P2X_7$-stimulated spinal microglia is important in the pathogenesis of hyperalgesia.

Recently, functional heterotrimeric $P2X_{4/7}$ receptors have been identified (Dubyak, 2007; Guo et al., 2007). It should be noted that ATP may not only bind to homotrimeric $P2X_4$ and $P2X_7$ receptors to bring about allodynia and hyperalgesia but also to these heteromeric receptors. It is yet to be determined if the evidence presented above results from ATP acting on the heterotrimeric or homotrimeric types of $P2X_4$ and $P2X_7$ receptors.

1.3.1.4 The role of the $P2X_3$ receptor in inflammatory and neuropathic hyperalgesia

A further P2X receptor subtype that may be involved in the pathogenesis of chronic pain is the $P2X_3$ receptor. The $P2X_3$ subtype is involved in the early stages of pain processing, being found both on peripheral and central (spinal cord) terminals of sensory neuronal afferents including nociceptive fibres (Chen et al., 1995; Jarvis et al., 2002; Lewis et al., 1995; Vulchanova et al., 1997). Additionally in the spinal cord, $P2X_3$ receptors are found in the dorsal commissure,

which is a tract involved in a visceral pain pathway, and also in the lateral collateral pathway of Lissauer with $P2X_3$ subunits found on cells projecting to visceral organs (Studeny et al., 2005). This subtype is also found in other areas of the CNS (Kukley et al., 2001; Seguela et al., 1996), however peripheral afferent and spinal cord $P2X_3$ receptors have been specifically linked to pain. Subcutaneous and intraplantar application of $P2X_3$ receptor antagonists decrease inflammatory hyperalgesia, highlighting a role for peripheral $P2X_3$ receptors (McGaraughty et al., 2003; Wu et al., 2004). A clear role for spinal $P2X_3$ receptors in neuropathic hyperalgesia is seen in a study involving intrathecal administration of siRNA to the $P2X_3$ receptor. This resulted in a decreased $P2X_3$ receptor expression in the spinal cord dorsal horn and subsequently reduced levels of neuropathic hyperalgesia (Dorn et al., 2004). Similarly, spinal administration of $P2X_3$ receptor antagonists had specific spinal cord neuronal effects, namely decreased Aδ– and C-fibre-evoked responses after neuropathic injury (Sharp et al., 2006), as seen in Figure 1.5C. Since the $P2X_3$ subunit can form heterotrimers such as the $P2X_{2/3}$ receptor and the antagonists used in the aforementioned studies block these heterotrimeric receptors, it is possible that both homotrimeric and heterotrimeric $P2X_3$-containing receptors play a role in chronic pain (Sharp et al., 2006). The pathways of involvement of spinal ATP in chronic pain are summarised in Figure 1.5D.

1.3.2 Interleukin-10 (IL-10)

1.3.2.1 The role of pro-inflammatory cytokines in chronic pain

The body responds to injury or disease with a series of cellular and vascular changes that can limit damage. This is called the inflammatory response. Cytokines are a group of diverse, small, soluble proteins which act to mediate inflammatory responses (Benveniste, 1992). Cytokines can be divided into two groups, namely, pro-inflammatory cytokines and anti-inflammatory cytokines on the basis of their effect on the inflammatory response. Pro-inflammatory cytokines start and maintain an inflammatory response whereas anti-inflammatory cytokines decrease the response (Lin, 2003). Three such pro-inflammatory cytokines are tumor necrosis factor-α (TNF-α), IL-1β and interleukin-6 (IL-6). In the CNS, these cytokines are released from a number of different cell types. The resident CNS immune cells microglia and astrocytes can release these cytokines. In response to substances involved in pain, both astrocytes and microglia are a source of TNF-α and IL-6 and microglia can also release biologically active IL-1β (Chao et al., 1992; Chauvet et al., 2001; Grimaldi et al., 1994; Lee et al., 2000; Martin et al., 1993; Millan, 1999; Sanz and Di Virgilio, 2000; Sawada et al., 1999; Tanga et al., 2005). Macrophages may also be a source of pro-inflammatory cytokines in the CNS in painful situations. Macrophages are phagocytic cells which do not natively reside in the CNS. After a peripheral nervous system injury, blood-bourne macrophages can infiltrate into the spinal cord and differentiate into microglia (Zhang et al., 2007a). Although virtually all macrophages differentiate into microglia (Zhang et al., 2007a), there is a possibility that prior to differentiation these infiltrating

macrophages can be a source of these cytokines in pain as they are known to release them (Benveniste, 1992). Finally, after peripheral inflammation, leptomeningeal cells which make up the meninges around the CNS, have been shown to release TNF-α (Wu et al., 2005).

The presence of spinal pro-inflammatory cytokines has been linked with chronic pain. Levels of TNF-α, IL-1β and IL-6 are known to increase in the spinal cord after induction of hyperalgesia and allodynia (DeLeo et al., 1996; DeLeo et al., 1997; Sweitzer et al., 1999). These cytokines can increase the efficiency of transmission of neurotransmitters involved in spinal nociceptive processing such as SP, glutamate and ATP (Millan, 1999). Specifically, IL-1β enhances the spinal release of SP (Malcangio et al., 1996), expression of the hyperalgesia-linked $P2X_7$ receptor (Narcisse et al., 2005) and increases N-methyl-D-aspartate (NMDA)-mediated Ca^{2+}-currents (Viviani et al., 2003). TNF-α can also make transmission via glutamate more efficient by increasing insertion of one of its receptors into neuronal membranes (Stellwagen et al., 2005). Additionally, these cytokines can further exacerbate pain states by increasing the release of each other, for example IL-1β and TNF-α increase IL-6 release from astrocytes (Aloisi et al., 1992) and IL-6 increases the release of TNF-α from microglia (Wanaka et al., 1997).

Considering the effects of these cytokines on nociceptive neurotransmission, it is not surprising that the introduction of IL-1β into the spinal cord is sufficient to produce hyperalgesia and allodynia (Reeve et al., 2000; Sung et al., 2004) and

introduction of IL-6 is sufficient to produce allodynia (DeLeo et al., 1996). Further, blocking spinal cytokine receptors with intrathecally administered antagonists during the induction of neuropathic hyperalgesia and allodynia (IL-1β, TNF-α, IL-6) and inflammatory hyperalgesia (IL-1β and not TNF-α) decreases the severity of these pain-related symptoms (Arruda et al., 2000; Milligan et al., 2003; Watkins et al., 1997). As such, one way to decrease the severity of chronic pain symptoms is to decrease the release of cytokines such as IL-1β, IL-6 and TNF-α by glia in the spinal cord and to combat the deleterious effects of released spinal cytokines.

1.3.2.2 An introduction to IL-10 and its anti-inflammatory actions

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, along with interleukin-4, interleukin-12, interleukin-13, interleukin-18 and interleukin-23 (Isomaki and Punnonen, 1997; Yamagata and Ichinose, 2006). IL-10 belongs to the helical structural group of cytokines, so named because the structure of cytokines in this class have right handed coils called α-helices collected in a bundle (Conklin, 2004). IL-10 is a helical cytokine which is encoded by a single gene and in the human, is made up of two polypeptide chains, each consisting of 160 amino acid residues. These chains are combined and intercalate in a dimer to produce a 36 kDa protein, as illustrated in Figure 1.6A and B (Donnelly et al., 1999; Zdanov, 2004). Rat IL-10 shares 73% of the human IL-10 amino acid sequence (Ball et al., 2001).

IL-10 exerts its cellular effects by binding the IL-10 receptor (IL-10R) which is found on cultured rat astrocyte and microglial cells in the CNS (Ledeboer et al., 2002). Additionally, although extensive studies identifying the presence of the IL-10R across a range of neuronal populations is yet to be carried out, the IL-10R has been identified on retinal ganglion cells where IL-10 increased the survival of retinal ganglion cells in culture (Boyd et al., 2003).

The IL-10R is a cell surface receptor consisting of 4 chains. The first two are high-affinity IL-10 binding helical α-chains that can recruit Janus Kinase 1 (JAK1) to the receptor complex. JAK1 is a type of protein tyrosine kinase, which are enzymes that can effect the function of a protein by phosphorylating it at a tyrosine residue. The second two chains in the IL-10R are accessory β-chains which can recruit tyrosine kinase 2 (Tyk2), another protein tyrosine kinase, to the receptor complex (Finbloom and Winestock, 1995; Ho et al., 1995; Moore et al., 2001). The first two chains are delineated IL-10R1 whilst the second two are commonly referred to as IL-10R2. Both chains are necessary for signal transduction (Kotenko et al., 1997). When IL-10 binds IL-10R1, a process demonstrated in Figure 1.6C, JAK1 and Tyk2 are phosphorylated. These proteins can then phosphorylate IL-10R1 which allow the transcription factors Signal Transducer and Activator of Transcription 1 and 3 (STAT1 and STAT3) to bind the receptor and be phosphorylated by JAK1 and Tyk2. In their phosphorylated form, the two STATs can translocate into the nucleus of cells and bind STAT binding elements of gene promoter sites, effecting the production of mRNA and protein

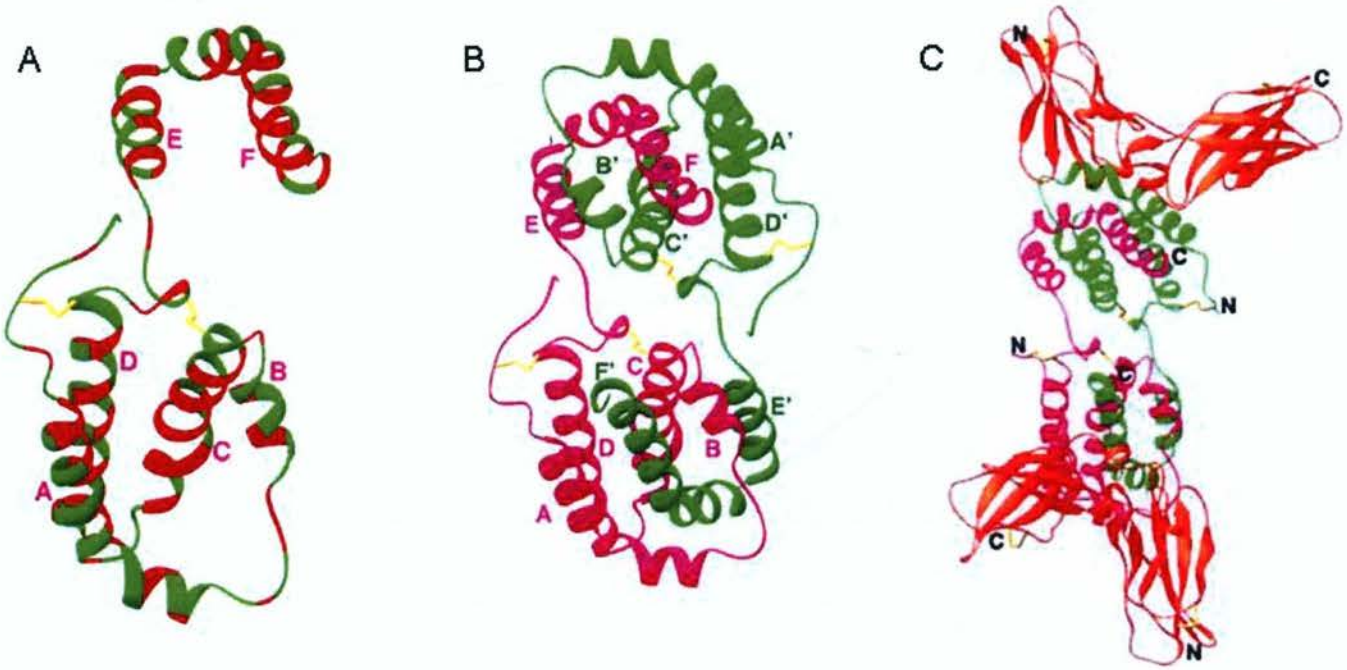


Figure 1.6 Structure of the IL -10 dimer and IL-10 receptor. A) Structure of one of the two polypeptide chains that compose the IL-10 dimer. The letters A to F indicate sections of the polypeptide chain that are classified as α -helices. Yellow segmen ts represent disulfide bonds. B) Structure of the IL -10 dimer showing how the two IL -10 polypeptide chains fit together. One subunit is in pink with the o ther in green. C) Representation of how the IL -10 dimer in green and pink interacts with the two IL-10R1 chains represented in red. N and C represent the amino and carboxy terminals of the indicated proteins respectively. Adapted from Zdanov (2004).

from specific genes (Moore et al., 2001). In microglia, binding of the IL-10 receptor induces STAT1 but not STAT3 (Zocchia et al., 1997).

IL-10 was formerly known as cytokine synthesis inhibitory factor as typically the net outcome of its effect on mRNA and protein production is to decrease the release and action of pro-inflammatory cytokines. A TLR4 agonist induces the release of IL-6 and TNF-α from microglia and astrocytes (Benveniste et al., 1995; Heyen et al., 2000; Kremlev and Palmer, 2005; Ledeboer et al., 2000; Mizuno et al., 1994; Sawada et al., 1999) and additionally IL-1β from microglia (Sawada et al., 1999) although this release can be quite inefficient compared to when a TLR4 agonist acts in conjuction with other transmitters (Ledeboer et al., 2002; Martin et al., 1993; Sanz and Di Virgilio, 2000; Sawada et al., 1999). The anti-inflammatory effect of IL-10 is exemplified by the finding that IL-10 greatly diminishes the amount of IL-1β, IL-6 and TNF-α stimulated by the TLR4 agonist from microglia and astrocytes (Benveniste et al., 1995; Heyen et al., 2000; Kremlev and Palmer, 2005; Ledeboer et al., 2000; Mizuno et al., 1994; Sawada et al., 1999). As suggested by its links with STAT, there is evidence that IL-10 works at the level of transcription to produce these anti-inflammatory outcomes by decreasing the activation of transcription factors and the level of TLR4-stimulated pro-inflammatory cytokine mRNA (Heyen et al., 2000; Ledeboer et al., 2002; Zhou et al., 2007).

Furthermore, the anti-inflammatory action of IL-10 is not limited to decreasing the release of pro-inflammatory cytokines in inflammatory conditions, but it also acts to

reduce the impact of pro-inflammatory cytokines that do get released. IL-10 exposure decreases the density of IL-6 receptors on microglia (Sawada et al., 1999) and decreases the ability of IL-1β to activate the intracellular signaling pathways linked to its receptors in astrocytes (Pousset et al., 2000).

1.3.2.3 Studies demonstrating the effects of IL-10 in chronic pain states

Given that numerous spinal pro-inflammatory cytokines contribute to allodynia and hyperalgesia, and that IL-10 can decrease the release and impact of these cytokines in inflammatory conditions, it is not surprising that spinal introduction of IL-10 can reverse allodynia and hyperalgesia in chronic pain states. Initial studies of the effect of IL-10 on chronic pain symptoms utilized intrathecal injections of IL-10 protein 10 days after induction of chronic constriction injury. These injections were found to partially reverse neuropathic mechanical allodynia and thermal hyperalgesia 4 days after administration (Milligan et al., 2005a). However, the effects were only transient with chronic pain symptoms returning to pre-injection levels within 24 hours of injection, probably explained by the observation that injected IL-10 had a half life of 2 hours in the spinal cord (Milligan et al., 2005a).

To combat this limitation and prolong the time IL-10 could be administered in the spinal cord, subsequent studies took advantage of the technique of gene therapy where DNA encoding the desired gene is administered into relevant parts of the body, resulting in the production of the desired protein. Intrathecally administered adeno-associated virus expression vectors containing rat IL-10 complementary

DNA (cDNA) predominantly infected the leptomeningeal cells, leading to the production and release of IL-10 into the spinal cord. The result of this was partial reversal of neuropathy-induced mechanical allodynia and thermal hyperalgesia. The effect diminished two to three weeks after administration when symptoms returned to pre-administration levels (Milligan et al., 2005a; Milligan et al., 2005b). In an attempt to extend the duration and amplitude of impact of intrathecal gene therapy, naked plasmid DNA encoding IL-10 was administered into the spinal cord without a vector. This led to a prolonged (> 40 days) and complete reversal of neuropathic- and paclitaxel (a cancer drug)-induced allodynia (Ledeboer et al., 2007; Milligan et al., 2006a). Due to inefficient uptake of plasmid, the drawback of these studies was that reversal of symptoms required numerous high doses of plasmid, which was estimated to equate to a dose of 290 mg for a 81 kg person. This limits the therapeutic usefulness of naked plasmid delivery (Milligan et al., 2006b).

The most successful example of the benefit of IL-10 in chronic pain states came from a study which used a synthetic polymer to deliver IL-10 DNA intrathecally. Complete reversal of neuropathic allodynia occurred for over 40 days with only two injections of much lower doses of DNA to those used in the naked DNA study. These effects are shown in Figure 1.7A (Milligan et al., 2006b).

Not only can exogenous IL-10 influence chronic pain symptoms, but there is evidence that endogenous IL-10 levels influence the experience of chronic pain.

Patients that experience a painful neuropathy have on average half the blood IL-10 mRNA levels than those with a painless neuropathy (Uceyler et al., 2007). Although this does not necessarily mean that endogenous spinal IL-10 levels are imperative, it does suggest endogenous IL-10 plays a role in abrogating the symptoms of chronic pain. The anti-inflammatory and anti-nociceptive actions of IL-10 are summarized in Figure 1.7B.

1.3.3 Other molecules implicated in chronic pain

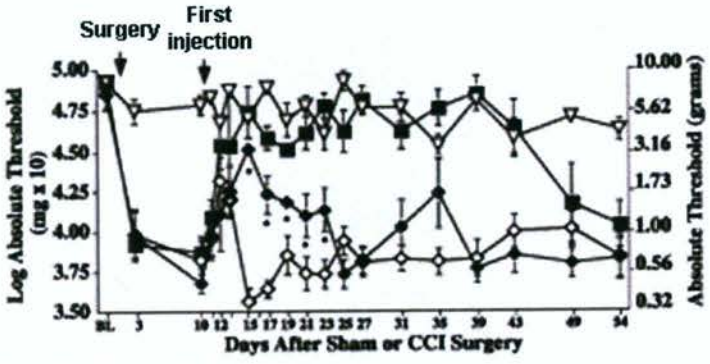
In addition to IL-10, pro-inflammatory cytokines, glutamate, ATP and its downstream mediator BDNF, a number of other molecular mediators are currently being explored as potential targets of chronic pain therapies. Subcutaneous injection of capsazepine, an antagonist to the transient receptor potential channel, vanilloid receptor 1 (TRPV1), produces a maximum 44% reversal of inflammatory hyperalgesia and 80% reversal of mechanical hyperalgesia in guinea pigs (Walker et al., 2003). Infusion of Na^+ channel blocker lidocaine results in a 45% reduction in mechanical allodynia in rats with neuropathic pain (Abdi et al., 1998). Gabapentin reduces neuropathic mechanical allodynia in rats by 27% through its inhibitory actions on voltage-gated Ca^{2+} channels (Abdi et al., 1998; Field et al., 2006). In addition, the synthetic cannabinoid WIN55,212-2 completely reversed neuropathic allodynia and hyperalgesia in rats for 20 min (Bridges et al., 2001).

When results of similar experiments targeting ATP and IL-10 are compared with the results of the aforementioned experiments targeting TRPV1, Na^+ channels and

Ca^{2+} channels, it can be seen that ATP and IL-10 may play a more central role in pain and analgesia than these other 3 potential molecular mediators. As highlighted in sections 1.3.1 and 1.3.2.3, interrupting the normal role of ATP in the spinal cord can completely prevent hyperalgesia (Coull et al., 2005) and introducing increased amounts of IL-10 in the spinal cord can completely reverse allodynia (Milligan et al., 2006b). Hence, the anti-chronic pain affects of targeting ATP and IL-10 in animals are much more powerful than targeting TRPV1, Na^+ channels and Ca^{2+} channels. Furthermore, in contrast to these latter three channels, a more central role of ATP can be conceptualized on the basis of the multiple pathways through which it may contribute to chronic pain, namely through the $P2X_3$ receptor, $P2X_4$ receptor and the $P2X_7$ receptor. Furthermore, ATP has demonstrated a contribution to both allodynia and hyperalgesia, whilst at present, evidence supports a role for TRPV1 primarily in hyperalgesia, and for Na^+ and Ca^{2+} channels primarily in allodynia.

Whilst targeting the endocannabinoid system can also completely reverse alldoynia and hyperalgesia in rats, numerous human clinical trials have shown that cannabis and synthetic cannabinoids either have no effect on the level of neuropathic pain experienced by patients or they are only as effective as codeine (Attal et al., 2004; Campbell et al., 2001). Furthermore these compounds produce unwanted side effects (Campbell et al., 2001). As such, the mediators ATP and IL-10 will be the selective focus of this thesis.

A


Surgery
First injection
Log Absolute Threshold (mg x 10)
Absolute Threshold (grams)
Days After Sham or CCI Surgery

B

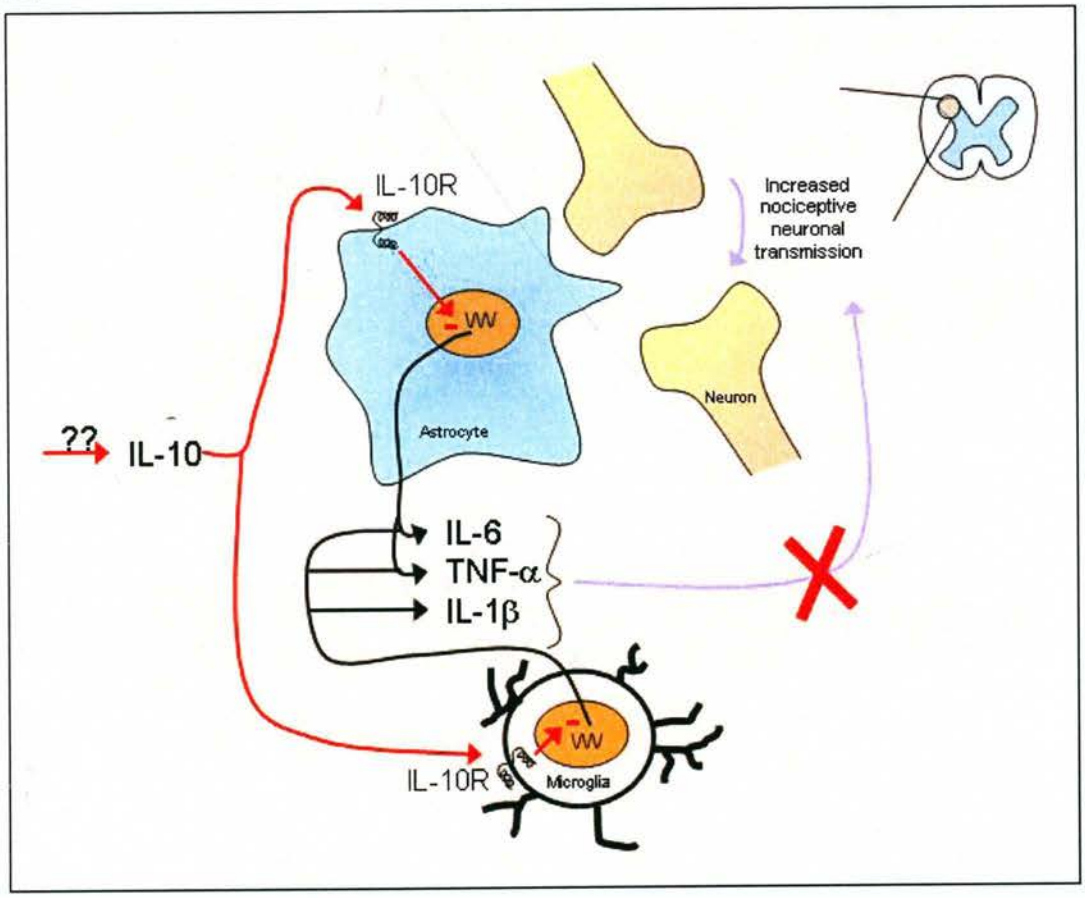

IL-10R
Increased nociceptive neuronal transmission
NW
Neuron
Astrocyte
??
IL-10
IL-6
TNF-α
IL-1β
NW
IL-10R
Microglia

Figure 1.7 The anti-nociceptive actions of interleukin-10 (IL-10). A) The amount of force that could be applied to a hindpaw before paw withdrawal was measured in rats subjected to a range of conditions. Black rectangles denote animals subjected to neuropathy (induced by chronic constriction injury or CCI) and subsequently injected twice with a synthetic polymer which delivers IL-10 DNA into the spinal cord. Black diamonds denote animals subjected to neuropathy and subsequently injected once with a synthetic polymer which delivers IL-10 DNA into the spinal cord. Empty diamonds represent animals subjected to neuropathy and injected twice with a synthetic polymer which did not deliver IL-10 DNA into the spinal cord. Empty triangles represent animals subjected to sham surgery which did not result in neuropathy and two subsequent injections of a synthetic polymer which did not deliver IL-10 DNA into the spinal cord. It can be seen that delivery of IL-10 DNA completely reverses neuropathic allodynia. BL = baseline. *$p<0.05$. Taken from Milligan et al (2006b). B) In the dorsal horn of the spinal cord, microglia and astrocytes release the pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (black arrows). These cytokines cause increased nociceptive neuronal transmission (purple arrows). Interleukin-10 (IL-10) derived from an unknown source decreases the release of these substances from glia by acting through the IL-10 receptor (IL-10R) and consequently reverses the cytokine-induced changes in neuronal transmission (red arrows, dashes and cross). The orange circle represents the nuclei of glial cells.

1.4 POSSIBLE SOURCES OF ATP AND IL-10 IN THE SPINAL CORD

In consideration of the evidence that ATP can contribute to neuropathic allodynia through spinal $P2X_4$ receptors and hyperalgesia through spinal $P2X_3$ and potentially $P2X_7$ receptors, knowledge of the sources of spinal ATP before and during chronic pain is needed to gain a greater understanding of the pathomechanisms of chronic pain. Whilst two possible sources of spinal ATP in chronic pain have been identified, it is possible that more sources exist. Additionally, given the evidence that spinal IL-10 is important in alleviating hyperalgesia and allodynia, knowledge of the endogenous spinal sources of IL-10 is important in understanding the endogenous protective mechanisms of the CNS in chronic pain. To date, however, it has not been examined which spinal cells release IL-10 in response to nociceptive substances. This present research aims to identify novel sources of ATP and IL-10 in the spinal cord in response to pain-related substances.

1.4.1 Possible sources of ATP in the spinal cord in response to pain-related substances

1.4.1.1 Documented sources of ATP in the spinal cord

Knowledge of spinal sources of ATP in response to pain-specific stimuli is limited. Release from dorsal horn neurons is a possible source and there are two studies documenting release from spinal cord glia. When dorsal roots of spinal cord slices are stimulated, many of the post-synaptic currents set up in the post-synaptic laminae II dorsal horn neurons are blocked by antagonists to glutamate, GABA and

glycine. However a subset of excitatory post-synaptic currents are blocked only by an ATP receptor antagonist (Bardoni et al., 1997), indicating that ATP is one transmitter released by afferents into the dorsal horn. It is not completely clear which neuronal fibre types are the source of this ATP and hence it is not completely clear whether this ATP release is in response to nociceptive stimuli. Lesion of C fibres does not effect depolarization-stimulated release of ATP in the dorsal horn, indicating these nociceptive fibres are not the source of ATP (Sawynok et al., 1993). Dorsal rizhotomy, which kills all afferent neurons, does not completely eliminate the release of ATP when synaptosomes are exposed to depolarizing K^+ concentrations, indicating some ATP is released from interneurons originating in the dorsal horn (Sawynok et al., 1993). Further support for this comes with the observation that stimulation of cultured dorsal horn neurons results in a co-release of both GABA, a common interneuron transmitter, and ATP (Jo and Schlichter, 1999). Hence, at the very least it appears dorsal horn interneurons are a source of ATP in the spinal cord subsequent to peripheral afferent action potentials. The ability of the non-nociceptive Aβ and the nociceptive Aδ fibres to release ATP can not be ruled out and is yet to be investigated. Hence, although neuronal ATP release is seen in the dorsal horn, the extent to which it occurs subsequent to painful stimulation is yet to be discovered.

There is evidence that glia may be a source of spinal ATP. In addition to the immune surveillance role outlined in section 1.2.2, microglia have recently been shown to respond to neurotransmitters. In particular, spinal microglia have been

shown to release ATP on exposure to glutamate (Liu et al., 2006). As glutamate release occurs in increased amounts in chronic pain conditions (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992), it is conceivable that microglia could be a source of ATP on induction of chronic pain. Furthermore, spinal cord astrocytes have been documented to release ATP on exposure to the $P2Y_2$ and $P2Y_4$ agonist uridine triphosphate (Abdipranoto et al., 2003). This suggests that when astrocytes are exposed to a source of ATP in the spinal cord they can release more ATP in response but does not shed light on the initial source of ATP in the spinal cord after nociceptive stimulation.

1.4.1.2 Glutamate-stimulated ATP release from cortical astrocytes

Apart from glutamate-stimulated ATP release from spinal cord microglia, UTP-stimulated ATP release from spinal cord astrocytes and potentially release from peripheral afferent neurons, there are no other reported sources of ATP release in response to pain-related compounds from spinal cells in the literature. It is important, however, to identify other potential spinal sources to get a clear idea of what could be contributing to the pain-problematic spinal pool of ATP.

One place to start in this exploration may be to identify which cells are known to release ATP in response to pain-related transmitters in other areas of the CNS and examine if this release also occurs in the spinal cord. Queiroz et al (1997) demonstrated that application of 1 mM of the neurotransmitter glutamate to cultured

cortical astrocytes led to a nineteen-fold increase in ATP release above basal level from these astrocytes. ATP release was obtained using glutamate concentrations as low as 100 μM. The receptors, intracellular pathways and secretion mechanisms that were involved in this process were investigated.

1.4.1.2.1 Receptors and intracellular pathways involved in glutamate-stimulated ATP release from cortical astrocytes

There are four known subtypes of glutamate receptors in the CNS, classified on the basis of their pharmacological, physiological and structural properties. The first subtype is the G-protein linked metabotropic glutamate receptor (mGluR). These exist as homodimers, which is unusual as G-protein linked receptors are commonly found as monomers (Abe et al., 1992; Aramori and Nakanishi, 1992; Pin et al., 2006). mGluRs are classified into three groups. Group I mGluRs activate phospholipase_C (PLC) (Pin and Duvoisin, 1995). Figure 1.8A shows this results in inositol triphosphate (IP_3) and diaclyglycerol (DAG) liberation from phosphatidylinositol-4.5-bisphosphate (PIP_2) and consequently increases in the intracellular level of Ca^{2+} from the opening of IP_3 gated Ca^{2+} stores. Group II and III mGluRs inhibit adenylyl cyclase (AC) and consequently inhibit the production of cyclic adenosine monophosphate (cAMP), as shown in Figure 1.8B (Pin and Duvoisin, 1995). There are eight subunits of mGluRs, namely mGluR1 up to mGluR8 classified on the basis of their protein structure and pharmacology. An example of their structure is shown in Figure 1.9A. Group I mGluRs consist of

subtypes mGluR1 and 5, group II mGluRs consist of mGluR2 and 3 and finally group III mGluRs consist of mGluR 4,6,7 and 8 (Pin and Duvoisin, 1995).

The remaining three subtypes of glutamate receptors are ionotropic receptors, which can depolarise the cell through the influx of ions and the activation of voltage-dependent Ca^{2+} channels, which open on depolarization, resulting in Ca^{2+}-influx. This further depolarises the cell and can activate Ca^{2+}-sensitive PLC (Haydon, 2001). The subunits of the ionotropic glutamate receptor subtypes share some features including subunits with an extracellular glutamate binding domain which binds glutamate in a manner akin to the motion of a venus fly trap, an intracellular C-terminal domain and a three-transmembrane segment domain which contains a fourth section that arches inside the membrane. This segment contributes to the formation of the ion channel (Madden, 2002). The general structure of the subunits of these ionotropic receptors is shown in Figure 1.9B.

One form of ionotropic glutamate receptor is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, encoded by 4 different genes named GluR1 through to GluR4. Ionotropic glutamate receptors encoded by GluR5 through to GluR7 and KA1 and KA2 are termed kainate (KA) receptors. Ion channels are comprised of subunits that have the same name as the gene that encodes them and subunits are approximately 100 kDa in molecular weight (Bettler and Mulle, 1995). Each receptor contains four or five subunits (Dingledine et al., 1999).

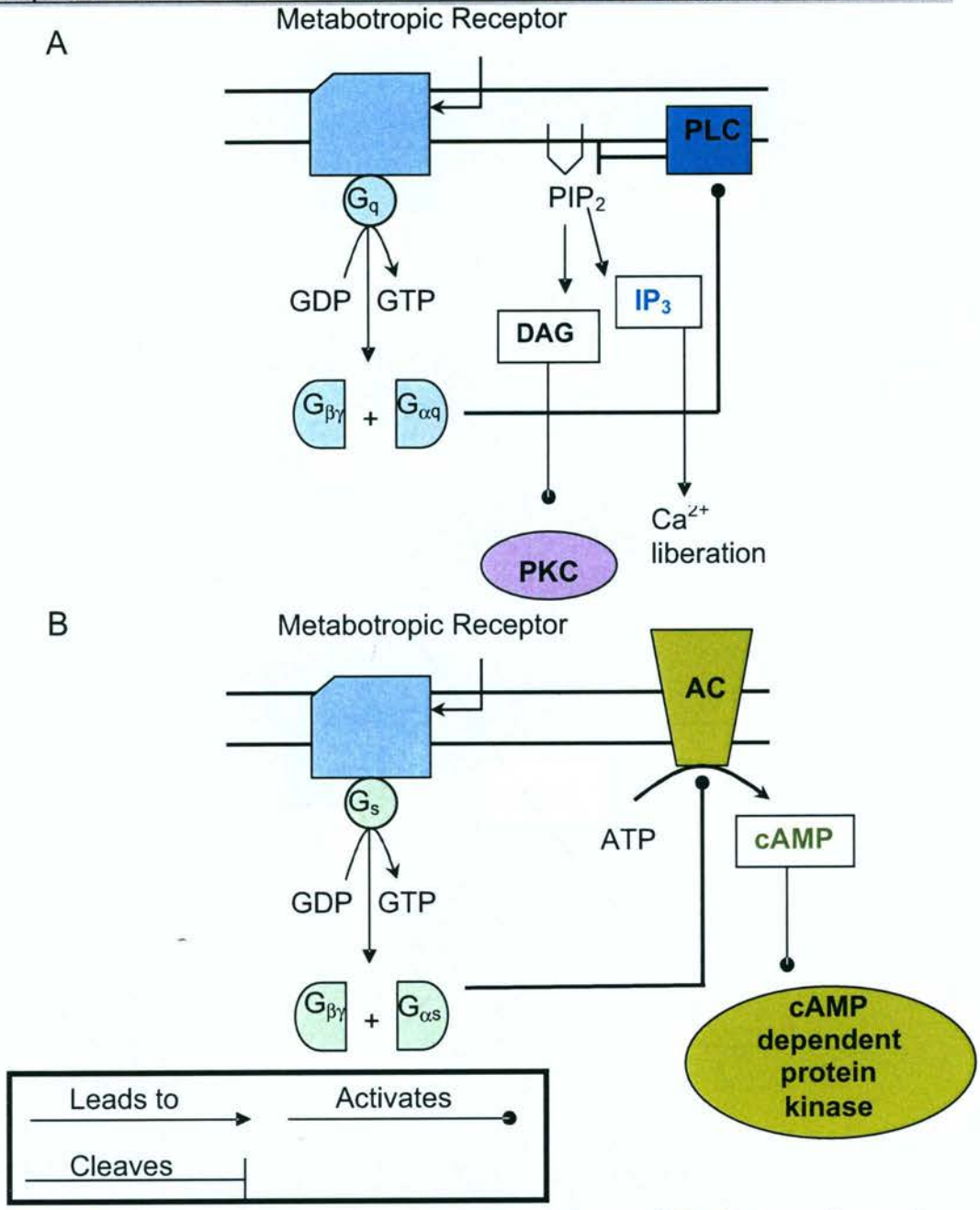


Figure 1.8 Common intracellular signaling pathways linked to metabotropic receptors. A) Phospholipase C intracellular signaling pathway. On binding of a G_q-protein coupled receptor, the G-protein cleaves and one of the products is $G_{\alpha q}$. This can then activate phospholipase C (PLC) which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2), resulting in diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 can open intracellular Ca^{2+} stores and DAG can activate protein kinase C (PKC). B) Adenylyl cyclase intracellular signaling pathway. On binding of a G_s-protein coupled receptor, the G-protein cleaves and one of the products is $G_{\alpha s}$. This can activate adenylyl cyclase (AC) which results in cyclic adenosine monophosphate (cAMP) production. This can then activate cAMP dependent protein-kinase.

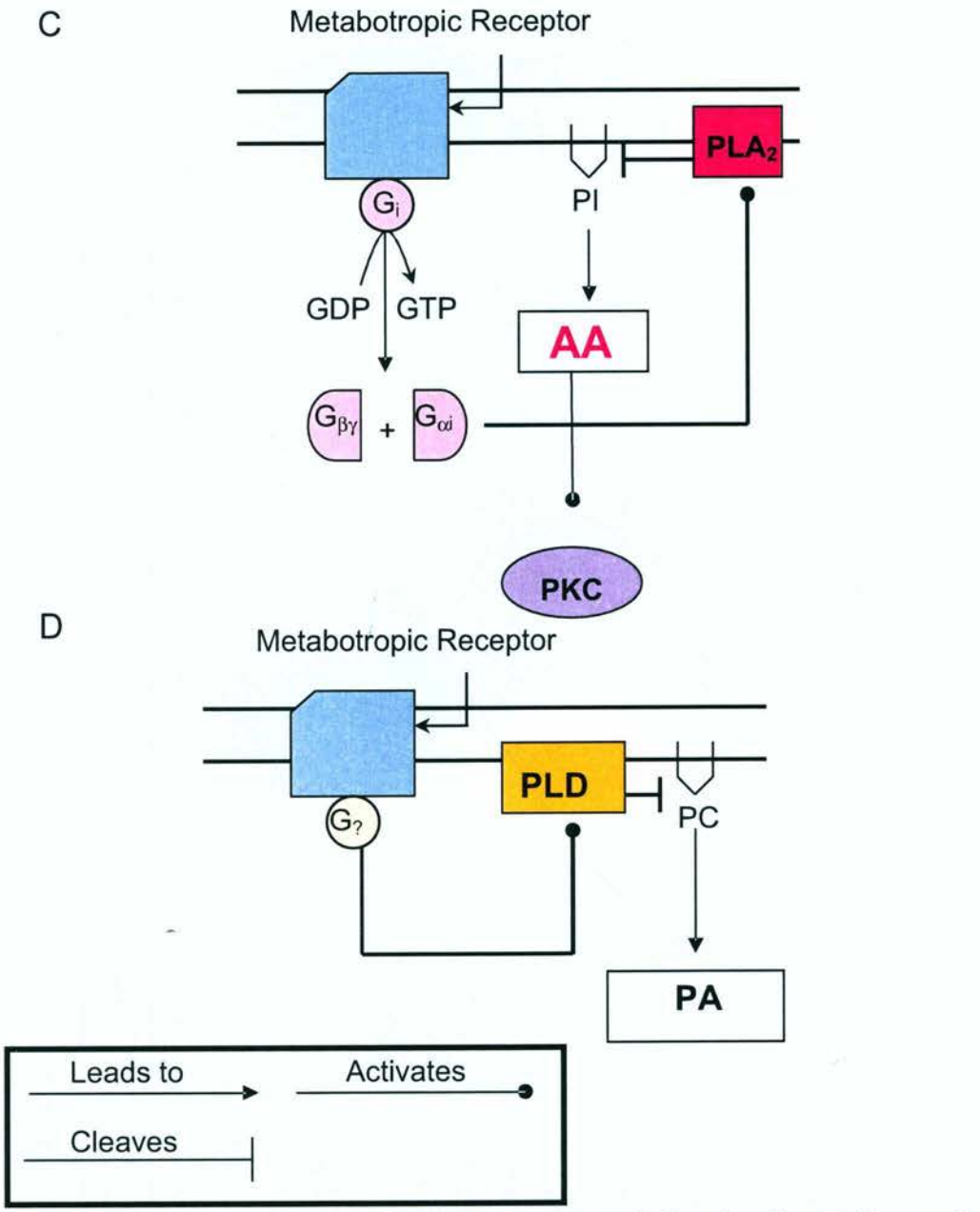


Figure 1.8 continued. C) Phospholipase A_2 intracellular signaling pathway. On binding of a G_i-protein coupled receptor, the G-protein cleaves and one of the products is $G_{\alpha i}$. This can then activate phospholipase A_2 (PLA_2) which cleaves phosphoinositide (PI), resulting in arachidonic acid (AA) release. D) Phospholipase D intracellular signaling pathway. On binding of a G-protein coupled receptor, a G-protein cleaves, which results in activation of phospholipase D (PLD) and subsequent cleavage of phosphatidylcholine (PC) to produce phosphatidic acid (PA). The nature of the G-protein that cleaves in the PLD pathway depends on the receptor and cell bound (for example Gayral et al (2006) and Everett and Senogles (2004)). This figure was adapted from Kandel and Schwartz (2000).

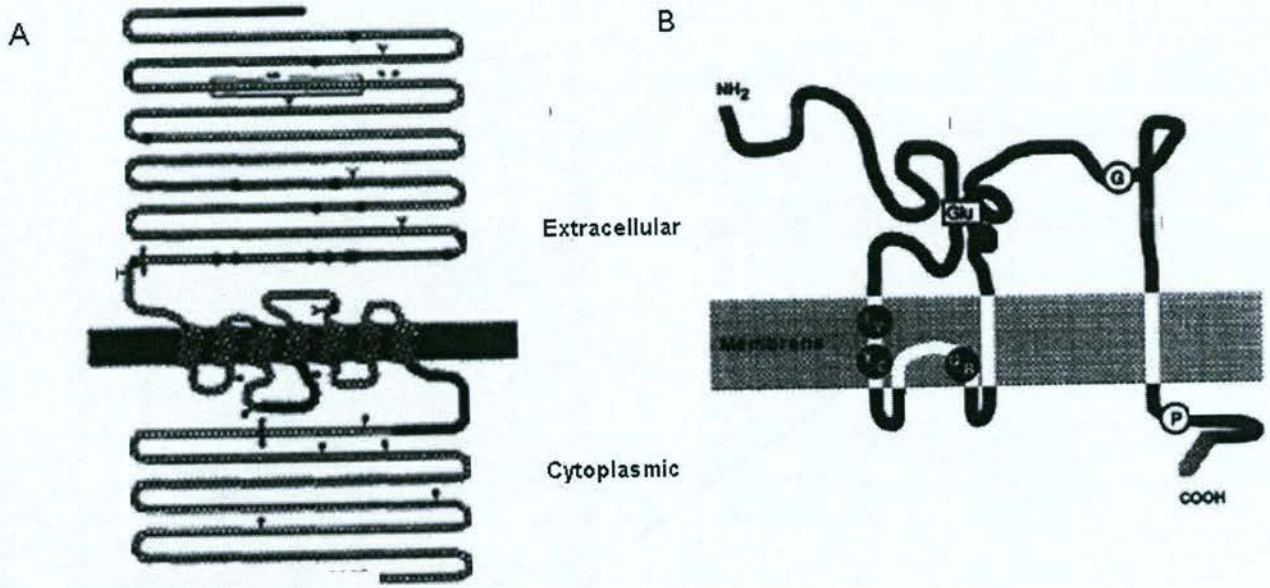


Figure 1.9 The structure of metabotropic and ionotropic glutamate receptors. A) The structure of a metabot ropic glutamate receptor 1 (mGluR1) subunit as an example of the structure of mGluR subunits. Note the large extracellular and intracellular domains and the seven transmembrane segments. The portions of the structure marked as black indicate amino acid r esidues that are conserved between all mGluR subunits. Taken from Pin & Duv oisin (1995). B) The general structure of ionotropic glutamate receptor subunits. Note the large extracellular domains and the three transmembrane domai ns with a further fourth segment which arches inside the membrane. Glu indicates the proposed site for glutamate binding, G indicates a proposed site for glycosylation of the GluR6 subunit and P indicates a proposed site for NR1 phosphorylation. The blac k circles labeled IV, YC and QR indicate sites of possible RNA editing. Taken from Bettler and Mulle (1995).

As their name suggests, AMPA receptors have a high affinity to the agonist AMPA, whereas kainate receptors have a high affinity to the agonist kainate. When glutamate or synthetic agonists bind AMPA or kainate receptors, they become permeable to ions such as Na^+ and K^+. These receptors are also permeable to Ca^{2+} ions, except AMPA receptors that contain the subunit GluR2 (Bettler and Mulle, 1995; Hollmann et al., 1991). A feature of AMPA and kainate receptors is their quick de- and re-sensitisation (Zorumski and Thio, 1992).

Another ionotropic glutamate receptor subtype, the N-methyl-D-aspartate (NMDA) receptor, named because of its affinity for the agonist NMDA, is a tetramer composed of a combination four out of seven different subunits, which are named NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B (Cull-Candy et al., 2001; Schuler et al., 2008). NMDA receptors are usually comprised of NR1/NR2 subunits or NR1/NR3 subunits. NMDA receptors comprised of NR1 and NR2 subunits require both glutamate to be bound to the NR2 subunit and a co-agonist such as glycine or a neutral amino acid like D-serine or D-alanine to be bound to the NR1 subunit for the receptor to be fully activated (Brugger et al., 1990; Johnson and Ascher, 1987; Laube et al., 1997; White et al., 1989) whilst receptors built of NR1 and NR3 subunits are activated by glycine alone (Chatterton et al., 2002). In addition, NMDA receptors can be comprised of a mixture of NR1, NR2 and NR3 subunits and these receptors have a reduced response to glutamate as compared to receptors composed of only NR1 and NR2 subunits (Das et al., 1998).

The NMDA channel differs from the AMPA and KA ionotropic glutamate receptors in several important ways. Firstly, in neurons the NMDA receptor is both ligand and voltage-gated. In neurons, it is blocked by a Mg^{2+} ion which is only removed after partial depolarisation of the cell, leading to the voltage-gated nature of the channel. Subsequent to depolarisation, glutamate or a similar agonist is needed to open the ion channel, exemplifying the ligand-gated nature of the channel. Once opened, the NMDA ion channel has a much higher Ca^{2+} permeability and results in a longer lasting depolarisation than the AMPA or KA receptors. Additionally, the NMDA receptor has a higher affinity for glutamate than the other ionotropic glutamate receptors (Mori and Mishina, 1995). The nomenclature for the glutamate receptor subtypes discussed above is summarised in Table 1.1.

There is evidence for the presence of all four types of glutamate receptors on freshly isolated (*in situ*) brain astrocytes. AMPA and KA expression (Conti et al., 1994; Petralia et al., 1994) and activity (Clark and Mobbs, 1992; Jabs et al., 1994; Porter and McCarthy, 1995) is highly documented in astrocytes in brain regions such as the hippocampus, retina, cerebellum and cortex. The most common AMPA subunits localised to these astrocytes are the GluR1 and 4 subunits, with less than 10% of astrocytes expressing the GluR2 and 3 subunits. This suggests that most astrocytes with AMPA receptors are of the Ca^{2+} permeable variety (Conti et al., 1994). There is also immunohistochemical (Aoki et al., 1994; Van Bockstaele and Colago, 1996) and functional evidence (Lehmann, 1987; Porter and McCarthy, 1995; Ziak et al., 1998) for NMDA receptors on *in situ* astrocytes. The mGluR3

and 5 subtypes of mGluR have been identified on astrocytes *in situ* (Cohen et al., 1968; Fotuhi et al., 1994; Romano et al., 1995), although there is evidence that the mGluR1, 2 and 4 subtypes are lacking in cortical astrocytes (Fotuhi et al., 1994; Martin et al., 1992; Shigemoto et al., 1992; Tanabe et al., 1993). To date, evidence for the presence of other *in situ* mGluR subtypes on brain astrocytes is lacking (Porter and McCarthy, 1997).

To isolate the glutamate receptors that were responsible for glutamate-stimulated ATP release, Queiroz et al (1997) applied 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX), a claimed AMPA specific antagonist and found it abolished glutamate's ability to stimulate ATP release. This observation in isolation seemed to suggest that glutamate-mediated ATP release was through AMPA receptors. However, they also found that NBQX decreased the magnitude of ATP release elicited by NMDA and kainate receptor stimulation, suggesting that NBQX also antagonised these receptors. Additionally, they found that agonists of all four glutamate receptors produced ATP release from cortical astrocytes (Queiroz et al., 1997; Queiroz et al., 1999). These observations suggest that all four glutamate receptor subtypes may be involved the release of ATP from cultured cortical astrocytes.

It was found that at high concentrations of glutamate (1 mM), the influx of extracellular Ca^{2+} through voltage-dependant Ca^{2+} channels was necessary for ATP release. Conversely, the blockade of voltage-dependant Ca^{2+} channels and external

Ca^{2+} availability at lower concentrations (300 μM) of glutamate did not reduce ATP release. This suggests the intracellular signaling pathway involved in glutamate-mediated ATP release depends on the concentration of glutamate that is in the vicinity of astrocytes. A possible explanation of this concentration dependency could be that different receptors mediate glutamate-elicited ATP release at different concentrations of glutamate. Queiroz et al (1999) found that AMPA-stimulated ATP release is not disrupted by removing extracellular Ca^{2+} and voltage-dependant Ca^{2+} channels function, whereas NMDA and KA-stimulated ATP release was. Hence, it may be that at lower concentrations of glutamate, ATP release subsequent to stimulation of the AMPA receptor is the predominant mechanism of ATP release, whereas at the higher concentration of 1 mM, the NMDA and KA subtypes are the predominant subtypes acted upon.

Queiroz et al (1999) found that LiCl significantly attenuated ATP release stimulated by glutamate, NMDA, AMPA and kainate. LiCl is an inositol monophosphatase inhibitor. Inositol monophosphatase dephosphorylates inositol monophosphate, resulting in the production of inositol. Levels of inositol are important for regenerating PIP_2, so in effect LiCl functions as a PIP_2-synthesis inhibitor and consequently reduces the amount of IP_3 and DAG generated prior to PLC activation (del Rio et al., 1998; Murray and Greenberg, 1997; Murray and Greenberg, 2000). Hence, this result suggests that ATP release from ionotropic glutamate receptor stimulation on cortical astrocytes involves activation of PLC.

Table 1.1 Nomenclature of glutamate receptor subtypes. Information from Bettler and Mulle (1995), Mori and Mishina (1995), Pin and Duviosin (1995) and Schuler et al (2008).

Metabotropic glutamate receptors

Subtype	*Group*	*Subunits in group*	*Intracellular signaling pathway name*
mGluR	I	mGluR1, 5	+ PLC/IP_3
	II	mGluR2, 3	- AC/cAMP
	III	mGluR4, 6, 7, 8	- AC/cAMP

Ionotropic glutamate receptors

Subtype	*Subunit composition*
AMPA	GluR1-4
Kainate	GluR5-7, KA1-2
NMDA	NR1, NR2A, NR2B, NR2C, NR2D, NR3A, NR3B

As some isoforms of PLC are Ca^{2+}-dependent (Haydon, 2001), this is congruent with the findings discussed above that NMDA and KA receptors bring about ATP release by creating an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, which would then activate PLC, resulting in ATP release. However, how PLC activation is evoked by AMPA, which was not found to use extracellular Ca^{2+} to bring about ATP release, is not clear. AMPA might bring about ATP release through activation of PLC by an unknown, Ca^{2+}-independent mechanism.

1.4.1.2.2 Secretion mechanisms involved in glutamate-mediated ATP release from cortical astrocytes

Work on cortical astrocytes suggests there are at least four methods of ATP release from these cells. These include release by cell lysis (Queiroz et al., 1999), exocytosis (Araque et al., 2001; Coco et al., 2003), transporter proteins and connexin hemichannels (Stout et al., 2002).

When the membrane of a cell becomes irreversibly permeable or lyses, molecules such as ATP can leave the cell. In this way, when an astrocyte lyses, ATP can be released (Queiroz et al., 1999).

Exocytosis is the Ca^{2+}-dependent (Nicholls, 1998) process by which vesicle-soluble N-ethylmaleimide-sensitive factor attachment receptor (v-SNARE) proteins on neurotransmitter-containing vesicles interact with target (t)-SNARE proteins on the membrane of a cell, causing the vesicle to fuse with the membrane. Consequently

the vesicle's contents are released into the extracellular space (Calakos and Scheller, 1996). A number of different types of vesicles have been found inside astrocytes which can undergo exocytosis – analogues of small synaptic vesicles which contain glutamate and other classical neurotransmitters (Calakos and Scheller, 1996), large dense core vesicles which contain ATP and other peptides (Coco et al., 2003) and lysosomes containing ATP (Zhang et al., 2007b). Astrocytic release of ATP by exocytosis of large dense core vesicles has been shown after stimulation with uridine triphosphate (UTP), a metabotropic ATP receptor agonist (Abdipranoto et al., 2003; Coco et al., 2003).

There are three types of transporters in astrocytes that are involved in allowing the passage of ATP from inside the cell to outside and all are members of the ATP binding cassette protein family (Queiroz et al., 1999). They are the cystic fibrosis transmembrane conductance regulator, which is also an ion channel, multidrug resistance-associated-proteins and P-glycoprotein. It has been shown that antagonising these ABC proteins decreases the amount of ATP released from astrocytes in response to mechanical stimulation and stimulation with UTP, the metabotropic purinergic receptor antagonist (Ballerini et al., 2002; Queiroz et al., 1999). However, it should be noted that the effects of the cystic fibrosis transmembrane conductance regulator on ATP efflux may only be indirect, as ATP does not appear to flow through the cystic fibrosis transmembrane conductance regulator on stimulation, despite the fact that antagonising the cystic fibrosis

transmembrane conductance regulator decreases ATP release after stimulation (Wu et al., 2001).

Proteins known as connexin hemichannels, which can join together to form a gap junction, have been suggested to have a functional role as an ion channel in their unjoined form. Evidence is mounting that inhibition of these hemichannels with octanol and flufenamic acid leads to decreased release of ATP from astrocytes after mechanical stimulation (Cotrina et al., 1998; Stout et al., 2002). However, doubt has recently been cast on this evidence as these hemichannel blockers have also been shown to block $P2X_7$ receptors (Suadicani et al., 2006), hence further work with more specific antagonists is needed to conclude about the role of connexin hemichannels in ATP release.

Queiroz et al. (1999) examined the role that some of these possible secretion mechanisms played in glutamate-mediated ATP release. There was no lysis of cells on exposure to glutamate, thus cell lysis was not an explanation of ATP release. They also found that inhibiting the CFTR reduced glutamate-stimulated ATP release by 23%, which was not a significant difference from the uninhibited state. On the other hand, (Pangrsic et al., 2007) used a fluorescent dye to image vesicles in astrocytes that contained ATP co-localised with atrial naturetic peptide and observed these vesicles underwent exocytosis on exposure to glutamate. Presumably these vesicles were large dense core vesicles, given their peptidergic contents. In further support of an exocytotic release from astrocytes, Zhang et al

(2007b) found that on exposure to glutamate, ATP-containing lysosomes underwent partial exocytosis and selective lysis of lysosomes abolished glutamate-stimulated ATP release from cultured cortical astrocytes. The effect of hemichannel disruption on glutamate-stimulated ATP release from cortical astrocytes has not been examined.

1.4.1.2.3 Glutamate uptake and ATP degradation: the final steps in glutamate-stimulated ATP release from cortical astrocytes

The final steps in glutamate-stimulated ATP release are removal of extracellular glutamate and degradation of ATP. Excess glutamate in the extracellular space must be removed. Found on the surface of murine astrocytes are the glutamate and aspartate transporter (GLAST) and the glutamate transporter (GLT) (Rothstein et al., 1994). These proteins uptake glutamate into astrocytes across a Na^+ gradient, with a stoichiometry of one glutamate molecule uptaken per three Na^+ ions that enter the astrocyte (Anderson and Swanson, 2000). Astrocytes can also uptake glutamate in a Na^+-independent manner through chloride-dependent glutamate/cystine antiporters, although this uptake only accounts for less than five percent of total glutamate uptake (Cho and Bannai, 1990; Tsai et al., 1996). When uptaken glutamate has entered the astrocyte, it is metabolised to the amino acid glutamine by glutamine synthetase, an enzyme found exclusively in astrocytes (Martinez-Hernandez et al., 1977). Neurons also express GLAST along with three other glutamate transporters, referred to as excitatory amino acid transporter 3, 4 and 5. The excitatory amino acid transporter 4 is primarily found on neurons in the

cerebellum and the excitatory amino acid transporter 5 is primarily found on neurons in the retina (Furuta et al., 1997; Pow and Barnett, 2000; Rothstein et al., 1994). These transporters, however, only clear an insignificant fraction of the total glutamate cleared from the extracellular space (Anderson and Swanson, 2000). Furthermore, under pathological conditions such as ischaemia, the excitatory amino acid transporter 3 can reverse its normal operation and lead to release of glutamate into the extracellular space (Rossi et al., 2000)).

When ATP is released from an astrocyte, it is degraded to ATP by ecto-ATPase and then to AMP by ecto-ADPase and finally to adenosine by ecto-nucleotidase (Trapido-Rosenthal et al., 1990). Ecto-ATPases are found on the same areas of the cell surface from which ATP is released from astrocytes (Joseph et al., 2003), although not all released ATP is immediately decomposed, as some released ATP can diffuse to effect other cells (Fam et al., 2003; Koizumi et al., 2003; Scemes et al., 2000).

The work reviewed above and summarised in Figure 1.10 suggests that glutamate-stimulated ATP release from cultured cortical astrocytes is possibly mediated by all four subtypes of glutamate receptor, leading to both extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels and activation of the PLC/PIP_2 second messenger system. The result of this is ATP release through exocytosis of lysosomes and large-dense core vesicles. Glutamate is subsequently taken up by

GLAST and GLT and ATP is degraded by ecto-ATPase, ecto-ADPase and ecto-nucleotidase.

1.4.1.3 Potential of spinal cord astrocytes to release ATP in response to glutamate

Given that a focus of this present research is to identify novel sources of spinal ATP, it is appealing to hypothesise glutamate-stimulated ATP release from spinal cord astrocytes as a potential source of ATP in the spinal cord for numerous reasons. Firstly, glutamate is the most common excitatory neurotransmitter and is released in the dorsal horn in increased amounts during chronic pain (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992). Astrocytes are ideally positioned to be exposed to this synaptically released glutamate as their processes enwrap synapses (Ventura and Harris, 1999). In addition to this, there are more astrocytes in the CNS then there are neurons or other glial cell types suggesting that they could be a large contributor to the total pool of ATP if they were to release ATP to glutamate stimulation (Peinado et al., 1998; Stevens, 2003). Finally results from cortical astrocytes suggest astrocytes could be a powerful source of ATP compared to other cell types. Glutamate-stimulated ATP release from cortical astrocytes was a much more dominant source of ATP than glutamate-stimulated cortical neuronal ATP release as mixed astrocyte-neuronal cultures containing 10% of neurons did not differ in the amount of glutamate-stimulated ATP release than those with the same amount of astrocytes but further neurons added to increase the total percentage of

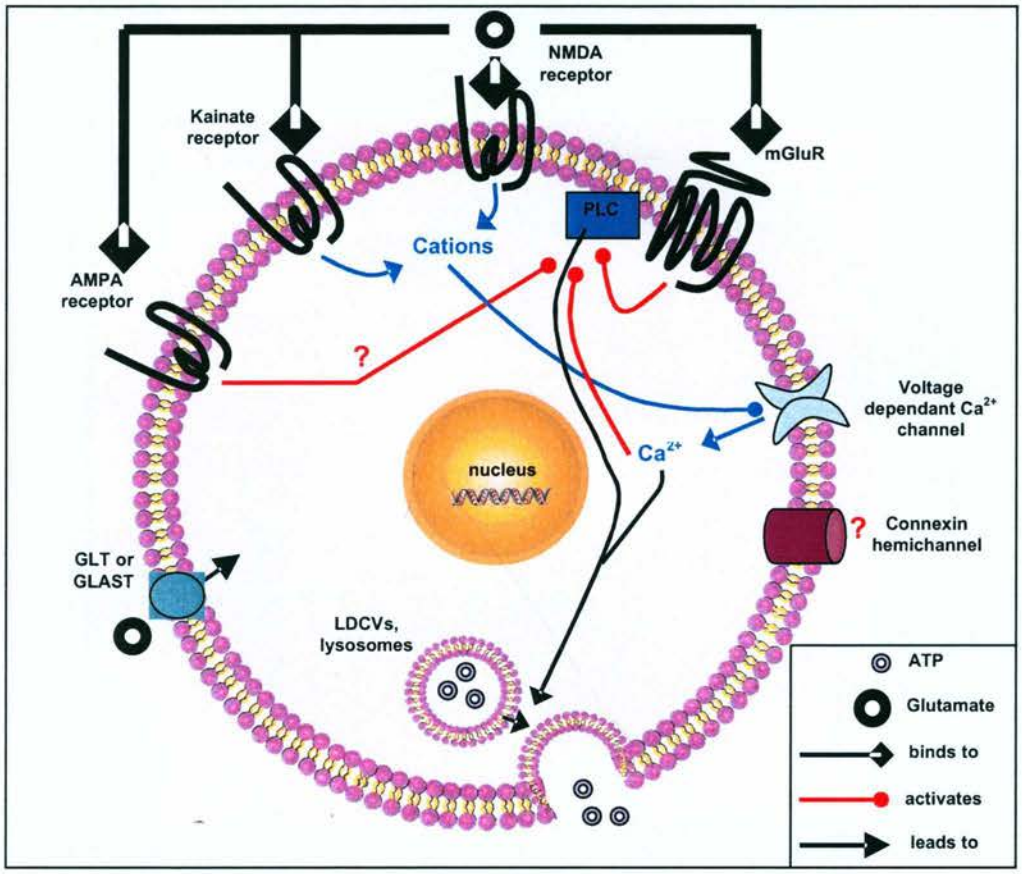


Figure 1.10 Mechanisms of glutamate-stimulated ATP release from cortical astrocytes. Glutamate (1 mM) brought about ATP release by binding to N-methyl-D-aspartate (NMDA), kainate, metabotropic (mGluR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. On glutamate binding, cations entered the cell through NMDA and kainate receptors, and this subsequently activated voltage dependent Ca^{2+} channels, causing further Ca^{2+} influx. The Ca^{2+} ions then activated phospholipase C (PLC). PLC was also activated when glutamate bound mGluRs and AMPA receptors, although the mechanism by which AM PA receptor binding leads to PLC activation is unknown. The result of PLC activation was ATP release by exocytosis of lysosomes and large dense core vesicles (LDCVs). Extracellular glutamate was uptaken by the glutamate transporter (GLT) and the glutamate and aspartate transporter (GLAST).

neurons to 30% (Queiroz et al., 1999).

It cannot be assumed that because astrocytes from the cortex display glutamate-stimulated ATP release, that the same will be true for spinal cord astrocytes because a regional heterogeneity amongst astrocytes has been reported. There are numerous examples of this. SP can stimulate phosphoinositide turnover and prostaglandin release in spinal cord astrocytes but not in cortical astrocytes (Cholewinski et al., 1988; Marriott et al., 1991). Conversely, the neuropeptides vasopressin and oxytocin can stimulate phosphoinositide turnover in cortical but not spinal cord astrocytes (Cholewinski et al., 1988). A β-adrenergic agonist can increase cAMP in spinal cord and cortical astrocytes, however the hormone somatostatin blocks this response from cortical astrocytes but does not block it from spinal cord astrocytes (Cholewinski and Wilkin, 1988). Finally, another hormone, thyrotropin releasing hormone, causes phosphoinositide turnover in spinal cord but not cortical astrocytes (McDermott et al., 1992). This raises the question of what evidence exists to suggest glutamate may be able to stimulate ATP release from spinal cord astrocytes. Spinal cord astrocytes have some of the cellular machinery needed for glutamate-stimulated ATP release. For example immunocytochemical experiments have shown cultured spinal cord astrocytes express group I and II mGluRs (Silva et al., 1999) and *in situ* spinal cord astrocytes express NMDA receptors and the GluR1, 4 and 5 sub-units of the AMPA and kainate receptors (Aicher et al., 1997; Brand-Schieber et al., 2004). Additionally, as mentioned above, spinal cord astrocytes are able to release ATP to another transmitter, UTP (Abdipranoto et al., 2003).

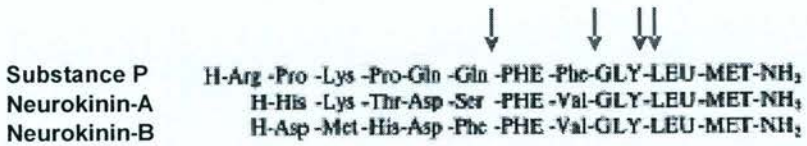
Exposure of spinal cord astrocytes to glutamate results in an increase in intracellular Ca^{2+} and, as mentioned in section 1.4.1.2.1, intracellular Ca^{2+} elevations are involved in the process of ATP release (Ahmed et al., 1990; Queiroz et al., 1999). This demonstrated ability of spinal cord astrocytes to release ATP and to have receptors to respond to glutamate suggests that glutamate-stimulated ATP release may be a viable process in spinal cord astrocytes. Given the appealing potential of spinal cord astrocytes to release ATP to glutamate, *one objective of the current research is to explore whether glutamate can stimulate ATP release from spinal cord astrocytes and if release is found, to explore the mechanisms involved.*

1.4.1.4 The potential for SP to be involved in ATP release from spinal cord astrocytes

Glutamate is co-released with other neurotransmitters at many CNS synapses so that when glutamate is released by neurons and binds to astrocytes, these other transmitters will also be present in the extracellular environment surrounding astrocytes (Millan, 1999). This opens up the possibility for these simultaneously released transmitters to be potential modulators of glutamate-stimulated ATP release, if not releasing agents themselves. The neurotransmitter SP is found co-localised with glutamate in spinal cord terminals of nociceptive fibres (De Biasi and Rustioni, 1988) and although only a small amount of these fibres have been reported to contain proteins required for loading of glutamate into exocytotic vesicles and for the exocytosis of these vesicles, SP-containing fibre terminals are localized directly next to fibres that contain both glutamate and the proteins

required for its exocytosis (Morris et al., 2005). Consequently upon noxious stimulation both glutamate and SP are simultaneously released in laminae I, II, IV and V of the dorsal horn (Allen et al., 1999; Sakamoto and Atsumi, 1989; Sasaki et al., 1998; Womack et al., 1988). In the spinal cord SP and glutamate are also released together in the interomediolateral column and the ventral horn bringing about increases in blood pressure and motor neuron excitability respectively (Helke et al., 1982; Hokfelt et al., 2000; Kapoor et al., 1992; Nicholas et al., 1992). SP is a peptide containing 11 amino acid residues (Maggio, 1988) and once released SP is degraded by a range of enzymes including angiotensin converting enzyme and neutral endopeptidase 24.11 (Borson, 1991; Khawaja and Rogers, 1996; Skidgel et al., 1984) which cleave SP at the sites indicated in Figure 1.11A. Prior to deactivation, however, SP is known to bind at least three distinct receptors. These are the neurokinin-1 (NK1), neurokinin-2 (NK2) and neurokinin-3 (NK3) receptors (Saria, 1999). A fourth receptor was proposed in the human (Krause et al., 1997) however this receptor has since been shown to have a very similar pharmacology and amino acid composition to the human NK3 receptor and a search for a separate gene for this fourth receptor has failed, suggesting this fourth receptor is actually a variant of NK3 (Krause et al., 1997; Sarau et al., 2000). NK1, NK2 and NK3 receptors exist as monomers that contain seven transmembrane domains with an extracellular amino terminal and an intracellular carboxy terminal as illustrated in Figure 1.11B and differ in the residues indicated in the figure (Khawaja and Rogers, 1996; Meyer et al., 2006). Of these three neurokinin receptors, SP has the highest

A



B

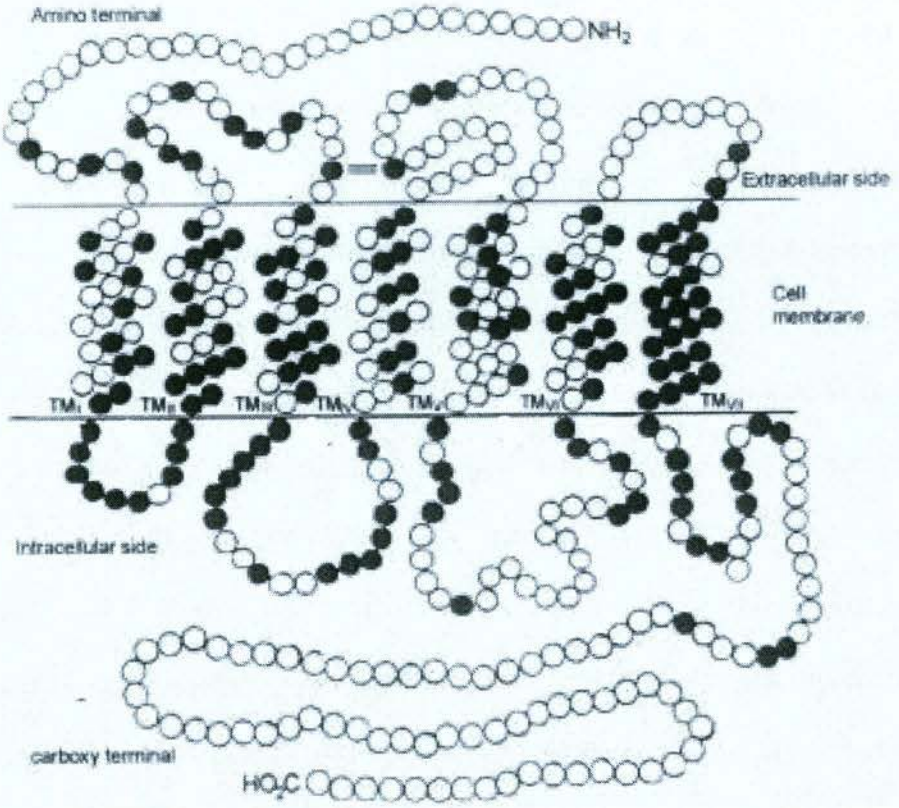


Figure 1.11 Structure of substance P and its receptors. A) The amino acid composition of substance P. Blue arrows indicate the bonds which are cleaved by neutral endopeptidase 24.11 and the red arrows indicate the bonds which are cleaved by angiotensin converting enzyme. The amino acid compositions of neurokinin-A and neurokinin-B are also displayed, with amino acids common to these neuropeptides indicated in capitals. Adapted from Maggio (1988). B) The secondary structure of the neurokinin-1 receptor as an example of the structure of neurokinin receptors. The amino acid residues conserved between the three subtypes of neurokinin receptor are indicated by black circles. TM $_{1\text{-}7}$ denotes the seven transmembrane segments of the receptor. Adapted from Khawaja and Rogers (1996).

relative affinity to the NK1 receptor and primarily exerts its biological effects through this receptor (Harrison and Geppetti, 2001) although it will act on all neurokinin receptor classes (Mussap et al., 1993; Regoli et al., 1994). Two peptides with similar amino acid sequences to SP (see Figure 1.11A) are neurokinin-A and neurokinin-B and these have a higher affinity than SP at the neurokinin-1 and neurokinin-2 receptors respectively (Cascieri et al., 1992). There is both immunohistochemical and functional evidence that astrocytes in the murine spinal cord express all three types of SP receptor (Palma et al., 1997; Wienrich and Kettenmann, 1989; Yashpal et al., 1990; Zaratin et al., 2000; Zerari et al., 1997; Zerari et al., 1998). Consequently, when SP is released at the same time as glutamate in the spinal cord, the receptor machinery is present for SP to bind to astrocytes.

All three neurokinin receptors are G-protein coupled receptors which are known to stimulate the PLC/IP_3 signaling pathway, the phospholipase A_2 (PLA_2)/arachidonic acid pathway, the cAMP/AC pathway and the phospholipase D (PLD) pathway (Garcia et al., 1994; Montuschi et al., 1996; Nakajima et al., 1992; Regoli et al., 1994; Yau et al., 1991). By acting through these pathways, which are summarised in Figure 1.8, SP has been shown to cause membrane potential depolarisation (Wienrich and Kettenmann, 1989), phosphoinositide turnover, prostaglandin release (Hartung et al., 1988; Marriott et al., 1991) and increases in intracellular Ca^{2+} levels (Heath et al., 1994) in astrocytes from the spinal cord. Some of these events, such as phosphoinositide turnover (Abdipranoto et al., 2003) and increases in

intracellular Ca^{2+} concentration (Queiroz et al., 1999), have been shown to increase ATP release from astrocytes. Hence, when SP and glutamate are co-released from neurons onto astrocytes, not only will glutamate bind to astrocytes to effect them, but SP can potentially bind to astrocytes and bring about changes linked with ATP release, allowing the possibility that the effects of SP on astrocytes may include either release of ATP itself or interference with the process of glutamate-stimulated ATP release.

SP has been postulated to be a modulatory peptide for a range of neurotransmitter systems. For example, Bourque and Robitaille (1998) found that SP on its own caused increases in intracellular Ca^{2+} in perisynaptic Schwann cells. Conversely, when combined with muscarinic acetycholine receptor agonists or ATP, SP reduced the percentage of cells that responded to these agonists. SP has also been shown to potentiate noradrenaline-induced cAMP activation in astrocytes (Rougon et al., 1983) and enhanced NMDA-induced currents in spinal cord dorsal horn neurons (Randic et al., 1990; Rusin et al., 1993).

Considering this evidence, it can be seen that SP is co-released with glutamate onto spinal astrocytes that have both SP and glutamate receptors, SP has been shown to bring about changes in astrocytes linked to ATP release and has been shown to modulate the response to neurotransmitters in glial cells and spinal cord cells. Put together, these suggest that SP is a promising neurotransmitter to examine in the search for either a substance to elicit ATP release from spinal astrocytes or a

potential endogenous modulator of glutamate-stimulated ATP release. *Hence, a further objective of this present research will be to examine if SP elicits ATP release from spinal cord astrocytes or if it modulates any observed glutamate-stimulated ATP release from spinal cord astrocytes.*

1.4.2 Possible sources of IL-10 in the spinal cord in response to pain-related substances

1.4.2.1 Documented sources of IL-10 in the brain and evaluation of candidates for sources of the spinal pool of IL-10 in chronic pain

Apart from discovering novel sources of ATP in the spinal cord, the second main aim of this present research is to identify novel sources of IL-10 in the spinal cord in chronic pain. Although it has not been investigated which cells in the spinal cord release IL-10, release of IL-10 has been shown from a number of cell types in other areas of the CNS. One way to generate candidate spinal stimulating substances and cellular sources of IL-10 may be to review what is known about IL-10 release in these non-spinal areas of the CNS. The most studied cellular sources of IL-10 in the CNS are astrocytes and microglia. In the brain, they have been recorded to release IL-10 on exposure to a range of agents. One group of such agents are pathogens. Parasites, such as the abortion-causing Neospora caninum, elicit IL-10 release from astrocytes (Pinheiro et al., 2006), neonatal Borna disease virus, which causes chronic infection of neurons elicits IL-10 release from mixed glia (Ovanesov et al., 2006) and bacteria such as Borrelia burgdorferi and Neisseria meningitidis elicit IL-10 release from both microglia and astrocytes (Rasley et al., 2006).

Athough the most common result of exposure to pathogens is an increase in IL-10 release from these glia, some pathogens such as Japanese encephalitis virus decrease IL-10 release levels from microglia (Swarup et al., 2007).

Pathogens have molecular structures on their surface which are invariant. These pathogen-associated molecular patterns are recognized by Toll-like receptors on cells of the mammalian innate immune system (Cristofaro and Opal, 2006). Thirteen different Toll-like receptors (TLR) have been described in mice and eleven in humans. Agonists to two different types of Toll-like receptors also elicit IL-10 release from glia. A TLR4 agonist stimulates the release of IL-10 from microglia and astrocytes of the brain (Ledeboer et al., 2002; Mizuno et al., 1994; Seo et al., 2004; Williams et al., 1996) and when stimulated with a TLR3 agonist such as double stranded RNA or double stranded messenger RNA, astrocytes taken from the subcortical white matter release IL-10 (Alexopoulou et al., 2001; Bsibsi et al., 2006; Kariko et al., 2004). Double stranded RNA can be released extracellularly on lysis of virus-infected cells (Majde et al., 1998).

Binding of infiltrating T-cells to microglia results in IL-10 release (Chabot et al., 1999). Infiltration of T-cells can occur in blood-brain barrier breakdown or in diseases such as multiple sclerosis (Chabot et al., 1999). Finally, cytokines such as TNF-α and IL-6 can elicit IL-10 release from human brain microglia (Sheng et al., 1995). Interestingly, on induction of peripheral inflammation, the level of IL-10 increases in the CNS near microglia and astrocytes

close to leptomeningeal cells (Wu et al., 2005). When exposed to peripheral inflammation leptomeningeal cells were shown to release TNF-α, possibly explaining why adjacent cells such as microglia showed an increased staining for IL-10 (Wu et al., 2005).

Other non-glial cells have been documented to release IL-10 in the brain. Cultured brain endothelial cells have been shown to spontaneously release IL-10 on their luminal side and hence could be a basal source of IL-10 in the brain (Verma et al., 2006). Little is known about how pain-related substances effect the secretion of IL-10 by brain endothelial cells, however an agonist of the TLR4 does not significantly increase brain endothelial cell-stimulated IL-10 release (Verma et al., 2006).

As mentioned in section 1.3.2.1, after the induction of peripheral neuropathic pain blood-borne macrophages can infiltrate into the spinal cord and differentiate into microglia. Peripheral macrophages secrete IL-10 in the blood both spontaneously and on exposure to a TLR4 agonist (Hagiwara et al., 1995; Kim et al., 2004). It is not known if macrophages infiltrating into the spinal cord secrete IL-10 in a similar manner before they differentiate into microglia.

If all the brain cellular sources described above were to be sources of IL-10 in the spinal cord, it is probable that microglia and astrocytes would play a more dominant role in chronic pain than endothelial cells and macrophages. Although endothelial

cells are in contact with perivascular microglia, endothelial cells are located quite a distance from the majority of the pro-inflammatory cytokine releasing microglia and hence any potential release of IL-10 may be limited in its effectiveness in preventing pro-inflammatory cytokine release. Also macrophages are only transiently in the spinal cord during chronic pain before they differentiate into microglia (Zhang et al., 2007a), temporally restricting the impact of any possible IL-10 release. On the other hand, if spinal microglia and astrocytes could be shown to release IL-10 this may have a large impact in chronic pain as it presents the opportunity for IL-10 to act in an autocrine manner to shut down the action of pro-inflammatory cytokines released by these cells. This makes it appealing to investigate if microglia and astrocytes may be a source of IL-10 in the spinal cord.

Of the factors documented to stimulate IL-10 from brain microglia and astrocytes, most pertinent to the situation of chronic pain are reports that an agonist for the TLR4 can increase the production of IL-10 from microglia and astrocytes. Pathogens, T-cells or extracellular dsRNA/mRNA in the spinal cord occur in infection and certain disease states and it would not be expected that these IL-10 inducers would be present in a person whose only pathology is chronic pain (Chabot et al., 1999; Majde et al., 1998). In contrast, the TLR4 is known to be activated in neuropathic pain (Tanga et al., 2005). It has not been investigated whether it is also activated in inflammatory pain. Additionally, although IL-6 and TNF-α are present in painful situations, at least in neuropathic hyperalgesia and allodynia their production appears to be subsequent to TLR4 stimulation as TLR4

antisense oligodeoxynucleotide administration completely removes the increase in mRNA of these cytokines seen on induction of neuropathic pain (Tanga et al., 2005). Consequently, any effect on IL-10 levels that IL-6 and TNF-α may have in neuropathic pain will occur after effects of TLR4 stimulation.

Taking into consideration the above-mentioned possible autocrine anti-inflammatory mechanism and the early TLR4 activation in neuropathic pain, it is appealing to hypothesise that TLR4-stimulated IL-10 release from microglia and astrocytes could be a source of IL-10 in the spinal cord in chronic pain. What is known about TLR4-stimulated IL-10 release is detailed below in order to explore whether spinal cord astrocytes and microglia possess any of the proteins known to be involved in the process of TLR4-stimulated IL-10 release from glia and other cells in other areas of the CNS and body.

1.4.2.2 TLR4-activated IL-10 release from brain microglia and astrocytes

The TLR4 agonist lipopolysaccharide (LPS) stimulates the release of IL-10 from human, mouse and rat brain microglia (Ledeboer et al., 2002; Mizuno et al., 1994; Seo et al., 2004; Williams et al., 1996) and from rat brain astrocytes (Ledeboer et al., 2002). In microglia, LPS concentrations of 0.1 – 1 μg/ml applied for 24 h elicits an accumulation of IL-10 in the extracellular medium that is 9 to 11 times higher than that which accumulates in the medium without stimulation (Ledeboer et al., 2002; Mizuno et al., 1994; Seo et al., 2004). The amount of accumulated IL-10 continues rising with an increased time of exposure to LPS and

even at 96 h of exposure the level of IL-10 continues to accumulate (Seo et al., 2004). The amount of accumulated IL-10 in the medium of cortical astrocytes stimulated with 0.1 μg/ml of LPS for 24 h rises 17-fold above the unstimulated amount. The timecourse of release from astrocytes has not been mapped (Ledeboer et al., 2002). Details of the TLR4 and intracellular mechanisms of TLR4-stimulated IL-10 release from immune cells have been investigated.

1.4.2.2.1 The Toll-like receptor 4 and its agonists

The TLR4 is a complex receptor with both an intracellular and extracellular domain and exists as a homodimer as can be seen in Figure 1.12A. As mentioned in section 1.4.2.1, Toll-like receptors respond to certain types of pathogen. The pathogen that the TLR4 responds to is gram-negative bacteria (Takeuchi et al., 1999). In addition, this receptor also responds to a range of other endogenous and exogenous substances. Gangliosides are a gylocophospholipid-containing component of mammalian cell-membranes. Gangliosides can be released into the extracellular space for example after exposure to oligomeric amyloid-β protein (Matsuzaki and Horikiri, 1999; Michikawa et al., 2001). Gangliosides have been shown to activate glial TLR4s leading to an upregulation of cytokines such as IL-1β and TNF-α (Jou et al., 2006). Biglycan, a proteoglycan rich in leucine which accumulates in the culture medium of astrocytes also signals through the TLR4 (and TLR2) leading to the release of cytokines from macrophages (Koops et al., 1996; Schaefer et al., 2005). It is unknown what stimulates biglycan release from astrocytes. Saturated fatty acids also signal through the TLR4 (Hwang, 2001).

Although a number of other proteins have been suggested as TLR4 agonists one problem in the identification of TLR4 agonists is that recombinantly produced compounds are often applied to cells to test whether a substance acts on the TLR4. Recombinant products contain residual bacterial cell wall components such as LPS and these contaminants can produce false positive results, such as what was found for heat shock protein 60 and 70 and surfactant protein-A (for a review see Tsan and Gao, 2004).

Apart from these endogenous ligands, the most commonly used exogenous TLR4 ligand is LPS which is often used to experimentally mimic inflammatory conditions. LPS is a component of gram-negative bacterial walls which enables the immune system to respond to gram negative bacteria. LPS from wild type gram-negative bacteria such as *Escherichia Coli*, *Porphyromonas gingivalis* or *Salmonellae typhimurium* is referred to as smooth LPS (S-LPS) and is composed of three major regions. The first is a polysaccharide side chain region with antigens (Osborn et al., 1964). This region is often referred to as the O-antigen region and its structure can vary depending on the bacterial source of LPS (Dixon and Darveau, 2005). The next portion is a core polysaccharide linking the side chain portion to the final lipid component called lipid A, which is unusual compared to most lipids as it contains glucosamine instead of glycerol (Osborn et al., 1964). In gram negative bacteria, lipid A is embedded in the cell membrane of

the bacteria while

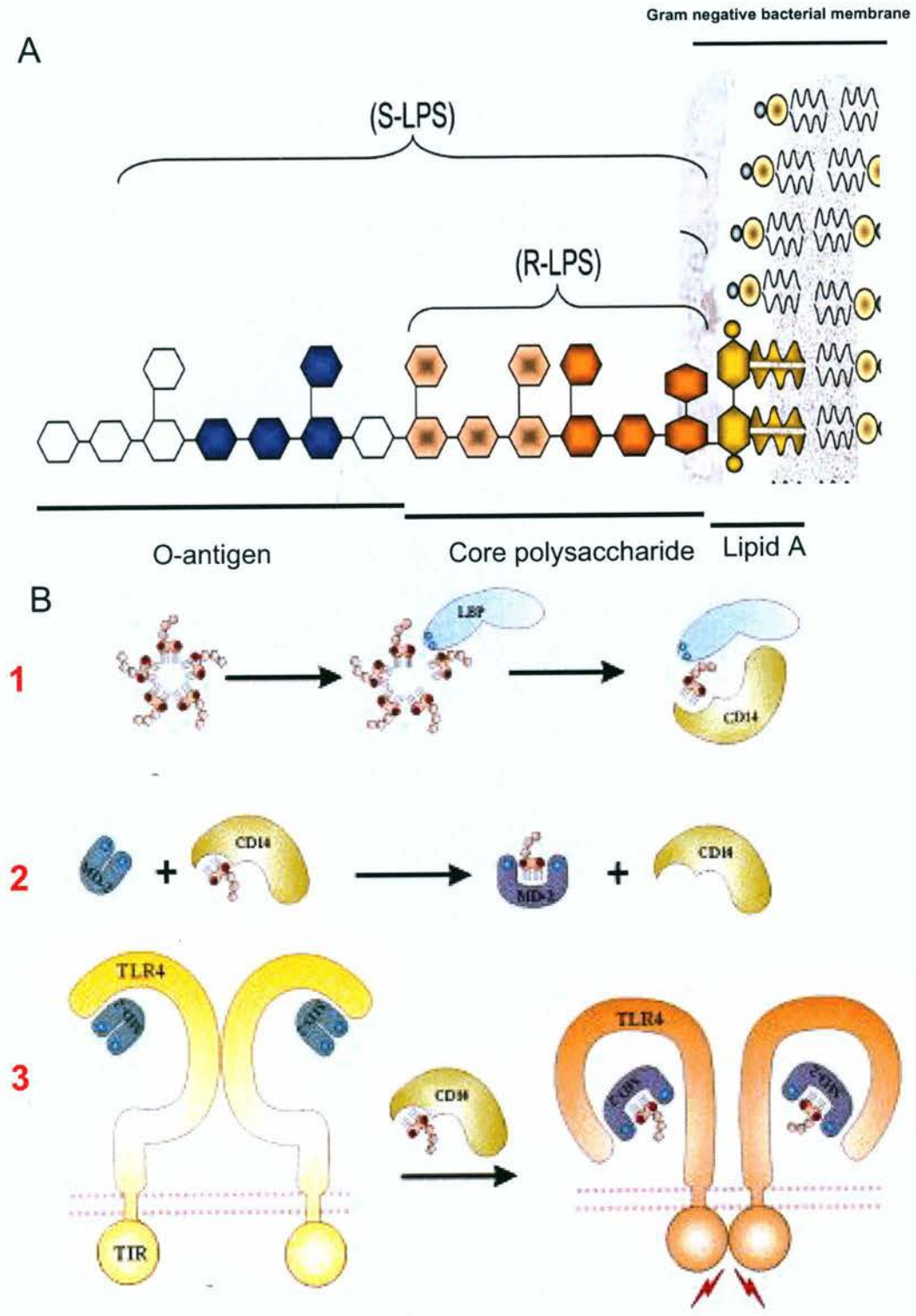


Figure 1.12 The structure of lipopolysaccharide (LPS) and its receptor. A) A schematic of the three different domains of LPS when it is embedded in the cell membrane of gram negative bacteria. The final extracellular segments of S-LPS and R-LPS are indicated. Adapted from Dixon and Darveau (2005). B) Schematic of the binding of LPS to the Toll-like receptor 4 (TLR4) complex. 1. A single LPS molecule is removed from an aggregate of LPS by LPS binding protein (LBP) and is presented to CD14. 2. CD14 then presents LPS to myeloid differentiation Ag (MD2). 3. The binding of LPS by MD2 brings the two Toll/interleukin-1 receptor (TIR) domains of the TLR4 homodimer together. Adapted from Jerala (2007).

the 2 polysaccharide components are extracellular (Dixon and Darveau, 2005). It is the lipid A portion of lipopolysaccharide that is the main biologically active component as administration of just this portion can elicit the same immune effects as LPS (Chaby, 2004). The structure of S-LPS can be seen in Figure 1.12A. Additionally, rough LPS (R-LPS) is produced by rough mutants of gram-negative bacteria. R-LPS does not contain the O-antigen region (Caroff and Karibian, 2003).

Considering the different origins of the endogenous and exogenous TLR4 ligands, it can be seen that this receptor is activated during infection with gram negative bacteria, after ganglioside-releasing cell damage and potentially on exposure to non-stimulated astrocytes. Additionally, as detailed in section 1.3.2.1, there is evidence that the TLR4 is activated during neuropathic pain although it is currently unknown which endogenous ligands contribute to its activation (Tanga et al., 2005).

1.4.2.2.2 Recognition of LPS by the Toll-like receptor 4 and subsequent signaling mechanisms

Numerous steps are required for LPS to activate intracellular signaling pathways through the TLR4. The TLR4 on its own is not responsive to LPS and requires a protein named myeloid differentiation Ag (MD2) to first bind LPS. The MD2/LPS complex can then bind to the extracellular portion of TLR4 (Kennedy et al., 2004; Shimazu et al., 1999). Hence, MD2 is in effect the ligand binding segment of the TLR4 receptor complex (Visintin et al., 2005).

Evidence suggests that, at least in part, LPS works at the transcriptional level to increase IL-10 release as all studies that have found a release of IL-10 from LPS-exposed microglia and astrocytes report a concomitant upregulation of IL-10 mRNA (Ledeboer et al., 2002; Mizuno et al., 1994; Seo et al., 2004; Williams et al., 1996). There are a number of different intracellular signaling pathways through which activation of the TLR4 can lead to changes in transcription. The intracellular segment of each TLR4 monomer contains a sequence of amino acids known as a Toll/interleukin-1 receptor (TIR) domain and when the TLR4 homodimer is bound by MD2/LPS, the TLR4 changes conformation bringing the TIR domains of each TLR4 monomer closer together. This enables one of two proteins that also contain TIR domains to attach to the TLR4. Depending on which TIR protein attaches to the TLR4, two distinct signaling pathways can be activated. One is a fast response pathway and the other is a delayed response pathway. The quick response myeloid differention 88 (MyD88)-dependent pathway is characterized by attachment of the MyD88-adaptor-like (M AL) protein to the TLR4 while the delayed response MyD88-independent pathway is characterized by the attachment of TIR-containing adaptor molecule-1 (TRAM -1) to the TLR4 (O'Neill and Bowie, 2007).

After attachment to the TLR4, MAL binds PIP_2 in the plasma membrane which recruits MyD88 (Fitzgerald and Chen, 2006). MyD88 recruits IL-1 receptor associated kinase 4 (IRAK4) which then recruits IRAK1. The target of IRAK1 is tumor-necrosis-factor-receptor-associated factor-6 (TRAF6). TRAF6 recruits a range of intracellular signaling molecules such as TGFβ-activated kinase 1 (TAK1),

TAK1-binding protein 2 (TAB2), ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) (O'Neill and Bowie, 2007). These enzymes activate 2 main pathways. The first is initiated by inhibitor of nuclear factor-kB (IkB) kinase complex (IKK). In its inactive form the transcription factor nuclear factor-kB (NF-kB) is bound to IkB. This interaction prevents NF-kB from interacting with DNA. IKK is a complex of several IKK subunits which acts to phosphorylate IkB, resulting in its degradation and detachment from NF-kB. This allows the translocation of NF-kB into the nucleus then subsequent attachment of it to NF-kB binding motifs on genes resulting in the production of a variety of pro-inflammatory cytokines.

NF-kB exists as a dimer made of a combination of p65 and p50 subunits. The most abundant form of NF-kB is the p50/p65 heterodimer. In a macrophage-like cell line, when the p50 homodimer form of NF-kb is stimulated by TLR4 activation IL-10 promoter activity is increased. No other form of NF-kB has IL-10 promoter activity (Cao et al., 2006). It is as yet unclear whether TLR4 stimulation in glia activates a p50 homodimer form of NF-kB and hence whether the TRAF-6/NF-kB pathway contributes to LPS-induced IL-10 release from glia.

Additionally, on activation of TAK1, TAB2, UEV1A and UBC13 by TRAF6, mitogen-activated protein kinase (MAPK) kinase (MKK) 3 and 7 are activated. MKK3 then activates the p38 MAPK whilst MKK7 activates the JUN N-terminal kinase (JNK) MAPK pathway (O'Neill and Bowie, 2007). In glia, LPS-induced p38

and JNK increase the activity of the transcription factor c/EBPβ (CCAAT/enhancer binding protein β). Also, in macrophages it has been demonstrated that LPS-stimulated p38 activation increases the activity of the transcription factor Simian virus 40 promoter factor 1 (Sp1) (Cao et al., 2006; Ejarque-Ortiz et al., 2007; Kelicen and Tindberg, 2004; Ma et al., 2001). Whether LPS activates Sp1 in glia has not been investigated although it has been shown that Sp1 is present in glia (Mao et al., 2007). The promoter site on the IL-10 gene has two areas responsive to c/EBPβ and Sp1 respectively and distrupting both sites almost completely blocks LPS-activated IL-10 expression in macrophages (Chiang et al., 2006; Liu et al., 2003).

Apart from MAL, the other protein that can attach to the intracellular segment of LPS/MD2-bound TLR4 is TRAM -1. Once attached, TRAM -1 binds the protein TIR-domain -containing adaptor inducing IFN-β (TRIF) which activates two pathways. The first pathway activated by TRIF is via tumor-necrosis-factor-receptor-associated factor-3 (TRAF3) and leads to activation of the transcription factor interferon regulatory factory-3 (IRF-3), which when combined with the NF-kB subunit p65 can bind interferon-stimulated response elements (ISRE) on genes, leading to the production of the cytokine interferon-β (O'Neill and Bowie, 2007). The second pathway that TRIF activates is via TRAF6 and leads to activation of NF-kB (Fitzgerald et al., 2003). Activation of the MyD88/TRAF6/IKK pathway leads to the production of NF-kB in a faster manner than does the TRIF/TRAF6 pathway.

Prior to MD2 binding of LPS, LPS is presented to MD2 by either a serum or glycol-phosphatidylinositol anchored membrane protein named cluster determinant 14 (CD14), which is necessary for some of the physiological effects of LPS (Tobias et al., 1995; Wright et al., 1990). S-LPS cannot induce MyD88-dependent signaling through the TLR4 without CD14, while R-LPS does not need CD14 to elicit the MyD88-dependent pathway (Huber et al., 2006; Jiang et al., 2005). Both R- and S-LPS require CD14 to signal through the TRIF pathway (Jiang et al., 2005).

Another protein that can increase the sensitivity of the response to LPS is LPS binding protein (LBP), although LPS can bind MD2 without LBP (Viriyakosol et al., 2001; Visintin et al., 2005; Visintin et al., 2001). LBP can either be present in the plasma or be associated with the cell membrane. In the blood LPS often exists in aggregates and usually the first step towards binding of the TLR4 by LPS involves binding of a single LPS molecule by LBP (Roes et al., 2006; Schumann et al., 1990). When it is present in low concentrations, the role of plasma LBP has been proposed as binding individual LPS monomers to separate them from aggregates (Jerala, 2007; Schumann et al., 1990). It is unclear if the role of membrane-associated LBP is the same as plasma LBP since membrane-associated LBP appears to bind whole aggregates (Roes et al., 2006). Once bound, LBP then delivers LPS to CD14. The ease of transfer of LBP-bound LPS to CD14 depends on the bacterial source of the LPS (Cunningham et al., 1996). The role of CD14 and LBP is illustrated in Figure 1.12B.

Work has been carried out to investigate whether endogenous ligands bind to the TLR4 and bring about intracellular changes in a similar manner to LPS. It is known that gangliosides require CD14 to bind TLR4 and a subsequent intracellular event is NF-kB activation (Jou et al., 2006). Additionally, there is evidence that biglycan signals through the TLR4 mainly via the MyD88 pathway, resulting in activation of MAPKs and NF-kB. Also, saturated fatty acids activate NF-kB (Hwang, 2001; Schaefer et al., 2005). Studies providing a more in-depth knowledge of how endogenous ligands activate the TLR4 are needed if interventions in TLR4 signaling are to be made to control outcomes of endogenous stimulation of this receptor.

A summary of the literature reviewed above shows that after CD14-dependent binding of S-LPS (the type of LPS used in most experimental studies) to MD2, this complex can attach to the TLR4 homodimer stimulating a fast MyD88-dependent and a more delayed MyD88-independent signaling pathway. The result of these pathways is interferon-β release, pro-inflammatory cytokine release, from a quick or delayed activation of NF-kB potentially accompanied by NF-kB stimuated IL-10 release and activation of p38 and JNK MAPK, resulting in activation of c/EBP and consequent upregulation of IL-10 mRNA and IL-10 release. This pathway is summarized in Figure 13.

1.4.2.2.3 Mechanisms of IL-10 secretion

The mechanisms of IL-10 secretion from cells are unknown. It is proposed that another cytokine, IL-1β may be released via secretory lysosomes, which are organelles that can be exocytosed, or microvesicles, which are small blebs of membrane (MacKenzie et al., 2001; Mambula and Calderwood, 2006). In some lysosomal storage disorders like Gaucher disease and Globoid cell leukodystrophy an upregulation of IL-10 is seen (Allen et al., 1997; Wu et al., 2000), perhaps providing circumstantial support for a lysosomal release of IL-10, however a co-localisation and co-secretion of IL-10 with lysosomal enzymes will have to be shown in further experiments to conclude a lysosomal mechanism. Further, other secretion mechanisms such as exocytosis of classic vesicles and microvesicles will also have to be explored.

1.4.2.2.4 Removal of LPS and degredation of IL-10

The final steps in TLR4-mediated IL-10 release are removal of the TLR4 agonist and released IL-10 from the extracellular space. There are two main documented methods of LPS deactivation. LPS can bind membrane-anchored CD14, be internalized, and then degraded, for example, by lysosomal acid phosphate, which is an enzyme identified in the CNS among other areas (Chaby, 2004; Gegner et al., 1995; Geier et al., 1992; Luchi and Munford, 1993). The second documented method of LPS deactivation utilises phospholipid transfer proteins and LBP. Phospholipid transfer proteins are plasma proteins with 24% sequence homology to

LBP, which bind the lipid A portion of LPS and can transfer LPS from aggregates to high density lipoprotein particles, neutralizing its biological activity. These high density lipoprotein particles are then phagocytosed (Chaby, 2004; Hailman et al., 1996). Addition of phospholipid transfer proteins to blood prevents LPS-stimulated cytokine release (Hailman et al., 1996). As well as its ability to transfer LPS to CD14, LBP can also transfer LPS to high density lipoprotein particles (Wurfel et al., 1994). Phospholipid transfer proteins have been documented in the brain (Jiang and Zhou, 2006) but it is as yet unknown if LBP is present in the CNS.

Although cytokines can be degraded by bacterial proteases (Wilson et al., 1998) and in the periphery can be degraded by proteases released from mast cells (Zhao et al., 2005) there are currently no delineated endogenous spinal cord mechanisms for IL-10 degredation (Milligan et al., 2005a). Although a bolus intrathecal injection of IL-10 was found to have a half life of 2 hours in the spinal cord, the rate of IL-10 clearance fell within the range of cerebral spinal fluid production rates and hence this half life was attributed to bulk cerebral spinal fluid flow as opposed to breakdown or degredation (Milligan et al., 2005a).

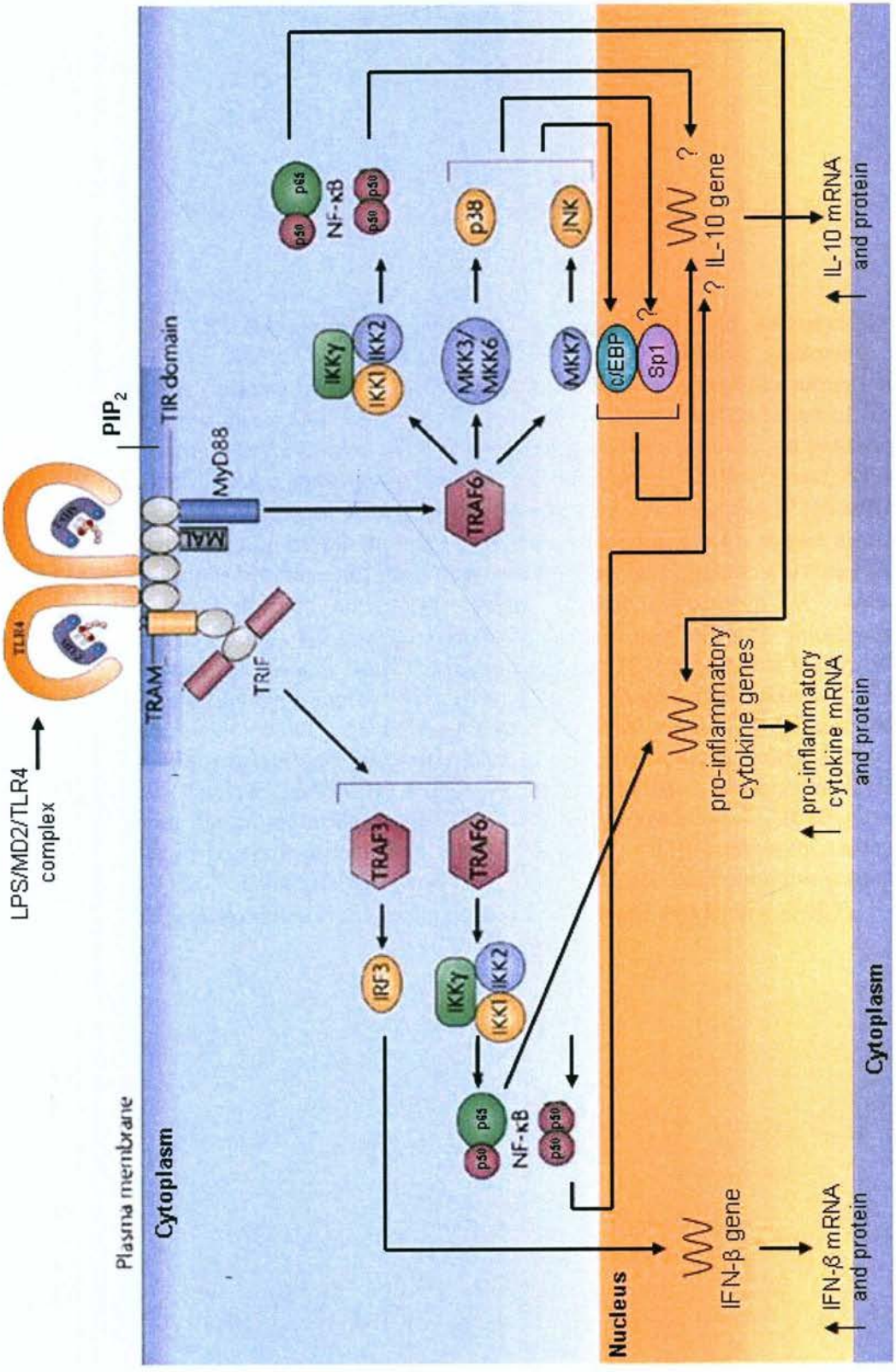

LPS/MD2/TLR4 complex
TLR4
PIP2
TIR domain
MyD88
MAL
TRAM
TRIF
Plasma membrane
Cytoplasm
TRAF3
IRF3
TRAF6
IKKγ
IKK1
IKK2
NF-κB
p65
p50
p50
p50
IKKγ
IKK1
IKK2
NF-κB
p65
p50
p50
p50
TRAF6
MKK3/
MKK6
p38
MKK7
JNK
c/EBP
Sp1
?
?
?
IL-10 gene
IL-10 mRNA
and protein
pro-inflammatory
cytokine genes
pro-inflammatory
cytokine mRNA
and protein
Nucleus
IFN-β gene
IFN-β mRNA
and protein
Cytoplasm

Figure 1.13 TLR4 signaling pathways in microglia and astrocytes. On binding of the MD2/TLR4 complex by LPS, two signaling pathways are activated. The first is a MAL/MyD88/TRAF pathway which leads to activation of the transcription factors NF-kB, p38 and JNK and results in upregulation of IL-10 and pro-inflammatory cytokine mRNA and protein levels. The second pathway is a TRAM/TRIF/TRAF pathway which leads to activation of IRF3 and NF-kB, resulting in upregulation of IFN-β, pro-inflammatory cytokine and IL-10 mRNA and protein. Question marks indicate processes which have been shown to occur in other cell types but have not been explored in glia. It is unclear whether MKK, p38, JNK and c/EBP activation occurs in the cytoplasm or nucleus. Abbreviations: c/EBP, CCAAT/enhancer binding protein; IFN-β, interferon-β; IKK, inhibitor of nuclear factor-kB kinase complex; IL-10, interleukin-10; IRF3, interferon regulatory factory-3; JNK, JUN N-terminal kinase; LPS, lipopolysaccharide; MAL, MyD88-adaptor-like; MD2, myeloid differentiation Ag; MKK, mitogen-activated protein kinase kinase; MyD88, myeloid differention 88; NF-kB, nuclear factor-kB; PIP_2, phosphatidylinositol bisphosphate; Sp1, Simian virus 40 promoter factor 1; TLR4, Toll-like receptor 4; TRAF, tumor-necrosis-factor-receptor-associated factor; TRAM, TIR-containing adaptor molecule; TIR, Toll/interleukin-1 receptor; TRIF, TIR-domain containing adaptor inducing IFN-β. Adapted from Jerala (2007) and O'Neill and Bowie (2007).

1.4.2.3 Potential of spinal cord astrocytes and microglia to release IL-10 on TLR4 activation

As mentioned in section 1.4.1.3, a regional heterogeneity between spinal cord and cortical astrocytes has been described and it cannot be assumed that, because cortical astrocytes display TLR4-stimulated IL-10 release, spinal cord astrocytes will also display this. Additionally, there is a lack of studies comparing the responses of spinal cord and cortical microglial cells, hence it cannot be assumed that spinal cord microglia react to neurotransmitters in a similar fashion to cortical transmitters.

This raises the question of what evidence there is that spinal cord astrocytes and microglia can release IL-10 in reponse to LPS. Spinal cord astrocytes and microglia have some of the cellular machinery that is needed for TLR4-stimulated IL-10 release. There is evidence that spinal cord microglia and astrocytes express functional TLR4s. Cultured spinal cord astrocytes and microglia release substances in response to LPS, with astrocytes shown to release nerve growth factor (Pehar et al., 2004) and microglia shown to release TNF-α, IL-1β and IL-6 (Ogata et al., 2003). Additionally, it is known that spinal cord astrocytes contain JNK and spinal cord microglia contain p38 (Svensson et al., 2003; Zhuang et al., 2006), which as detailed above are two key MAPKs involved in IL-10 transcription activation.

Given i) it has not been examined which cells release IL-10 in the spinal cord despite the possible anti-nociceptive impact of potential IL-10 release and ii) in consideration that there is evidence for the presence of some of the key proteins involved in TLR4-stimulated IL-10 release on spinal cord microglia and astrocytes, *one objective of this thesis will be to examine if TLR4 stimulation can elicit IL-10 release from spinal cord microglia and astrocytes.*

1.4.2.4 The potential of glutamate to be involved in IL-10 release from spinal cord microglia and astrocytes

As noted in section 1.3.1, when the TLR4 is being activated in chronic pain conditions, astrocytes and microglia are simultaneously exposed to many other neurotransmitters (Millan, 1999; Tanga et al., 2005). This suggests that to gain a full knowledge of the spinal sources of IL-10 it should be examined whether these substances elicit IL-10 release and/or affect the process of TLR4-stimulated IL-10 release if this process occurs. As time is restricted, the effects of every relevant substance can not be explored in the present research, however glutamate is an attractive starting point in the identification of further spinal sources of IL-10 and possible modulators of TLR4-stimulated IL-10 release. Glutamate shows potential for a number of reasons. Firstly, as discussed in section 1.3.1, glutamate is found in increased amounts in the spinal cord in chronic pain (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992). In conditions such as chronic pain where there is TLR4 activation and hence subsequent pro-inflammatory cytokine release, cytokines such

as TNF-α contribute to these increased levels of glutamate by stimulating glutamate release from astrocytes and microglia, and inhibiting astrocytic glutamate uptake (Bezzi et al., 2001; Takeuchi et al., 2006; Tilleux and Hermans, 2007). Secondly, spinal cord glia can respond to these increased levels of glutamate as they have glutamate receptors in their membranes. Spinal cord microglia have been shown to have NMDA receptors, AMPA receptors and mGluRs on their cell surface (Liu et al., 2006). Although the presence of kainate receptors has not been examined in spinal cord microglia, there is evidence for these receptors on cortical and hippocampal microglia (Christensen et al., 2006; Yamada et al., 2006). As discussed in section 1.4.1.3, spinal cord astrocytes express all subtypes of glutamate receptors on their cell surface. Hence when the TLR4 is being bound on these cells, glutamate receptors are also being bound, opening the possibility that glutamate may modulate any TLR4-stimulated IL-10 release. Furthermore, one outcome of the ligation of astrocytic and microglial glutamate receptors is the activation of key signaling molecules involved in IL-10 mRNA upregulation. NDMA elicits p38 activation in microglial cultures (Tikka and Koistinaho, 2001) and glutamate increases c/EBPβ mRNA and protein levels in astrocytes (Yano et al., 1996). Both these proteins are involved in the ability of LPS to bring about IL-10 release (as discussed in section 1.4.2.2.2).

A final reason why glutamate is an attractive starting point in the search for possible IL-10 stimulating agents is that glutamate has a predominantly anti-inflammatory effect on astrocytes and activated microglia. Exposure of resting microglia to

glutamate results in pro-inflammatory outcomes such as TNF-α release and subsequent neurotoxicity (Noda et al., 2000; Taylor et al., 2005). In contrast, in activated microglia glutamate decreases microglial activation, as evidenced by a decrease in cell surface expression of the activation marker ED1. Additionally, activated microglia can be neurotoxic but the degree of neurotoxicity induced by these microglia is decreased if they are exposed to glutamate (Taylor et al., 2003). Further examples of the anti-inflammatory nature of glial glutamate exposure can be seen when astrocytes are exposed to glutamate. When neurons are exposed to high levels of an excitotoxin such as NMDA, they undergo apoptosis or programmed cell death. If these neurons are exposed to astrocytes that have been stimulated by glutamate, the amount of NMDA-elicited neuronal death is greatly decreased (Bruno et al., 1998; D'Onofrio et al., 2001). Similarly, astrocytes exposed to LPS cause neuronal death but this neuronal death can be greatly decreased if astrocytes are co-exposed to glutamate (Zhou et al., 2006). Furthermore, one outcome of the exposure of astrocytes to cytokines is the induction of nitric oxide synthase, an enzyme that is involved in the production of the reactive oxygen species and inflammatory mediator nitric oxide. If glutamate is added to astrocytes simultaneous to cytokine exposure, the extent of nitric oxide synthase induction is reduced (Levy and Zochodne, 1998; Murphy et al., 1995). As the release of IL-10 is an anti-inflammatory action and glutamate often has an anti-inflammatory effect on these glial cells, it suggests glutamate is a promising potential source of IL-10 or a modulator of TLR4-stimulated IL-10 release.

In summary, it can be seen that in chronic pain there is increased levels of glutamate and glutamate receptors are present on spinal cord glia to bind this glutamate whilst the TLR4 is being bound. Given this, and also that the ligation of glutamate receptors results in anti-inflammatory effects and activation of key molecules involved in the production of IL-10, it is conceivable that glutamate may elicit IL-10 release from spinal cord glia or may modulate any TLR4-stimulated IL-10 release. For these reasons, *the final objective of the present research will be to examine if glutamate can elicit IL-10 release and modulate any TLR4-stimualted IL-10 release from spinal cord astrocytes and microglia.*

1.5 AIMS AND OBJECTIVES

Given that ATP in the spinal cord has been shown to play a role in bringing about the symptoms of chronic pain and IL-10 in the spinal cord has been shown to play a role in bringing about the alleviation of these symptoms and considering that the spinal sources of these remain largely unexplored, this work aims:

i) to investigate whether glutamate can stimulate ATP release from spinal cord astrocytes in Chapter 3 and if it can,

ii) to investigate whether SP can either cause ATP release itself or modulate glutamate-stimulated ATP release from spinal cord astrocytes in Chapter 3;

iii) to investigate whether stimulation of the TLR4 on spinal astrocytes results in IL-10 release in Chapter 4;

iv) to investigate whether glutamate can either cause IL-10 release itself or modulate any TLR4-activated IL-10 release from spinal cord astrocytes in Chapter 4;

v) to investiage whether stimulation of the TLR4 on spinal microglia results in IL-10 release in Chapter 5;

vi) to investigate whether glutamate can either cause IL-10 release itself or modulate any TLR4-activated IL-10 release from spinal cord microglia in Chapter 5.

If any of the above processes are found to occur, mechanisms involved in these will be explored.

CHAPTER 2

METHODS AND MATERIALS

Contents

2.1 OVERVIEW

A number of methods have been employed to explore and characterise the release of ATP and IL-10 from spinal cord microglia and astrocytes. First, tissue culture was used to isolate and purify spinal cord microglia and astrocytes, and also cortical astrocytes. ATP release was measured by online-bioluminescence. Membrane currents induced by agonist exposure were measured by electrophysiology. The presence of glutamate receptor subtypes, TLR4s and cell specific markers on cultured glia were probed with immunocytochemistry. Release of IL-10 was detected by enzyme-linked immunosorbent assay (ELISA). Cell membrane integrity was investigated with a lactate dehydrogenase assay and finally, levels of IL-10 mRNA were quantified with a reverse transcription-polymerase chain reaction (RT-PCR).

All experiments were approved by the University of Sydney Animal Ethics Committee and were conducted in accordance with the Australian Government National Health and Medical Research Council's Code of Practice for the Care and Use of Animals for Scientific Purposes. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified and experiments were performed at room temperature (22 – 24°C) unless otherwise specified.

2.2 PREPARATION OF GLIAL CELL CULTURES

Tissue culture is the process of sterile dissection of tissue from a specimen, dissociation of that tissue, propagation of dissociated cells in an artificial growth environment and removal of unwanted cells (Phelan, 2006). Tissue culture is a method useful in examining the function of an isolated cell type which would otherwise be impossible *in situ* due to the presence of other cell types.

2.2.1 Initial culture of mixed spinal cord glial cells

Mixed spinal cord glial cells were cultured according to Cole and de Vellis (1997) with modifications. Whole spinal cords were removed from 0–2 day old Sprague-Dawley rats. Meninges and peripheral nerves were removed and cords were incubated in porcine trypsin (0.125%) dissolved in Hank's balanced salt solution (HBSS, in mM: 136.9 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 0.4 KH_2PO_4, 0.3 Na_2HPO_4, 5.6 D-glucose, pH 7.4) for 20 min at 37°C. Subsequently the cords were washed twice in 2 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT, USA) and 1% penicillin/ streptomycin/glutamine (Invitrogen, Carlsbad, CA, USA; referred to as DMEM+) to inactivate the trypsin. Cells were then dissociated by mechanical trituration and were plated onto poly-D-lysine (20 µg/ml)-coated flasks at 37°C for 4 h. Media was aspirated to remove detached cells and flasks washed twice with 2 ml DMEM+. Glial cells were maintained at 37°C in 5% CO_2 in DMEM+ and culture medium was replaced with DMEM+ every three to four days.

2.2.2 Purification of spinal cord microglial cell cultures

Microglia were purified according to Liu et al (2006) with modifications. To isolate microglia from mixed spinal cord glial cultures, fourteen days after initial plating, flasks containing mixed glial cells were shaken at 400 rpm at 37°C for 40 min on a Unimax100 shaker and incubator (Heidolph, Schwabach, Germany). Microglia and oligodendrocytes do not attach to the base of the flask as strongly as astrocytes and consequently detach upon prolonged shaking (Cole and deVellis, 1997). Medium containing detached glial cells was removed and centrifuged at 1500 rpm for 5 min. The supernatant medium layer contained substances released by cultured glia and this glial conditioned medium supernatant was kept at 37°C. The cell pellet layer was washed twice with 1 ml DMEM+ and then plated at a density of 1 x 10^5 cells per well in a plastic 24-well plate (Sarstedt, Nümbrecht, Germany), at a density of 1 x 10^5 cells per coverslip on a 13 mm diameter circular glass coverslip (Menzel-Glaser, Braunschweig, Germany) or 1 x 10^6 cells per dish on a 35 mm diameter circular plastic Petri dish. Cell-adherent surfaces of these were pre-coated with 20 µg/ml poly-D-lysine. After 15 min, any unattached cells (predominantly oligodendrocytes) were removed and the remaining plated cells were maintained in a 1:1 mixture of DMEM+ and glial conditioned medium at 37°C in 5% CO_2. The purity of cultures was verified with immunocytochemistry. Cells were used for experiments 1 day after initial plating.

2.2.3 Purification of spinal cord astrocyte cell cultures

After removal of detached cells in section 2.2.2, flasks containing adherent cells were maintained in DMEM+ at 37°C in 5% CO_2 for a further 2 days. After *in vitro* day 16 the numbers of remaining microglia and oligodendrocytes were further reduced by replacing DMEM + with sorbitol medium (DMEM without glucose, 10% horse serum (Trace Bioscientific, Castle Hill, Australia), 1% penicillin/ streptomycin/glutamine and 25 mM sorbitol. Oligodendrocytes and microglia do not have aldose reductase and sorbitol dehydrogenase and consequently cannot use sorbitol for energy. Accordingly if sorbitol is substituted for glucose in the culture medium, oligodendrocytes and microglia die off, leaving astrocytes which have the enzymes necessary to use sorbitol for energy (Wiesinger et al., 1991). Twenty days after initial plating, flasks were shaken by the Unimax100 shaker and incubator at 400 rpm and 37°C for 16 h. Twenty-one days after initial plating, the age at which there becomes only one predominant (> 90%) subtype of cultured spinal cord astrocytes (Black et al., 1993), remaining astrocytes were washed twice with 3 ml HBSS and removed from the flask after incubation for 5 min at 23°C in 0.125% porcine trypsin. Suspended cells were centrifuged at 1500 rpm for 5 min and washed twice with 1 ml DMEM+. Subsequently cells were plated either on 13 mm glass coverslips, the base of wells in a plastic 24-well plate or on the base of a plastic 35 mm diameter Petri dish. Cells were plated at a density of 1×10^5 cells per well or coverslip, or 6.77×10^5 cells per Petri dish. Cell-adherent surfaces were pre-coated with 20 µg/ml poly-D-lysine. The purity of cultures was verified by immunofluorescent staining for markers of neurons and other glial cell classes. The

heterogeneity of the astrocyte population was examined based on morphological characteristics and over 90% of astrocytes resembled spinal cord astrocyte subtype 1a as described by Black et al. (1993). It is unclear whether these astrocytes are grey matter or white matter astrocytes. Cells were used for experiments 1-2 days after initial plating.

2.2.4 Culture of cortical astrocytes

The cortices of 0–2 day old Sprague-Dawley rats were removed and meninges were separated away. Preparations of mixed cortical glial cells were then prepared in the same manner as for mixed spinal cord glial cells in section 2.2.1. Cortical astrocytes were purified and plated in the same manner as for spinal cord astrocytes in section 2.2.3 and were used for experiments 1 to 2 days after initial plating.

2.3 ONLINE BIOLUMINESCENCE

Online bioluminescence is a technique that allows the real-time measurement of ATP release from cells. Using a luciferin-luciferase enzymatic ATP assay mix (firefly luciferase, luciferin, $MgSO_4$, ethylenediaminetetraacetic acid, dithiothreitol and bovine serum albumin (BSA) in a Tricine buffer according to manufacturer's proportions, Sigma-Aldrich) developed by Taylor et al. (1998), for every molecule of ATP that is evolved in solution, a photon of light is produced according to the equations:

$$\text{ATP} + \text{Luciferin} \underset{Mg^{2+}}{\overset{\text{Firefly Luciferase}}{\rightleftharpoons}} \text{Adenyl-luciferin} + PP_i$$

$$\text{Adenyl-luciferin} + O_2 \longrightarrow \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{light.}$$

Consequently, the amount of ATP afforded by cells can be indirectly calculated by measuring in real-time the number of photons produced by cells incubated in the luciferin-luciferase assay with a photomultiplier.

2.3.1 Preparation

Coverslips prepared in section 2.2 were placed in HEPES buffered salt solution (in mM; 140 NaCl, 5 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.4) for 5 min to remove DMEM+. Excess solution was removed from cell-free sides of the coverslip and it was placed on an 18 mm diameter round plastic photomultiplier stage constructed by cutting individual wells out of a plastic 24-well plate. Eighty µl of ATP assay mix (1 mg/ml; Sigma-Aldrich) dissolved in HEPES buffered salt solution was

immediately added to the coverslip. The photomultiplier stage was placed inside a light-impermeable chamber constructed by the University of Sydney Physics Workshop (Camperdown, Australia).

2.3.2 ATP release measurement

Photons were counted with a P30CWAD5-45 Photodetector Package (Electron Tubes, Coventry, UK) and displayed using an RS232 Photon Counting program (Electron Tubes). Photons were collected every second. Initial photon count was high as there was an imbalance between the basal amount of ATP produced by cells and the amount of ATP consumed by the ATP assay mix and the ecto-ATPases. Sixty to eighty min later the amount of ATP produced by cells and the amount of ATP consumed by the assay and ecto-ATPase reached equilibrium, illustrated by the photon count readings not varying by more than 10%. When this stable photon count extended for 10 min, the average photon count over this period of time was recorded as baseline. After the baseline was reached, recording of photon counts was stopped and agonists of interest were applied at 10% of the volume of liquid on the coverslip. Recording was immediately recommenced (within 20 s) after addition of the chemicals. Chemicals were not washed out during experiments. At the end of each experiment, 1% Triton X-100 (Bio-Rad, Hercules, CA, USA) was added to lyse cells, allowing an estimate of the number of cells on the coverslip. Each experimental condition was tested at least three times and in experiments using antagonists, all antagonists were applied 1 h prior to agonist addition.

2.3.3 Controls

To investigate the effects of chemicals and their solvents (HEPES buffered solution, DMSO, NaOH, HCl and acetonitrile + trifluoroacetic acid as per section 2.9) on the intrinsic luciferin-luciferase activity, chemicals or solvents were added to non-cell coated coverslips. To examine the effect of solvents on glutamate-stimulated ATP release, solvents were added to cell-coated coverslips after baseline ATP release had been reached. The effect of antagonists on the baseline ATP release by cells was investigated by addition of the antagonist and subsequent recording of photon release. The possibility that the mechanical sensitivity of cells caused any observed ATP release was also investigated by addition of HEPES without chemicals to cells. These treatments did not elicit ATP release.

2.3.4 Construction of a standard curve

Raw data was obtained showing the change in photon count every second. To enable the conversion of photon counts to moles of ATP, a standard curve was constructed. Eighty µl of ATP assay mix were added to a glass coverslip without cells on it. The baseline photon emission was recorded from this ATP assay mix and then ten known amounts of ATP between $3.3x10^{-15}$ and $1x10^{-10}$ moles were added to the ATP assay mix in increasing concentrations. The baseline photon emission was subtracted from the peak photon count values obtained from each amount of ATP, and the points plotted on a log scale. The gradient was computed and the photon counts for all experiments were divided by the reciprocal of this gradient value to enable conversion of photon count readings to moles of ATP. A

standard curve was constructed for each batch, both at the start and towards the end of use of each batch of luciferase to identify the degree of assay activity loss over time. No loss over time was found. An example of a standard curve is displayed in Figure 2.1.

2.3.5 Analysis of data

ATP release values were analysed to find the peak increase in ATP release relative to baseline (referred to as 'relative peak ATP level'). Results were expressed as mean relative peak ATP level ± SEM. Means were analysed for statistically significant differences with an unpaired Student's two-sample two-tailed *t*-test. Statistical significance was considered at $p < 0.05$.

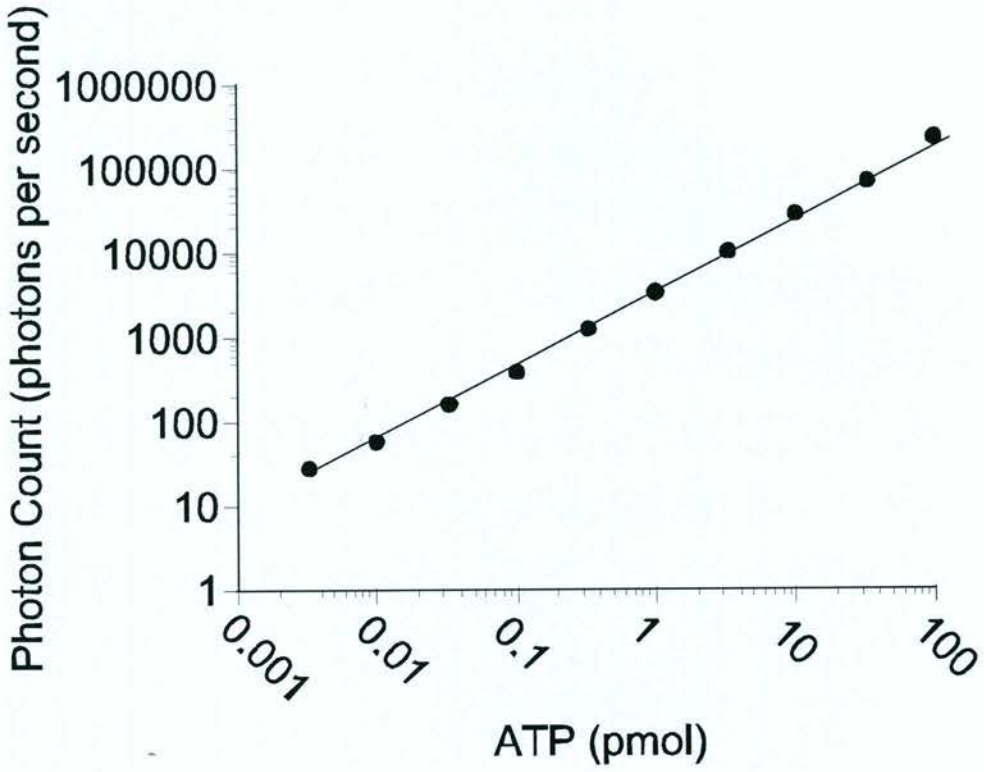


Figure 2.1 ATP standard curve. A representative image of the variation of photon counts on application of known amounts of ATP.

2.4 PATCH-CLAMP RECORDING

The opening of ion channels and receptors on a cell can lead to the flow of ions into and out of a cell. Whole cell patch-clamp recording is a technique that can be used to measure cellular currents set up by such ion flows. It involves the placement a glass pipette with a very small diameter tip (often about 1 - 2 μm) on the surface of a cell. Negative pressure is applied to the electrode interior resulting in the formation of a seal between the pipette and cell. This seal has a resistance in the order of magnitude of gigaohms. Exposure to the antibiotic nystatin perforates the patch of membrane encircled by the electrode tip. On perforation of this patch of membrane, ions in the intracellular fluid of the pipette can exchange with ions in the cytoplasmic fluid of the cell, allowing the pipette to sense changes in voltage in the patched cell (Jackson, 1997).

2.4.1 Identification of astrocytes

Astrocytes were identified according to the morphological features of type A astrocytes, illustrated in Figure 1.3C, such as their large flat cell body in comparison to the small, rounded cell bodies of microglia and oligodendrocytes. The morphology of these astrocytes corresponded with the morphology of cells that stained positive for glial fibrillary acidic protein (GFAP) in immunocytochemistry experiments (see section 2.5) and differed from the smaller CDllb positive microglia.

2.4.2 Patch-clamp recordings

Resistances of pipettes were 4 - 8 M Ω when the pipette was filled with internal pipette solution (in mM ; 145 CsCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES and 250 µg/mL nystatin, pH 7.3). The external bath solution consisted, in mM , of 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES with pH 7.4. Mg^{2+} was omitted from the external bath solution when NMDA was used as an agonist. Whole-cell currents in astrocytes were recorded using a Multiclamp 700A amplifier (Axon Instruments, Sunnyvale, CA, USA) under voltage-clamp mode at a holding membrane potential of -60 mV. The currents were sampled on-line using a Digidata 1322A interface and pClamp8 software (Axon Instruments). Glutamate, its analogs and SP were applied through pressure ejection via a picosprizer (General Valve, Fairfield, NJ, USA).

2.4.3 Analysis

The number of cells that displayed inward currents in response to agonists was identified with pClamp8. For those cells that displayed currents, the peak amplitude of the currents were recorded and averaged. Means were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant and values were expressed as mean ± SEM.

2.5 FLUORESCENCE IMMUNOCYTOCHEMISTRY

Fluorescence immunocytochemistry allows the identification and localization of cellular proteins through the attachment of fluorescent molecules to proteins of interest. In indirect fluorescence immunocytochemistry, cells are fixed, non-specific binding of antibodies is blocked and cells are permeabilised. Cells are then incubated in an antibody constructed to bind to the protein of interest. This is the primary antibody. A secondary antibody conjugated to a fluorescent protein is bound to the primary antibody. When mounted on a microscope and exposed to a wavelength of light that excites the fluorescent protein, filters can be used to visualize light emitted by the fluorescent molecule. Different primary and conjugated secondary antibody pairs can be used in the one preparation allowing the simultaneous investigation of several proteins (Goldstein and Watkins, 2008).

2.5.1 Fixation, blockade of non-specific binding and permeabilisation

Cells grown on coverslips described in section 2.2 were washed twice for 5 min each wash with the HEPES buffered salt solution described in section 2.3.1. Cells on coverslips were then fixed with 2% paraformaldehyde solution (BDH chemicals, Kilsyth, Australia) in phosphate buffered saline (PBS in mM; 136.89 NaCl, 2.68 KCl, 10.14 Na_2HPO_4, 1.76 KH_2PO_4, pH 7.4) for 10 min. Coverslips were washed twice for 5 min each wash with PBS and then incubated in PBS containing 1% BSA and 0.05% saponin for 30 min to block non-specific binding and permeabilise cells.

2.5.2 Antibody incubation and coverslip mounting

Cells on coverslips were then incubated either overnight at 4°C (in Chapter 3) or for 2 h at room temperature (in Chapters 4 and 5) with primary antibodies diluted in PBS containing 1% BSA and 0.05% saponin. Primary antibodies were washed out three times for 10 min each wash with PBS and then cells were incubated in secondary antibodies diluted in PBS containing 1% BSA and 0.05% saponin for 1 h at room temperature. Secondary antibodies were washed out 3 times with PBS for 10 min each wash. Excess moisture was removed from the back of coverslips and they were mounted on rectangular glass coverslips using ProLong Gold anti-fade mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Slides were sealed with nail polish and subsequently stored at 4°C until viewing.

To verify the results obtained using the above method, for every primary antibody used, cells were stained with and without the primary antibody and no positive staining was seen when the primary antibody was omitted. In double-labeling experiments, cells were also incubated in each single antibody to verify there was no cross-reactivity between primary antibodies. Patterns of antibody staining identified when cells were incubated in each single antibody matched patterns of antibody staining seen in double-labeling experiments. Furthermore, for Chapter 3, to verify the results obtained using the above method, experiments probing NR1 and GluR4 subunits were repeated with one of the following changes per experiment: omission of DAPI from the mounting media; substitution of the step incubating cells in saponin and BSA with three steps incubating cells in 1% Triton

X-100 which was washed out three times with PBS then a 1 hr incubation in 1% BSA; use of a different rabbit anti-rat NR1 antibody (Chemicon, Temecula, CA, USA; 1:50–1:200); incubation of cells in the rabbit anti-rat glutamate receptor antibodies prior to fixation of cells with paraformaldehyde solution. Results using these different methods were consistent.

2.5.3 Fluorescence microscopy

Fluorescence was visualised with an Axioplan2 upright microscope (Zeiss, Jena, Germany) for experiments described in Chapter 3 and an Axio200M inverted microscope (Zeiss) for experiments described in Chapters 4 and 5. Alexa Fluor 488 was excited at 495 nm and emission was collected at 594 nm. Alexa Fluor 594 was excited at 590 nm and emission was collected at 617 nm. DAPI was excited at 345 nm and emission was collected at 458 nm. Images were acquired with an AxioCam HR digital monochrome charge-coupled device camera (Zeiss) using Axiovision 3.1 software (Zeiss). Experiments with each antibody were performed on at least three different batches of cultures and were repeated at least twice on each of the batches. For each coverslip at least three fields of view were imaged. No autofluorescence of primary antibodies or non-specific binding of secondary antibodies was observed.

2.5.4 Analysis

For analysis of experiments described in Chapters 4 and 5, a constant exposure time was used to acquire images assessed for brightness of TLR4 staining. Using Image

J (U.S. National Institutes of Health, Bethesda, MD, USA), an arbitrary brightness threshold was applied to these images and the number of cells with regions of brightness above this threshold as a percentage of total cells was counted per field of view. This percentage was averaged over a minimum of three different fields of view per condition and mean percentages were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Percentages were expressed as mean ± SEM.

2.6 SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY

The release of protein by cells can be quantified using an enzyme-linked immunosorbent assay (ELISA). In a sandwich ELISA, a multi-well plate is coated with an antibody specifically designed to bind to the protein of interest. This antibody is termed the capture antibody. Wells are incubated in samples containing unknown amounts of the protein of interest, which bind the capture antibody. Subsequently, a detection antibody is added to the plate, which is specifically designed to bind to a different segment of the protein of interest to that of which the capture antibody is bound. This detection antibody has a biotin molecule added to it, the purpose of which is to react strongly with streptavidin conjugated to horseradish peroxidase. Effectively, the protein of interest is now labeled with horseradish peroxidase. The amount of horseradish peroxidase can be indexed by exposing the wells to hydrogen peroxide and tetramethylbenzidine (TMB). Horseradish peroxidase degrades hydrogen peroxide resulting in the oxidation of TMB. Oxidised TMB is a dark blue colour. The production of oxidized TMB can be ceased by addition of sulfuric acid, which turns the colour of oxidized TMB yellow. A spectrophotometer can be used to quantify the intensity of yellow in the wells, which can be converted to an amount of protein by construction of a standard curve (Hornbeck et al., 2001).

2.6.1 Sample preparation

Unless otherwise stated, 1 day after plating in 24-well plates, as per section 2.2, media was removed from wells and cells were incubated in either fresh DMEM+, 1 μg/ml LPS from *Escherecia Coli 055:B5* in DMEM+ or 1 mM glutamate in DMEM+ for 8 h. These solutions were then removed and cells were incubated in 500 μl of agonists and/or antagonists dissolved in DMEM stock with 2% BSA (DMEM/BSA) for 16 h. BSA was added to block non-specific binding to IL-10 capture antibodies (described in section 2.6.2). Plates were sealed on three of four sides to minimise loss of solution but to still allow gas exchange. Medium was then collected and centrifuged.

2.6.2 Assay

Ninety-six-well clear polystyrene microplates (R&D Systems, Minneapolis, MN, USA) were coated in anti-rat IL-10 monoclonal antibody (1:250; BD Biosciences, San Jose, CA, USA) diluted in a coating buffer consisting (in mM; 83 Na_2HPO_4, 134 NaH_2PO_4, pH 6.5) at 4°C overnight. Wells were washed three times for 5 min each wash with 0.05% Tween® 20 in PBS and incubated for 1 h in 1 % BSA in PBS. Wells were then washed for three periods of 5 min with 0.05% Tween® 20 in PBS. Immediately after centrifugation, medium samples were added to wells along with six standard concentrations of IL-10 (BD Biosciences) diluted in DMEM/2% BSA. After a 2 h incubation at room temperature wells were washed five times for 5 min each wash with 0.05% Tween® 20 in PBS. Wells were incubated for 2 h in a biotinylated anti-rat IL-10 antibody (1:250 diluted in 1% BSA in PBS, BD

Biosciences) and wells were washed 5 times for five min each wash with 0.05 % Tween® 20 in PBS. A streptavidin-horseradish peroxidase conjugate (1:250 diluted in 1 % BSA in PBS, BD Biosciences) was added to wells for 20 min and wells were washed 5 times for 5 min each wash with 0.05% Tween® 20 in PBS. Wells were incubated in a 1:1 mix of hydrogen peroxide and TMB for 20 min and 1 M sulfuric acid was added to stop the reaction. Absorbance of plates at 450 nm was read within 20 min of sulfuric acid addition using a Fluostar Galaxy Multiplate reader (BMG Labtechnologies, Offenberg, Germany) and wavelength correction was performed at 550 nm. A standard curve was constructed for each plate and was used to convert absorbance measurements for each condition into IL-10 concentration values. An example of a standard curve can be seen in Figure 2.2. Each experimental condition was tested on at least three different culture batches and at least two repetitions of measurement were made per batch giving a minimum n = 6, apart from select conditions in the time- and dose-response curves where n = 4.

Inhibitors were added 30 min prior to addition of agonists and left in for the duration of agonist exposure, in experiments involving inhibitors. To examine the effect of solvents on agonist-stimulated IL-10 release, solvents were added 30 min prior to addition of agonists. Solvents (dimethyl sulfoxide and NaOH as per section 2.9) at the final concentration used did not effect agonist-stimulated IL-10 release.

2.6.3 Analysis

A standard curve was constructed for each plate based on the absorbance readings of six known concentrations of IL-10. Absorbance readings of unknown samples were converted to concentrations of IL-10 using the reciprocal of the gradient of the standard curve. An example of a standard curve can be seen in Figure 2.2. Values in time- and concentration-dependency experiments and agonist and antagonist experiments were normalized to the average level of LPS-stimulated IL-10 release using the LPS-stimulated IL-10 release value for that batch. In most cases, results were presented as mean concentration of IL-10 (pg/ml) ± SEM. However, to enhance description of the time-dependency of the glutamate effect and the ability of glutamate receptor agonists to elicit IL-10 release, each repeat in these experiments was analysed according to the following statement:

$$\text{Ratio of agonist effect} = \frac{\text{IL-10 release stimulated by LPS + agonist}}{\text{IL-10 release stimulated by LPS}},$$

and results were reported as mean ratio ± SEM. To enhance description of the ability of glutamate receptor antagonists to effect LPS + glutamate-stimulated IL-10 release, each repeat in these experiments was analysed according to the following statement:

$$\text{Ratio of antagonist effect} = \frac{\text{IL-10 release stimulated by LPS + Glu + antagonist}}{\text{IL-10 release stimulated by LPS + antagonist}},$$

and results were presented as a mean ratio ± SEM. Statistically significant differences between means were analysed with a one-tailed Student's two-sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

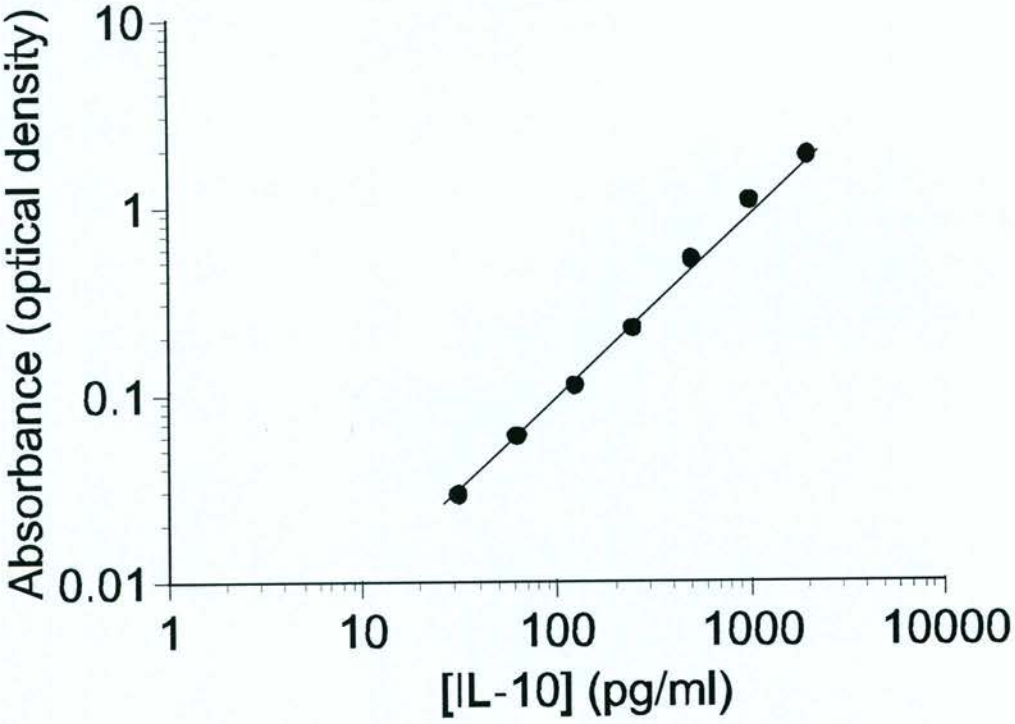


Figure 2.2 IL-10 standard curve. A representative image of the variation of absorbance on application of known amounts of IL-10.

2.7 LACTATE DEHYDROGENASE TOXICOLOGY ASSAY

When exposed to a toxic substance, cell membrane integrity is compromised leading to the release of cytoplasmic contents into the extracellular space. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme which catalyses the conversion of nicotinamide adenine dinucleotide (NAD^+) to its reduced form, NADH. The quantity of LDH in the extracellular medium as a percentage of the quantity of LDH in the intracellular medium is reflective of cell membrane integrity and can be quantified by exposure of LDH to NAD^+ (Decker and Lohmann-Matthes, 1988; Legrand et al., 1992). In the presence of LDH, NAD^+ is reduced to NADH. In the presence of NADH, flavin adenine dinucleotide (FAD^{2+}) is reduced to $FADH_2$. If a tetrazolium dye is present, $FADH_2$ can supply it with electrons and the reaction can be stopped with acid. The intensity of the colour produced can be measured spectrophotometrically.

2.7.1 Sample preparation

Cells were prepared and incubated as described in sections 2.2 and 2.6.1. When medium was collected for ELISA experiments, leftover medium was retained and stored at -80°C for up to 2 weeks. Additionally, to quantify total intracellular LDH levels for each batch, 1% Triton X-100 (Bio-Rad, Hercules, CA, USA) was added to the medium to lyse cells. After 5 min incubation, medium was collected and was stored at -80°C. Medium was then defrosted and used to quantify extracellular LDH for select incubation conditions of ELISA experiments.

2.7.2 Assay

Membrane integrity was assessed with a *In Vitro* Toxicology Assay Kit (Sigma-Aldrich) as per the manufacturer's protocol. Briefly, 75 µl of medium per condition was added to 150 µl of LDH Assay Mixture (Sigma-Aldrich) in separate wells of a 96-well plate. After 20 min incubation, 22.5 µl of 1 N HCl was added to stop the reaction. Within 30 min, absorbance was measured at 490 nm with a Fluostar Galaxy Multiplate reader (BMG Labtechnologies) and wavelength correction was performed at 650 nm.

2.7.3 Analysis

The absorbance reading corresponding to each extracellular LDH sample was divided by the absorbance reading for the corresponding total intracellular LDH sample to obtain a percentage of total LDH released per condition in accordance with Dringen and Hamprecht (1996). Statistically significant differences between means were analysed with a one-tailed Student's two-sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

2.8 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

DNA is transcribed into mRNA in the nucleus of cells. mRNA is transported into the cytoplasm where it is translated into protein. This process involves association of the mRNA with ribosomes (constructed of proteins and rRNA) as well as association with amino-acid bound tRNA. The level of expression of mRNA in a cell can be indexed with a reverse transcription-polymerase chain reaction (RT-PCR). Total RNA, consisting of mRNA, rRNA and tRNA is extracted from cells and is reverse transcribed to cDNA with the enzyme reverse transcriptase. A polymerase chain reaction (PCR) is then used to amplify the cDNA. The real time production of cDNA can be indexed using Sybr Green I, a dye which binds to double stranded DNA and when bound can be excited at 488 nm, consequently emitting at 522 nm (Nolan et al., 2006; Beverly, 2001)

2.8.1 Extraction of total RNA

Total RNA was extracted from cells plated onto Petri dishes in section 2.2.2 and 2.2.3 using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's protocol. Briefly, on ice, a 2-mercaptoethanol (1%)-containing lysis buffer was used to lyse cells. The lysate was filtered and 70% ethanol added to precipitate RNA. The lysate/ethanol mixture was added to a binding column to extract the RNA. The sample was treated with amplification grade DNase I (Invitrogen) for 15 min at room temperature to remove any contaminating DNA before purified RNA was eluted from the binding column. The

quantity of isolated RNA was determined spectroscopically using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.8.2 Reverse transcription of RNA

Extracted total RNA was reverse transcribed to cDNA using a SuperScript™ III First-Strand Synthesis System (Invitrogen) and oligo$(dT)_{20}$ primer (Invitrogen) according to the manufacturer's protocol. Briefly, a mixture of 1 µl oligo$(dT)_{20}$ (50 µM), 1 µl 2'-deoxynucleoside 5'-triphosphate (dNTP) mix (10 mM) and 40 ng astrocytic RNA (Chapter 4) or 3 ng microglial RNA (Chapter 5) extracted as per section 2.8.1, was made up to 10 µl with RNAse free water and was thermally denatured at 65°C for 5 min and placed on ice for at least 1 min. A reverse transcriptase master mix containing 4 µl 5x first strand buffer, 1 µl dithiothreitol (0.1 M), 2 µl $MgCl_2$ (25 mM), 1 µl RNaseOUT™ (40 U/µl) and 1 µl SuperScript™ III reverse transcriptase (200 U/ml) was made up. Residual DNA contamination was tested for by replacing the reverse transcriptase enzyme with RNAse free water. The master mix was added to the mixture on ice and the new mixture was heated at 50°C for 50 min to synthesise cDNA in a Hybaid Gradient PCR machine (Hybaid Limited, Hampshire, UK). The reaction was terminated by heating at 70°C for 15 min. The mixture was chilled on ice and then stored at -20°C until the PCR.

2.8.3 PCR

Aliquots of first strand cDNA (40 ng) were amplified in 10 µL of Platinum SYBR-Green qPCR SuperMix-UDG (Invitrogen) with 0.8 µL of forward primer and

0.8 µL of reverse primer made up to a total volume of 20 µL with diethylpyrocarbonate-treated H_2O. Primers were as follows:

- 18S forward primer: 5'-GCCGCTAGAGGTGAAATTCTTG-3';
- 18S reverse primer: 5'-AAAACATTCTTGGCAAATGCTTT-3';
- IL-10 forward primer: 5'-TGCCTTCAGTCAAGTGAAGAC-3';
- IL-10 reverse primer: 5'-AAACTCATTCATGGCCTTGTA-3'.

All were synthesized by Invitrogen.

PCR was carried out using the Rotor-Gene 3000 system (Corbett Life Science, Sydney, Australia). Incubation conditions were as follows: 2 min at 50°C, 2 min at 95°C, then thermo-cycling for 40 cycles of 20 s at 95°C, 20 s at 60°C then 20 s at 72°C. Melt curves were constructed at the end of the PCR run and any sample with a melt curve with multiple peaks suggesting contamination was discarded.

2.8.4 Analysis

The threshold cycle number, crossing point at threshold and reaction efficiencies of each condition in a reaction were identified with Rotor-Gene 3000 v3.1 software (Corbett Life Science). Relative quantification was carried out using the relative expression software tool (REST; Pfaffl et al., 2002) whereby IL-10 mRNA expression stimulated by the agonists glutamate, LPS or LPS + glutamate was standardised by the level of expression of the house-keeping gene 18S rRNA and compared to IL-10 mRNA expression of unstimulated cells according to the equation:

$$\text{Relative mRNA expression induced by an agonist} = \frac{(E_{\text{IL-10 agonist}})^{CP_{\text{IL-10 unstimulated}} - CP_{\text{IL-10 agonist}}}}{(E_{\text{18S agonist}})^{CP_{\text{18S unstimulated}} - CP_{\text{18S agonist}}}}$$

where $E_{\text{IL-10 agonist}}$ is the efficiency of the PCR performed on IL-10 cDNA derived from cells stimulated by an agonist, $E_{\text{18S agonist}}$ is the efficiency of the reaction performed on 18S cDNA taken from cells stimulated by an agonist; $CP_{\text{IL-10 unstimulated}}$ is the crossing point or cycle threshold of the PCR performed on IL-10 cDNA derived from unstimulated cells; $CP_{\text{IL-10 agonist}}$ is the crossing point or cycle threshold of the PCR performed on IL-10 cDNA derived from cells stimulated by an agonist, $CP_{\text{18S unstimulated}}$ is the crossing point or cycle threshold of the PCR performed on 18S cDNA derived from unstimulated cells; and $CP_{\text{18S agonist}}$ is the crossing point or cycle threshold of the PCR performed on 18S cDNA derived from cells stimulated by an agonist.

At least three batches were examined per condition and mean relative mRNA levels were determined. M ean levels were compared using a one-tailed Student's two-sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Values are expressed as mean relative IL-10 mRNA expression ± SEM.

2.9 PHARMACOLOGICAL MATERIALS

2.9.1 Agonists

Agonists used in experiments were: neurotransmitters glutamate (1 μM - 10 mM ; Chapters 3,4 and 5) and SP (3 - 300 μM; Chapter 3); neurokinin-1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P (100 μM; Chapter 3); neurokinin-2 receptor agonist [β-Ala^8]-neurokinin A Fragment 4-10 (64 μM; Chapter 3); neurokinin-3 receptor agonist succinyl-[Asp^6, N-Me-Phe^8]-Substance P Fragment 6-11 (senktide; 100 μM; Chapter 3); glutamate receptor subtype agonist kainate (Tocris Bioscience, Bristol, UK; 100 μM - 5 mM ; Chapters 3, 4 and 5), glutamate receptor subtype agonist α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA; 50 μM – 100 μM ; Chapters 3, 4 and 5), glutamate receptor subtype agonist N-methyl-D-aspartate (NMDA; 500 μM - 5 mM ; Chapters 3, 4 and 5), mGluRI and II agonist trans-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid (tACPD; 10 μM - 1 mM ; Chapters 3, 4 and 5), mGluRI agonist (*S*)-3,5-Dihydroxyphenylglycine hydrate (DHPG; 500 μM; Chapters 4 and 5); mGluRII agonist (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate (APDC; Tocris Bioscience; 5 μM; Chapters 4 and 5) and mGluRIII agonist L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4; 1 mM; Chapters 4 and 5), Ca^{2+} ionophore A23187 (10 μM; Chapter 3) and the TLR4 agonist lipopolysaccharides from *Escherichia Coli* 055:B5 (LPS; 1 μg/ml; Chapters 4 and 5). All agonists were dissolved in HEPES buffered salt solution in Chapter 3 or DMEM in Chapter 4 and 5, except A23187 which was dissolved in dimethyl sulfoxide (DMSO; maximum final concentration 0.17%).

2.9.2 Inhibitors

Inhibitors used in experiments in proceeding chapters were: NMDA receptor open channel antagonist (+)-MK-801 hydrogen maleate (MK-801; 40 μM; Chapters 3 and 5); kainate receptor antagonist γ-D-glutamylamino methylsulfonic acid (GAMS; 30 μM; Chapter 3); mGluRI and II antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG; 500 μM; Chapter 3); AMPA receptor antagonist 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI52466; 50 μM; Chapter 3); kainate and AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM; Chapters 3, 4 and 5); mGluR1 antagonist 7-Hydroxyiminocyclopropan[β]chromen-1α-carboxylic acid ethyl ester (CPCCOEt; 75 μM; Chapters 4 and 5); mGluR5 antagonist 6-Methyl-2-(phenylethynyl)pyridine (MPEP; 1 μM; Chapters 4 and 5); mGluRII antagonist (2S)-α-ethylglutamic acid (EGLU; 500 μM; Tocris Bioscience; Chapters 4 and 5); mGluRII and III antagonist (±)-α-Methyl-(4-sulfonophenyl)glycine (MSPG; 1 mM; Chapters 4 and 5) and NMDA receptor antagonist D(−)-2-Amino-5-phosphonopentanoic acid (AP5; 200 μM; Chapter 4); phosphatidylinositol-4,5-bisphosphate (PIP_2) synthesis inhibitor LiCl (1 mM ; Chapter 3); endoplasmic reticulum Ca^{2+}-dependent ATPase inhibitor thapsigargin (1 μM; Chapter 3); the inactive form of thapsigargin, called thapsigargin epoxide (Alomone Labs, Jerusalem, Israel; 1 μM; Chapter 3); PKC inhibitor chelerythrine chloride (20 μM; Chapter 3); voltage-dependent Ca^{2+} channel inhibitor $CdCl_2$ (1 mM ; Chapter 3); Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-

tetraacetic acid (EGTA; 5 mM ; Chapter 3); phospholipase D inhibitor 1-butanol (0.5% ; Chapter 3); cell-permeable AC inhibitor 2'5'dideoxyadenosine (DDA; 50 μM; Chapter 3); neurokinin-1 and neurokinin-2 receptor antagonist spantide I (3 μM; Chapter 3); neurokinin-1 antagonist cis-2-(Diphenylmethyl)-N-[(2iodophenyl)methyl]-1azabicyclo[2.2.2]octan-3-amine oxalate salt (L703606; 10 μM; Chapter 3); phospholipase A_2 (PLA_2) antagonist aristolochic acid (50 μM; Chapter 3); the NMDA receptor glycine co-binding site antagonist 7-chlorokynurenic acid (5 μM; Chapter 3), equilibrative nucleoside transporter 1 inhibitor *S*-(4-Nitrobenzyl)-6-thioinosine (NBMPR; 10 μM; Chapter 3), the adenosine kinase inhibitor iodotubercidin (EMD Biosciences, San Diego, CA, USA; 10 μM; Chapter 3), the ecto-ATPase inhibitor 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5-triphosphate (ARL 67156; 100 μM; Chapter 3), the glutamate transporter antagonist with preferential activity at excitatory amino acid carrier ł L-threo-β-benzyl-aspartate (TBOA; 10 μM; Chapter 3), transcription inhibitor actinomycin D (1 μg/ml; Chapters 4 and 5) and translation inhibitor cycloheximide (1 μg/ml; Chapters 4 and 5).

All inhibitors were dissolved in HEPES buffered salt solution in Chapter 3 or DMEM in Chapters 4 and 5, except thapsigargin, thapsigargin epoxide, aristolochic acid, DDA, 7-chlorokynurenic acid, CPCCOEt, NBMPR and iodotubercidin were dissolved in dimethyl sulfoxide (maximum final concentration 0.17%); MCPG and EGLU were dissolved in 0.1M NaOH (maximum final concentration 1 mM NaOH); GYKI52466 was dissolved in 2.5 mM HCl and [β-Ala^8]-Neurokinin A Fragment

4-10 was dissolved in 20% acetonitrile and 0.01% trifluoroacetic acid. As mentioned in sections 2.3.3 and 2.6.2, solvents were examined for effects on ATP or IL-10 release and no effects were found. Additionally, at 1 mM NaOH did not affect solution pH.

2.9.3 Primary antibodies

Primary monoclonal antibodies used were: the astrocyte intermediate filament marker mouse anti-rat GFAP (1:800; Chapters 3, 4 and 5; raised against GFAP from pig spinal cord), the microglia- and macrophage marker mouse anti-rat CD11b (1:100; Chemicon; Chapters 3, 4 and 5; raised against T cell-enriched splenocytes from B10 mice) and the neuron-specific marker antibody mouse anti-rat NeuN (1:150, Chemicon; Chapter 3; raised against purified cell nuclei from mouse brain). Primary polyclonal antibodies used were the oligodendrocyte marker rabbit anti-rat galactocerebroside (1:50 – 1:200; Chemicon; Chapter 3; raised against galactocerebroside from bovine brain), rabbit anti-rat glutamate receptor 2/3 (1:50 – 1:200; Chapter 3; immunogen information not available), rabbit anti-rat glutamate receptor 4 (1:50 – 1:200; Tocris, Bristol, UK, Chapter 3; synthetic peptide corresponding to amino acids 883 – 902 of rat precursor GluR4), rabbit anti-rat metabotropic glutamate receptor 2/3 (1:50 – 1:200; Chapter 3; synthetic peptide corresponding to the final 13 C-terminal amino acids of mGluR2/3), rabbit anti-rat mGluR1a and mGluR5 receptors (1:250; Chemicon; Chapter 3; no immunogen information available), rabbit anti-rat NR1 (1:50 – 1:200; Chapter 3; synthetic peptide corresponding to amino acids 918 – 938 of the NR1 protein), and rabbit

anti-rat Toll-like receptor 4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA; synthetic peptide corresponding to amino acids 339 – 638 of the TLR4 protein).

2.9.4 Secondary antibodies

Secondary antibodies used were goat anti-mouse IgG conjugated to Alexa Fluor 488 (1:100) to detect primary monoclonal antibodies and goat anti-rabbit IgG conjugated to Alexa Fluor 594 (1:100) to detect primary polyclonal antibodies. Both secondary antibodies were from Invitrogen.

CHAPTER 3

GLUTAMATE-STIMULATED ATP RELEASE FROM SPINAL CORD ASTROCYTES IS POTENTIATED BY SP

Contents

3.1 INTRODUCTION

It has recently been discovered that allodynia induced by nerve-injury involves upregulation of ATP $P2X_4$ receptors on microglia as a required molecular mediator (Tsuda et al., 2003). Furthermore, inflammatory and neuropathic pain are completely removed in mice lacking the ATP $P2X_7$ receptor which is found on microglial cells amongst others (Chessell et al., 2005; Sim et al., 2004). Activation of $P2X_3$ receptors on nociceptor nerve terminals in the spinal cord enhances transmission of chronic inflammatory and neuropathic pain (Kennedy et al., 2003; Sharp et al., 2006). Furthermore, inflammatory and neuropathic hyperalgesia is significantly reduced in mice lacking the ATP $P2X_3$ receptor (Dorn et al., 2004; McGaraughty et al., 2003; Souslova et al., 2000; Wu et al., 2004) as it is following intrathecal administration of compounds that disrupt glial function, such as fluorocitrate (Watkins et al., 1997). It appears that the level of ATP in the vicinity of peripheral afferent synapses in the dorsal horn is likely to be an important regulator of inflammatory and neuropathic pain.

Few studies have examined potential spinal sources of ATP in inflammatory and neuropathic pain. Depolarisation of peripheral afferent fibres results in dorsal horn ATP release however it is unclear whether noxious stimulation can elicit this release (Bardoni et al., 1997; Sawynok et al., 1993). Stimulation of ATP receptors elicits ATP release from spinal cord astrocytes, but this does not give insight into the original source of ATP in chronic pain (Abdipranoto et al., 2003; Salter and Hicks, 1994). Glutamate, a transmitter released from spinal nociceptive terminals in increased amounts during chronic pain (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992),

stimulates ATP release from spinal microglial cells (Liu et al., 2006). No other pathways of ATP release have been explored apart from these potential sources. Given the paucity of knowledge of spinal sources of ATP despite the important role of spinal ATP in chronic pain, further identification of spinal sources of ATP would contribute to the knowledge of the pathogenesis of chronic pain.

Spinal cord astrocytes are in intimate contact with nociceptor nerve terminals (Ventura and Harris, 1999; Zerari et al., 1998) and they possess receptors for two important nociceptive transmitters - glutamate (Agrawal and Fehlings, 1997) and SP (Marriott et al., 1991; Palma et al., 1997; Torrens et al., 1986; Wienrich and Kettenmann, 1989; Zerari et al., 1998). Binding of these receptors activates intracellular signaling processes which have been linked to ATP release, including Ca^{2+} liberation and phosphoinositide turnover (Abdipranoto et al., 2003; Ahmed et al., 1990; Hartung et al., 1988; Heath et al., 1994; Marriott et al., 1991; Queiroz et al., 1999). In addition, cortical astrocytes release ATP in response to glutamate and release much greater amounts of ATP than neurons, suggesting astrocytes could be a much more powerful source of ATP than other cell types (Queiroz et al., 1997; Queiroz et al., 1999). Considering this, it is natural to enquire whether spinal cord astrocytes, excited by the concomitant application of glutamate and SP, release ATP in relatively large amounts that could excite the range of ionotropic purinergic receptors, which have been so clearly implicated in neuropathic and inflammatory pain. We report here that glutamate can stimulate ATP release from spinal cord astrocytes and this release is greatly enhanced when SP is present. Mechanisms involved in this phenomenon are reported.

3.2 MATERIALS AND METHODS

3.2.1 Spinal cord and cortical astrocyte cell cultures

Detailed protocol for the preparation of astrocyte cultures is given in section 2.2. Briefly, spinal cords and cortices from 0 – 2 day old Sprague-Dawley rats were dissected out, and the meninges and, where appropriate, peripheral nerves were removed. The remaining spinal cord or cortical tissue was enzymatically and mechanically dissociated then washed with DMEM+. Dissociated cells were plated on a poly-D-lysine (20 μg/mL) coated culture flask for 4 h in a 5% CO_2 incubator at 37°C, were washed and then maintained at 5% CO_2 and 37°C in DMEM +, replaced every 3 – 4 days. To purify astrocytes, 14 days after initial plating, flasks were shaken for 40 min at 400 rpm and 37°C and this was repeated 20 days after initial plating for 16 h. Additionally, 16 days after initial plating DMEM+ was replaced with DMEM supplemented by 10% horse serum, 1% penicillin/streptomycin/ glutamine and 25 mM sorbitol. After 21 days, the age at which there remains only one predominant (> 90%) subtype of spinal cord astrocyte (Black et al., 1993), cells were transferred to 13 mm diameter round glass coverslips coated in 20 μg/ml poly-D-lysine. Cells were used for ATP release, electrophysiology and immunocytochemistry experiments 1 to 2 days after transfer. The purity of cultures was verified by immunocytochemistry.

3.2.2 Online bioluminescence

Detailed protocol for the measurement of ATP by online-bioluminescence is given in section 2.3. Briefly, coverslips were placed inside a light-impermeable chamber with ATP assay mix added (1 mg/ml) and the number of photons released were measured

every second by a P30CWAD5-45 Photodetector Package. When photon count readings did not varying by more than 10% over 10 minutes, the average photon count over this period of time was recorded as the baseline level. After the baseline was reached, recording of photon counts was stopped and agonists of interest were applied at 10% of the volume of liquid on the coverslip. Recording was immediately recommenced (within 20 s). Chemicals were not washed out during experimentation. At the end of each experiment, 1% Triton X-100 was added to lyse cells, allowing an approximation of the number of cells on the coverslip. For each batch of ATP assay mix used for experiments, a standard curve was computed using known concentrations of ATP allowing conversion of the photon count data to ATP concentration values. ATP release data was analysed to find the peak increase in ATP release relative to baseline (referred to as 'relative peak ATP level'). Experiments were repeated at least three times and results were expressed as mean relative peak ATP level ± SEM. Means were analysed for statistically significant differences with an unpaired Student's two-sample two-tailed *t*-test. $p < 0.05$ was considered statistically significant. All inhibitors were applied 1 h prior to addition of agonists.

3.2.3 Patch-clamp recording

Detailed protocol for the measurement of whole-cell currents by patch-clamp recording is given in section 2.4. Briefly, pipettes used had resistances between 4 - 8 M Ω when a patch pipette was filled with pipette solution detailed in section 2.4. Mg^{2+} was omitted from the bath solution when NMDA was used as an agonist. Whole-cell currents were recorded under voltage-clamp mode at a holding membrane potential of -60 mV. Currents were recorded with a Multiclamp 700A amplifier and

they were sampled on-line using a Digidata 1322A interface and pClamp8 program. Glutamate, its analogs and SP were applied through pressure ejection via a picosprizer.

3.2.4 Fluorescence immunocytochemistry

Detailed protocol for the detection of cellular proteins using fluorescent antibodies is given in section 2.5. Briefly, coverslips described in section 3.2.1 taken from the same culture batches used in bioluminescence experiments were washed twice with HEPES buffered salt solution, were fixed with 2% paraformaldehyde solution for 10 min, washed twice for 5 min each with PBS and then incubated in PBS containing 1% BSA and 0.05% saponin for 30 min. Cells on coverslips were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 1% BSA and 0.05% saponin. Primary antibodies were washed out three times for 10 min each with PBS and then cells were incubated in secondary antibodies diluted in PBS containing 1% BSA and 0.05% saponin for 1 h at room temperature. Secondary antibodies were washed out three times with PBS for 10 min each wash. Excess moisture was removed from the back of coverslips and they were mounted on rectangular glass coverslips using ProLong Gold anti-fade mounting media with DAPI. Slides were sealed with nail polish and subsequently stored at 4°C until viewing.

Fluorescence was visualised with an Axioplan2 upright microscope and images were acquired with an AxioCam HR digital monochrome CCD camera using Axiovision 3.1 software. Experiments with each antibody were performed on at least three different batches of cultures and were repeated at least twice on each of the

batches. For each coverslip at least three fields of view were imaged. As a control for each antibody, experiments were also run without a primary antibody and no non-specific binding of secondary antibodies was observed. As a further control, experiments were run without secondary antibodies and no autofluorescence of primary antibodies was observed.

To verify the results obtained using the method above, experiments probing the NR1 and GluR4 subunits were repeated with one of the following changes per experiment: omission of DAPI from the mounting media; substitution of the step incubating cells in saponin and BSA with three steps incubating cells in 1% Triton X-100 which was washed out three times with PBS then a 1 h incubation in 1% BSA; use of a different rabbit anti-rat NMDA receptor 1 antibody; incubation of cells in the rabbit anti-rat glutamate receptor antibodies prior to fixation of cells with formaldehyde solution. Results using these different methods were consistent.

3.3 RESULTS

3.3.1 SP enhances glutamate-stimulated ATP release from spinal cord astrocytes

We initially determined the ability of SP and glutamate to stimulate ATP release from spinal cord astrocytes when applied separately and together using online bioluminescence. Glutamate (1 mM) elicited approximately a two-fold increase in ATP release levels at the peak of its release compared to the baseline level (2.19 ± 0.11; $n = 67$; Figure 3.1A, B). This measure is referred to as relative peak ATP level. Peak ATP release was reached 5 - 7 min after application of glutamate and ATP release levels returned to baseline within 30 min after application (Figure 3.1B). When applied on its own at concentrations of 100 μM or less, SP did not change ATP release levels from the baseline level (1 μM, $n = 3$; 10 μM, $n = 12$; 30 μM, $n = 3$; 100 μM, $n = 15$; Figure 3.1A, B). However, when 100 μM SP and 1 mM glutamate were co-applied, ATP release levels significantly increased to approximately seven-fold above the baseline level (7.35 ± 0.68; $n = 38$; $p < 0.001$; Figure 3.1A, B). This equated to a 336% potentiation of glutamate-stimulated ATP release by SP compared to the effect of glutamate on its own. SP did not alter the time course of glutamate-stimulated ATP release (Figure 3.1B).

To verify the identity of the cells being acted upon by SP and glutamate to bring about the modulation of ATP release, immunocytochemistry was carried out. Over 90% of cells were positive for the astrocyte-specific intermediate filament marker GFAP, whilst less than 10% were positive for microglial and macrophage marker CD11b. Anti-galactocerebroside non-specifically labelled all cells. Consequently

oligodendrocyte presence was identified on the basis of morphological characteristics and oligodendrocytes were found to comprise < 1% of total cells. There were no neurons present in cultures as evidenced by a lack of NeuN staining. To examine a possible contribution of microglia and oligodendrocytes to the modulation of glutamate-stimulated ATP release by SP, the effect of SP on glutamate-stimulated ATP release from coverslips with total microglia and oligodendrocyte populations of ≤ 4% was compared to coverslips with the same number of astrocytes but with microglia and oligodendrocytes added until the total microglial and oligodendrocyte population reached 30%. In cells with ≤ 4% microglia and oligodendrocytes, SP (100 μM) increased glutamate (1mM)-stimulated ATP release by 412% with a glutamate-stimulated ATP release of 1.64 ± 0.16 (n = 3) fold above basal ATP release values and a SP and glutamate-stimulated ATP release of 6.78 ± 1.32 (n = 4) fold above basal levels. Similarly, on cells with 30% microglia and oligodendrocytes, SP (100 μM) increased glutamate (1mM)-stimulated ATP release by 379% with an ATP release of 1.61 ± 0.15 (n = 3) fold above basal ATP release values being elicited by glutamate only and of 6.10 ± 2.98 (n = 3) fold by co-application of SP and glutamate. There was no significant difference in values between the ≤ 4% contaminant cultures and the 30% contaminant cultures. Furthermore, glutamate (1 mM) stimulated an ATP release of 2.51 ± 0.30 (n = 5) fold above basal ATP release values from pure microglial cultures and substance P (100 μM) did not significantly increase this release (3.26 ± 1.67; n = 5). These results suggest microglia and oligodendrocytes do not contribute to the interaction of SP with glutamate-stimulated ATP release and that the potentiation results from SP and glutamate acting on astrocytes.

It was possible that ATP measured on application of agonists could be a consequence of adenosine release through the equilibrative nucleoside transporter 1 and subsequent anabolism by adenosine kinase then adenylate kinase to ATP. Neither the equilibrative nucleoside transporter 1 antagonist NBMPR (10 μM; 7.06 ± 1.78, n = 6) nor the adenosine kinase antagonist iodotubercidin (10 μM; 7.92 ± 1.01; n = 3) significantly changed SP and glutamate-stimulated ATP release. Similarly the ecto-ATPase inhibitor ARL 67156 did not have a significant effect on SP and glutamate-stimulated ATP release (100 μM; 6.27 ± 1.10; n = 4).

To examine if SP also potentiated glutamate-induced currents in spinal cord astrocytes, glutamate-induced whole cell currents were recorded and the effects of SP on them were determined. In 17 out of 19 cells tested, glutamate (1 mM) caused slow inward currents which took approximately 10 s to peak. The average peak amplitude of the inward currents was 49 ± 9 pA. SP (100 μM) did not cause inward currents in any cells examined (n = 4). However, on co-application of glutamate and SP, inward currents were significantly larger than that recorded from astrocytes exposed to glutamate alone (150 ± 29 pA; n = 3; $p < 0.05$; Figure 3.1C).

As cortical astrocytes express both glutamate (Janssens and Lesage, 2001) and SP receptors (Beaujouan et al., 1990), it was determined whether SP could also potentiate glutamate-stimulated ATP release from cortical astrocytes. When applied by itself, 100 μM SP did not cause ATP release (n = 5). Glutamate (1 mM) elicited ATP release from cortical astrocytes (1.80 ± 0.14; n = 14), but this relative ATP release level was not significantly enhanced by SP (2.17 ± 0.24; n = 12;).

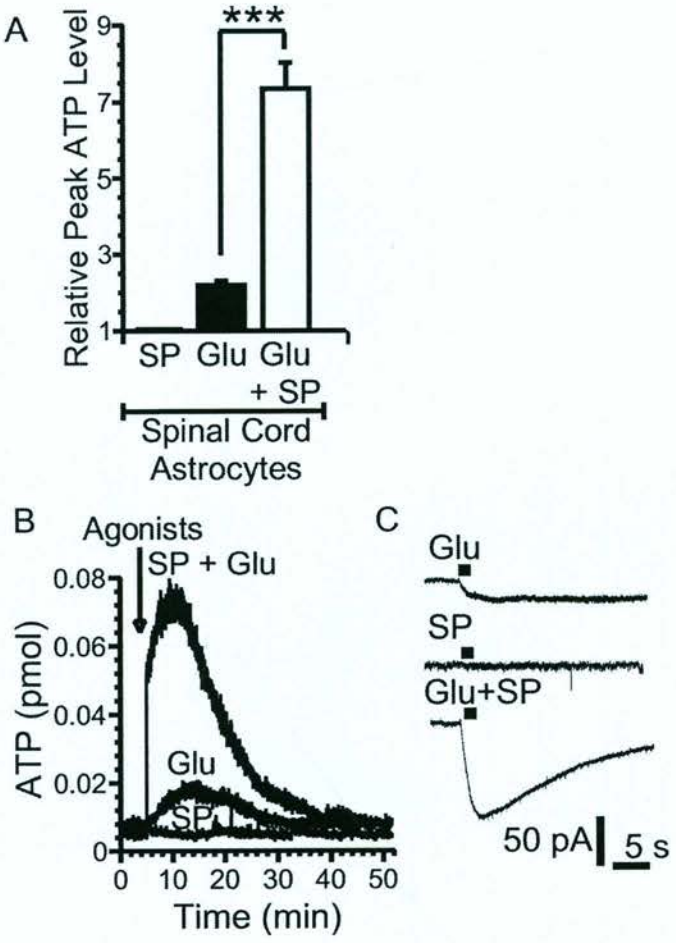


Figure 3.1 SP potentiates glutamate-stimulated ATP release from cultured spinal cord astrocytes. A) The effect of application of 100 μM SP (SP; n = 15), 1 mM glutamate (Glu; n = 67) and co-application of 1 mM Glu and 100 μM SP (Glu + SP; n = 38) on ATP release relative to the basal release level from cultured spinal cord astrocytes. In this figure and histograms in following figures, relative peak ATP level is defined as the peak ATP release divided by the baseline ATP value and is expressed as mean ± SEM in this and subsequent figures involving relative peak ATP level data. B) Representative time courses of ATP release induced by these agonists from spinal cord astrocytes. The arrow represents time of addition of these agonists to the bath. C) A representative trace of a glutamate (1 mM)-induced inward current from a spinal cord astrocyte, the amplitude of which was dramatically enhanced by 100 μM SP, while SP itself did not induce currents. The black bars above the current traces represent the duration of agonist application. ***$p < 0.001$.

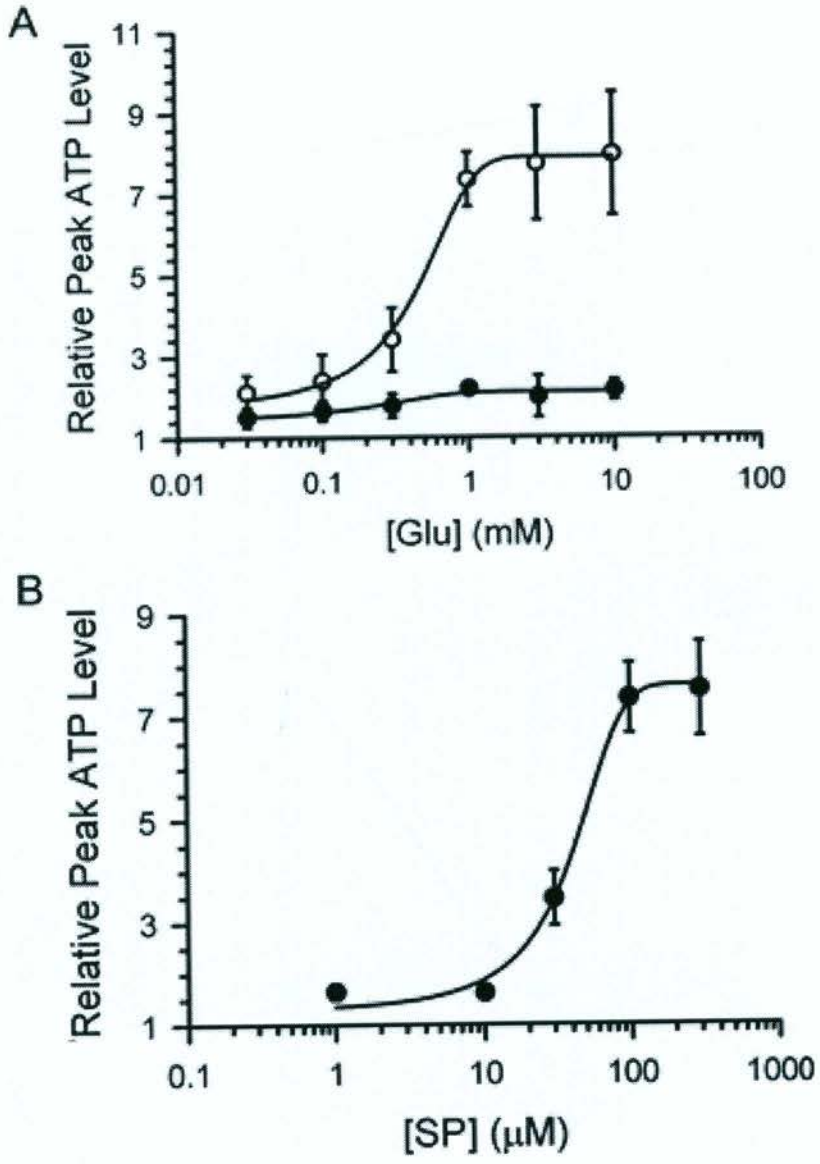


Figure 3.2 Glutamate-stimulated ATP release and the potentiation of this by SP is concentration-dependent. A) Glutamate dose-dependently stimulated ATP release (lower line/black circles; EC_{50} = 234 μM; E_{max} = 2.11) and the degree of modulation by SP (100 μM) increased with increasing concentrations of glutamate (top line/white circles; EC_{50} = 347 μM; E_{max} = 7.91). B) SP dose-dependently increased glutamate-stimulated ATP release when it was co-applied with a constant 1 mM glutamate (EC_{50} = 34 μM; E_{max} = 7.60). For each concentration, n is at least 3.

3.3.2 Glutamate-stimulated ATP release and potentiation of this by SP is concentration- and time-dependent

To examine if the interaction between SP and glutamate was dependent on the concentration of glutamate, ATP release was measured on co-application of increasing concentrations of glutamate with a constant 100 μM SP, and compared with the level of ATP release stimulated by the corresponding concentration of glutamate on its own. In the absence of SP, 30 μM glutamate elicited ATP release (1.51 ± 0.25; n = 4) and increasing concentrations augmented ATP release in a dose-dependent manner (100 μM, 1.66 ± 0.24, n = 4; 300 μM, 1.78 ± 0.29, n = 3; 1 mM, 2.19 ± 0.11, n = 67). Concentrations above 1 mM (3 mM, 1.99 ± 0.51, n = 4; 10 mM, 2.15 ± 0.25, n = 3) did not further increase ATP release. The EC_{50} was 234 μM and E_{max} was 2.11 when glutamate was applied alone (Figure 3.2A). When 100 μM SP was co-administered with glutamate, potentiation of the level of glutamate-stimulated ATP release was seen even at the lowest concentrations of glutamate examined (30 μM, 2.11 ± 0.43, n = 3; 100 μM, 2.39 ± 0.66, n = 4). Although ATP release stimulated by co-application of SP with 30 μM and 100 μM glutamate was not significantly larger than the ATP release by corresponding concentrations of glutamate in the absence of SP, their relative values were comparable with the E_{max} of glutamate alone. Statistically significant increases in levels of ATP release were seen at concentrations of 300 μM glutamate (3.41 ± 0.78, n = 6, $p < 0.05$) and above (1 mM, 7.35 ± 0.68, n = 38, $p < 0.001$; 3 mM, 7.74 ± 1.40, n = 4, $p < 0.01$; 10 mM, 7.96 ± 1.51, n = 3, $p < 0.05$). The EC_{50} was 347 μM which was comparable with that obtained on application of glutamate in the absence of SP,

however the E_{max} was 7.91 which was appreciably higher than that obtained with glutamate alone (Figure 3.2A).

To examine if the interaction between SP and glutamate was dependent on the concentration of SP, ATP release was measured on co-application of varied concentrations of SP with a constant 1 mM glutamate and compared with that elicited by 1 mM glutamate on its own. No significant increases above the glutamate-only level were seen with co-application of 1 μM (1.65 ± 0.16, n = 3) or 10 μM (1.63 ± 0.13, n = 3) SP. Significant increases were seen with higher concentrations of SP (30 μM, 3.47 ± 0.53, n = 4, $p < 0.05$; 100 μM, 7.35 ± 0.68, n = 38, $p < 0.001$; 300 μM, 7.52 ± 0.92, n = 7, $p < 0.001$). The EC_{50} of SP was 34 μM and the E_{max} was 7.60 (Figure 3.2B). Consequently, SP was used at a concentration of 100 μM and glutamate at 1 mM for the remaining studies.

The time-dependence of the potentiation of glutamate-stimulated ATP release by SP was investigated by applying glutamate at different intervals after SP application. SP potentiated glutamate-stimulated ATP release when the two agonists were co-applied (0 min, 7.35 ± 0.68, n = 38). However when glutamate was added 30 secs (2.10 ± 0.21, n = 3), 2 min (2.23 ± 0.29, n = 3), 5 min (2.13 ± 0.39, n = 3), 10 min (2.27 ± 0.35, n = 3) and 15 min (1.76 ± 0.29, n = 3) after SP, no potentiation was observed. Therefore SP and glutamate were co-applied in further experiments. An examination of the degree of potentiation when SP was applied at different intervals after glutamate application could not be performed. This was because levels of ATP release were increasing after application of glutamate, and this prevented comparison of potentiation between experiments. It was not examined whether the time-

dependency of the potentiation was inherent in the mechanisms leading to the modulation or whether it was attributable to the breakdown of SP by endopeptidases, such as neutral endopeptidase 24.11, known to be expressed on spinal cord astrocytes (Horsthemke et al., 1984; Lentzen and Palenker, 1983; Marcel et al., 1990; Vincent et al., 1994).

3.3.3 Neurokinin receptors mediate the potentiation of glutamate-stimulated ATP release by SP

It was investigated what subtypes of neurokinin receptor were acted on by SP to potentiate glutamate-stimulated ATP release. The selective NK1 agonist [Sar^9, $Met(O_2)^{11}$]-substance P (100 μM; 2.06 ± 0.45; n = 4), the selective NK2 agonist [β-Ala^8]-Neurokinin A Fragment 4-10 (64 μM; 1.74 ± 0.51; n = 3) and the selective NK3 agonist senktide (100 μM; 1.83 ± 0.21; n = 3) did not significantly potentiate glutamate-stimulated ATP release (Figure 3.3A). However when [Sar^9, $Met(O_2)^{11}$]-substance P and [β-Ala^8]-neurokinin A Fragment 4-10 were co-applied with glutamate, a significant potentiation of glutamate-stimulated ATP release was observed (3.51 ± 0.38; n = 3; $p < 0.05$; Figure 3.3A). Furthermore when all three neurokinin agonists were co-applied with glutamate, greater potentiation of glutamate-stimulated ATP release was recorded (4.85 ± 0.94; n = 6; $p < 0.05$; Figure 3.3A). The NK1 and NK2 preferring antagonist spantide I (3 μM) significantly reduced SP and glutamate-stimulated relative ATP release to levels seen when glutamate was applied alone (2.03 ± 0.31; n = 4; $p < 0.001$; Figure 3.3B). Similarly, the selective neurokinin-1 antagonist L703606 (10 μM) abolished the potentiating effects of SP (1.90 ± 0.25; n = 4; $p < 0.001$; Figure 3.3B, C).

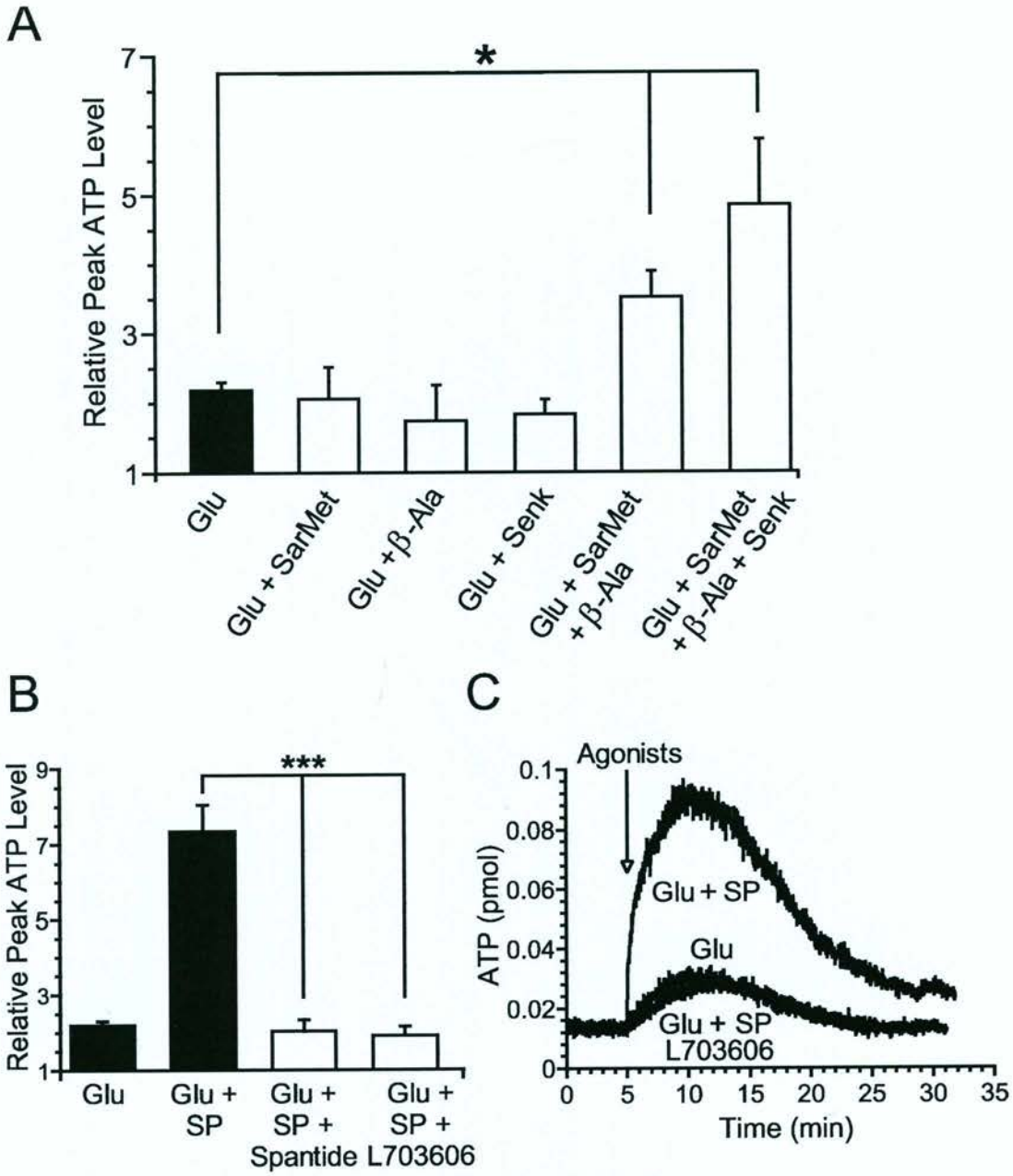


Figure 3.3. SP acts on neurokinin receptor subtypes to potentiate glutamate-stimulated ATP release. A) Summary histogram of the effect of neurokinin receptor agonists [Sar^9, $Met(O_2)^{11}$]-substance P (SarMet; NK1 receptor agonist; 100 μM; n = 4), [β-Ala^8]-Neurokinin A Fragment 4-10 (β-Ala; NK2 receptor agonist; 64 μM; n = 3) and senktide (Senk; NK3 receptor agonist; 100 μM; n = 3) on glutamate-stimulated ATP release. Significant potentiation of glutamate-stimulated ATP release was seen only on co-application of neurokinin receptor subtype agonists. B) Pre-incubation in Spantide I (Spantide; NK1 and NK2 receptor antagonist; 3 μM; n = 4) and L703606 (NK1 receptor antagonist; 10 μM; n = 4) abolished the potentiation of glutamate-stimulated ATP release by SP. C) Representative time courses of ATP release stimulated by addition of glutamate, glutamate and SP and glutamate and SP in the presence of L703606. * $p < 0.05$, *** $p < 0.001$.

3.3.4 Glutamate activates AMPA receptors to bring about ATP release

The contribution of NMDA, AMPA, kainate and metabotropic glutamate receptors to glutamate-stimulated ATP release in the absence of SP was examined. Application of 50 μM AMPA stimulated ATP release (2.41 ± 0.34; n = 9) whereas 5 mM kainate (n = 3), 1 mM tACPD (n = 4), an mGluR group I and II agonist, and 5 mM NMDA (n = 3) did not lead to ATP release (Figure 3.4A, B). In agreement with these results, glutamate-stimulated ATP release was significantly reduced by 50 μM GYKI52466 hydrochloride (AMPA receptor antagonist; 1.16 ± 0.16; n = 5; $p < 0.001$) and 20 μM CNQX (AMPA and kainate antagonist; 1.2 ± 0.07; n = 4; $p < 0.001$; Figure 3.4C, D). In contrast, it was not significantly affected by 30 μM GAMS, a kainate receptor antagonist (1.78 ± 0.22; n = 4), 500 μM MCPG, an mGluR group I and II antagonist (1.85 ± 0.21; n = 4) and 40 μM MK-801, an NMDA receptor antagonist (2.39 ± 0.44; n = 5; Figure 3.4C). Additionally, AMPA (1 mM) induced slow inward currents which were similar to those induced by glutamate in all spinal cord astrocytes tested (50 – 75 pA; n = 3; Figure 3.4E). NMDA (1 mM; n = 4) and tACPD (1 mM; n = 5) did not elicit inward currents in spinal cord astrocytes (Figure 3.4E).

To examine whether glutamate receptor subtypes were available on the membrane of spinal cord astrocytes to be bound by glutamate, immunocytochemistry was conducted. The AMPA GluR2/3 subunit was found in fine clusters throughout the cytoplasm and membrane of all GFAP-positive spinal cord astrocytes imaged (n = 35 astrocytes; Figure 3.5A). In contrast, the AMPA GluR4 subunit was localized specifically to an area overlapping and immediately surrounding the DAPI-stained nuclei of spinal cord astrocytes (n = 36 astrocytes; Figure 3.5B). An antibody

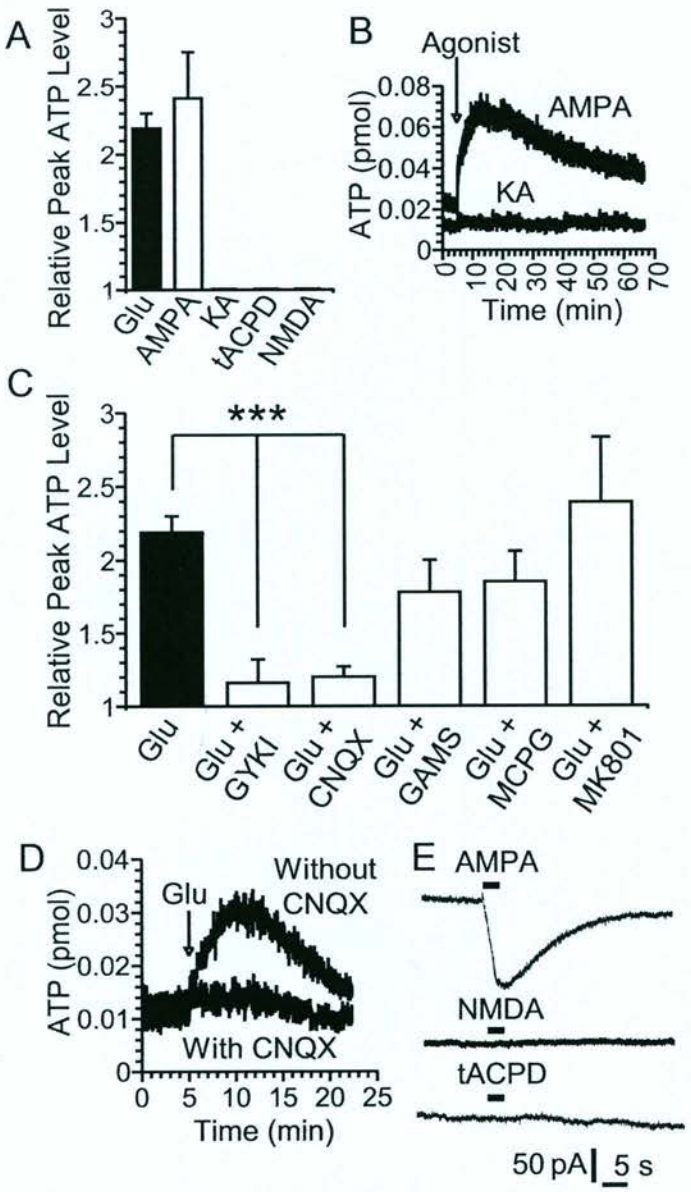


Figure 3.4 Glutamate stimulates ATP release by activating AMPA receptors. A) AMPA stimulated ATP release (50 μM; n = 7), however no ATP release was seen on application of kainate (KA; 5 mM; n = 3), tACPD (mGluRI and II agonist; 1 mM; n = 4) or NMDA (5 mM; n = 3). B) Time course of the effect of AMPA and kainate on ATP release. C) Glutamate-stimulated ATP release was significantly reduced by application of GYKI52466 (GYKI; AMPA receptor antagonist; 50 μM; n = 5) and CNQX (AMPA and kainate receptor antagonist; 20 μM; n = 4), but was not significantly reduced by application of GAMS (kainate receptor antagonist; 30 μM; n = 4), MCPG (mGluRI and II antagonist; 500 μM; n = 4) and MK-801 (MK801; NMDA receptor antagonist; 40 μM; n = 5). D) Time course of the effect of CNQX on glutamate-stimulated ATP release. E) Representative traces of patch clamp recordings from spinal cord astrocytes on application of AMPA (1 mM), NMDA (1 mM) and tACPD (1 mM). Inward membrane currents were induced by AMPA but not by NMDA or tACPD. The black bars above the current traces indicate the duration of addition of the agonists. ***$p<0.001$.

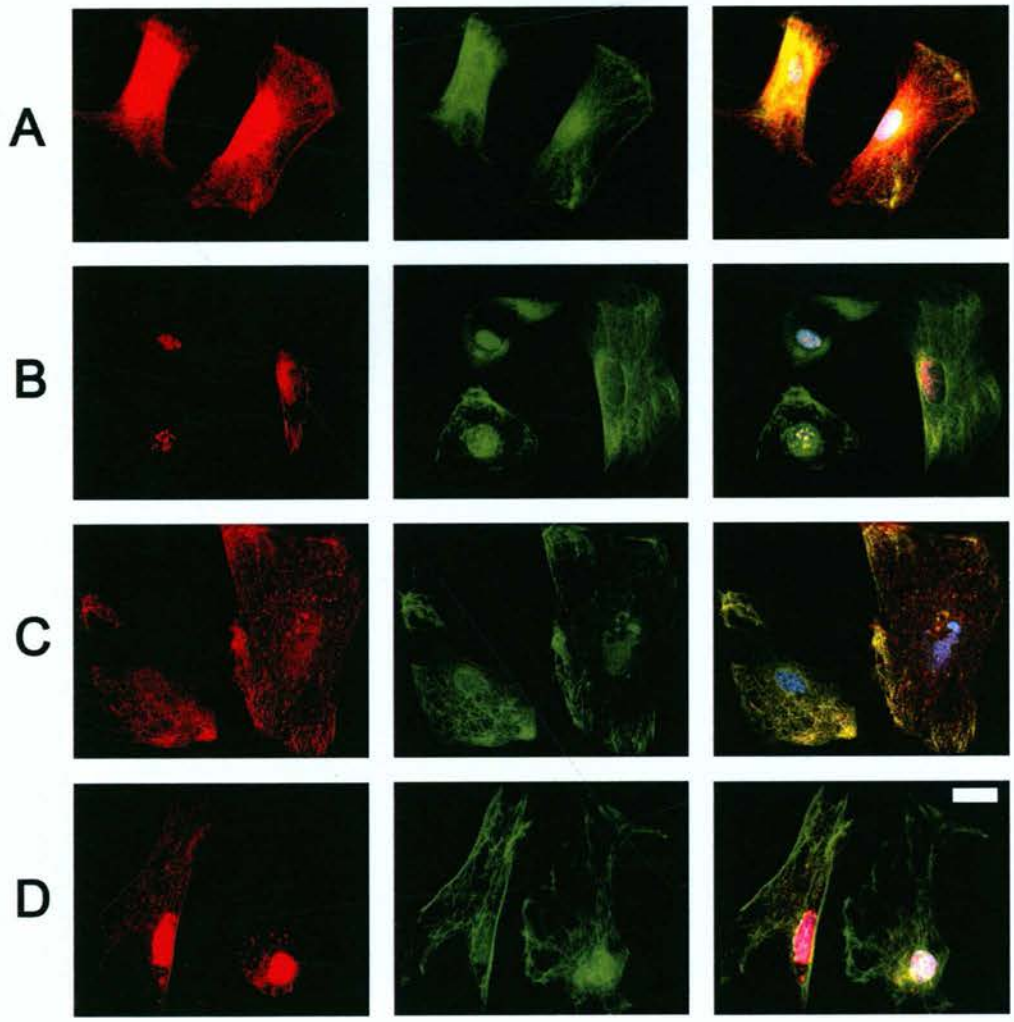


Figure 3.5 Expression of AMPA, NMDA and mGluRs in spinal cord astrocytes. Representative examples of AMPA receptor subunits GluR2 and GluR3 staining of spinal cord astrocytes (A), AMPA receptor subunit GluR4 staining (B), mGluR1a and mGluR5 staining (C) and NMDA receptor subunit NR1 staining (D). Images on the left display glutamate receptor subtype staining in red, the middle images display astrocyte intermediate filament marker GFAP staining in green and the right images are the previous two images merged with additional DAPI staining of the nucleus in blue. Co-localisation of glutamate receptor and GFAP staining appears yellow while co-localisation of glutamate receptor and nucleus staining appears violet. The white bar in the bottom right box represents 20 μm and holds for all images in the figure.

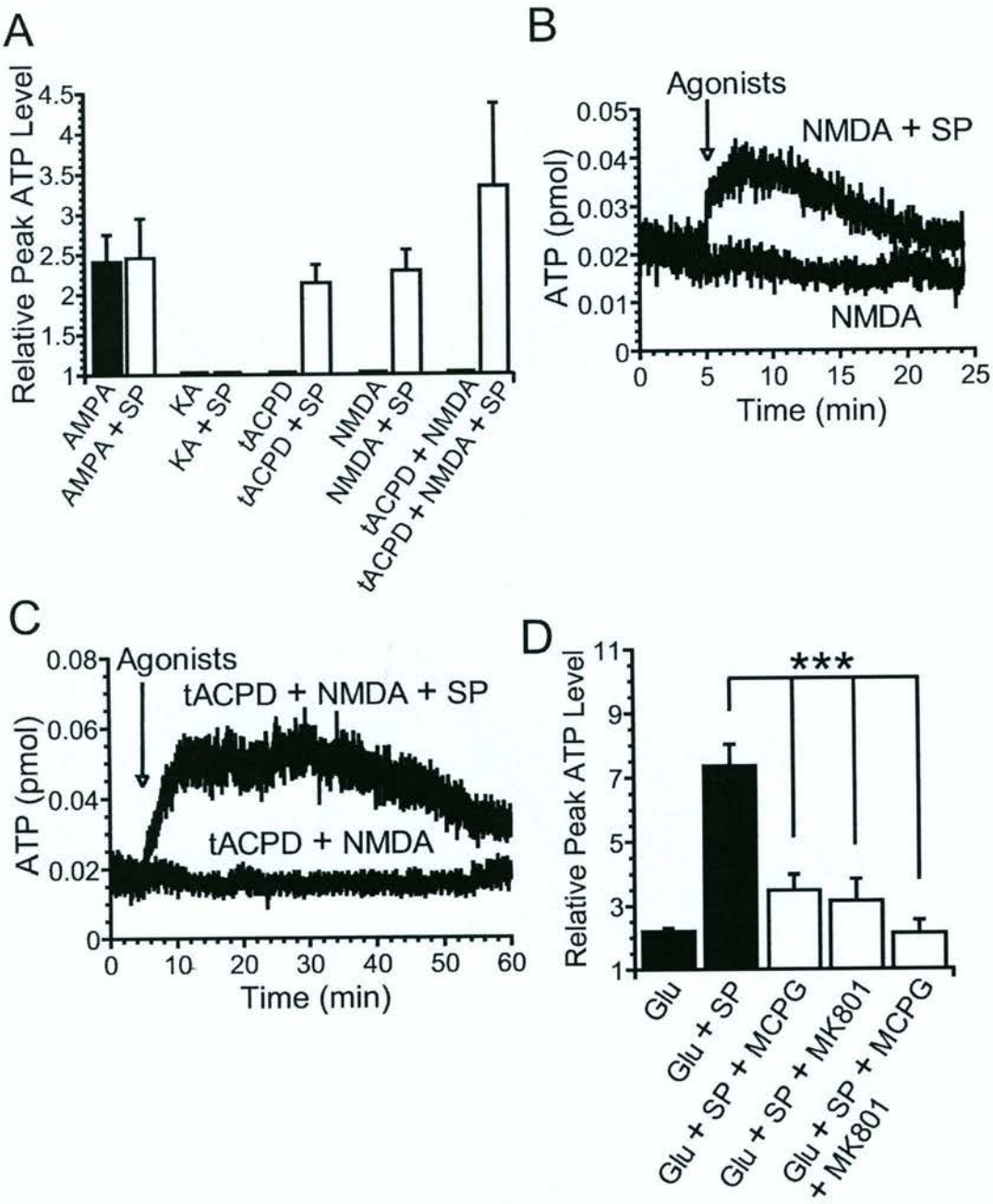


Figure 3.6 SP allows glutamate to stimulate ATP release through NMDA and mGluRs. A) SP (100 μM) did not affect the amount of ATP stimulated by glutamate receptor subtype agonists AMPA (50 μM; n = 9 without SP and n = 5 with SP) and kainate (KA; 5 mM; n = 3 without SP and n = 3 with SP). tACPD (1 mM; n = 4) and NMDA (500 μM, n = 3) did not lead to ATP release on their own but, when co-applied with SP, ATP release was seen (n = 7 for tACPD plus SP and n = 9 for NMDA plus SP). When NMDA and tACPD were co-applied with SP (n = 6), relative ATP release levels approximated that obtained by summating release levels from each agonist plus SP separately. B) Time course of the effect of SP on NMDA- and C) NMDA plus tACPD-stimulated ATP release. D) The potentiation of glutamate-stimulated ATP release was significantly reduced by application of MCPG (500 μM; n = 5) and MK-801 (40 μM; n = 6). Co-application of MCPG and MK-801 completely removed the effect of SP (n = 4). ***$p<0.001$.

recognising the mGluR1a (a splice variant of the mGluR1 receptor) and the mGluR5 receptor stained positively on 8.8% of imaged GFAP-positive astrocytes (n = 29 of 329 astrocytes) and was distributed in thick clusters throughout the membrane and cytoplasm of spinal cord astrocytes (Figure 3.5C). The NMDA receptor subunit NR1 was found localised to an area overlapping the DAPI-stained nuclei of spinal cord astrocytes in 64% of cells imaged (n = 74 of 115 astrocytes) whilst distribution of NR1 also extended throughout the membrane and cytoplasm in the remaining cells imaged (n = 41 of 115 astrocytes; Figure 3.5D). The co-localisation of GluR4 and NR1 with astrocytic nuclei persisted despite the use of a variety of immuno-cytochemical methods and antibodies.

3.3.5 SP selectively enables glutamate to stimulate ATP release by binding NMDA receptors and mGluRs

SP is known to enhance the activity of some neuronal glutamate receptor subtypes such as the NMDA receptor (Mjellem-Joly et al., 1991; Randic et al., 1990; Rusin et al., 1993). It was therefore determined whether SP changes the ability of glutamate receptor agonists to stimulate ATP release. Co-application of SP and 500 μM NMDA led to ATP release (2.29 ± 0.26; n = 9; Figure 3.6A, B), as did co-application of SP and 1 mM tACPD (2.15 ± 0.23; n = 7; Figure 3.6A). Application of 500 μM NMDA, 1 mM tACPD and 100 μM SP led to further increases in ATP release (3.34 ± 1.02; n = 6; Figure 3.6A, C). No ATP release was seen on co-application of SP and kainate (5 mM; n = 3; Figure 3.6A). Co-application of SP and AMPA (50 μM; 2.46 ± 0.49; n = 5) led to an ATP release which was not significantly different from application of AMPA alone (Figure 3.6A).

The potentiation by SP of glutamate-stimulated ATP release was significantly reduced by 500 μM MCPG (3.48 ± 0.50; n = 5; $p \leq 0.001$; Figure 3.6D) and 40 μM MK-801 (3.57 ± 0.64; n = 6; $p < 0.001$; Figure 3.6D). Furthermore, SP and glutamate co-stimulated relative ATP release levels were reduced to glutamate-only levels when pre-incubated with both 500 μM MCPG and 40 μM MK-801 (2.13 ± 0.41; n = 4; $p < 0.001$; Figure 3.6D).

The possibility that SP may have brought about the modulation of NMDA receptors by potentiating the function of the glycine co-binding site of the NMDA receptor or by increasing extracellular levels of NMDA receptor co-agonists glycine, D-serine or D-alanine was examined. The potentiation of NMDA (500 μM)-induced ATP release by SP was not significantly changed by pre-incubation in the glycine co-binding site antagonist 7-chlorokynurenic acid (5 μM; 1.76 ± 0.27; n = 6). Another possible mechanism by which SP may have modulated the NMDA receptor was by depolarizing the cell and removing the Mg^{2+} block, however because of the Mg^{2+} dependence of the activity of the bioluminescence enzymes (Wulff, 1983), it was not possible to conduct experiments in which the concentration of Mg^{2+} was varied.

3.3.6 Glutamate transporters are not involved in the potentiation by SP of glutamate-stimulated ATP release

SP is known to be able to inhibit glutamate transporters (Johnson and Johnson, 1993). It is possible, then, that inhibition of glutamate transporters may have led to increased extracellular glutamate concentrations, which may have contributed to the potentiation seen on co-application of SP with glutamate. However glutamate-

stimulated ATP release was not significantly affected by the glutamate transporter antagonist TBOA (10 μM; 2.30 ± 0.27; n = 3).

3.3.7 Multiple intracellular signaling pathways are involved in the potentiation of glutamate-stimulated ATP release by SP

As neurokinin receptors are G-protein coupled receptors linked to the phospholipase C, PLA_2, PLD and AC (Garcia et al., 1994; Hartung et al., 1988; Nakajima et al., 1992; Torrens et al., 1998) signaling pathways, potential roles of each of these cell signaling pathways in the effects of SP were explored. The PKC inhibitor chelerythrine chloride (20 μM) abolished the effects of SP on glutamate-stimulated ATP release (2.09 ± 0.53; n = 6; $p < 0.001$; Figure 3.7A), as did PLA_2 inhibitor aristolochic acid (50 μM; 2.40 ± 0.16; n = 4; $p < 0.001$; Figure 3.7A, B). The potentiation of glutamate-stimulated ATP release by SP was also significantly reduced by 0.5% 1-butanol (PLD antagonist; 3.44 ± 0.87; n = 5; $p < 0.01$) and 1 mM LiCl (inositol monophosphatase inhibitor; 3.93 ± 0.73; n = 8; $p < 0.01$), however these antagonists did not fully abolish the effects of SP (Figure 3.7A). Thapsigargin (1 μM), an endoplasmic reticulum Ca^{2+} pump activator inhibitor, also decreased the potentiation (5.43 ± 0.56; n = 6; $p < 0.05$). However thapsigargin reduced the potentiation to the same levels of ATP release as the inactive form of thapsigargin, thapsigargin epoxide (1 μM; 5.55 ± 1.16; n =4). The cell-permeable AC antagonist DDA (50 μM; 8.63 ± 1.66; n = 5) did not significantly affect SP and glutamate-stimulated ATP release (Figure 3.7A). It should be noted that none of these inhibitors significantly decreased glutamate-stimulated ATP release (see section 3.3.8). Furthermore, the potentiation of NMDA-stimulated ATP release

by SP was significantly decreased by 20 μM chelerythrine chloride (1.12 ± 0.33; n = 4; $p < 0.05$; Figure 3.7C), but was not significantly affected by 0.5% 1-butanol (2.54 ± 0.68; n = 4; Figure 3.7C).

As NMDA receptors are Ca^{2+} permeable, the role of extracellular Ca^{2+} influx on NMDA and SP-stimulated ATP release was examined. The potentiation of NMDA-stimulated ATP release by SP was significantly decreased in Ca^{2+}-free bath solution containing 5 mM EGTA compared with the relative peak ATP release level seen when NMDA and SP were added to cells in bath solution containing 500 μM Ca^{2+} (1.10 ± 0.14; n = 4; $p < 0.001$; Figure 3.7D). Influx of extracellular Ca^{2+} was shown to be sufficient to induce ATP release as the Ca^{2+} ionophore A23187 triggered ATP release (10μM; 2.38 ± 0.64; n = 3; Figure 3.7D).

3.3.8 An influx of extracellular Ca^{2+} is needed for glutamate-stimulated ATP release

As some AMPA receptors are Ca^{2+} permeable, the role of extracellular Ca^{2+} influx in glutamate-stimulated ATP release was examined. Astrocytes incubated in Ca^{2+}-free bath solution containing the Ca^{2+} chelator EGTA (5 mM) had significantly reduced glutamate-stimulated ATP release levels (1.30 ± 0.07; n = 5; $p < 0.001$; Figure 3.7D) compared with glutamate-stimulated ATP release from astrocytes in bath solution which contained 500 μM Ca^{2+}, the concentration used throughout the studies described previously. A possible non-receptor source of Ca^{2+} influx on application of glutamate may have been voltage-dependent Ca^{2+} channels which are known to be expressed on astrocytes (D'Ascenzo et al., 2004). However a voltage-dependent Ca^{2+} channel blocker, $CdCl_3$, did not significantly affect glutamate-stimulated ATP release

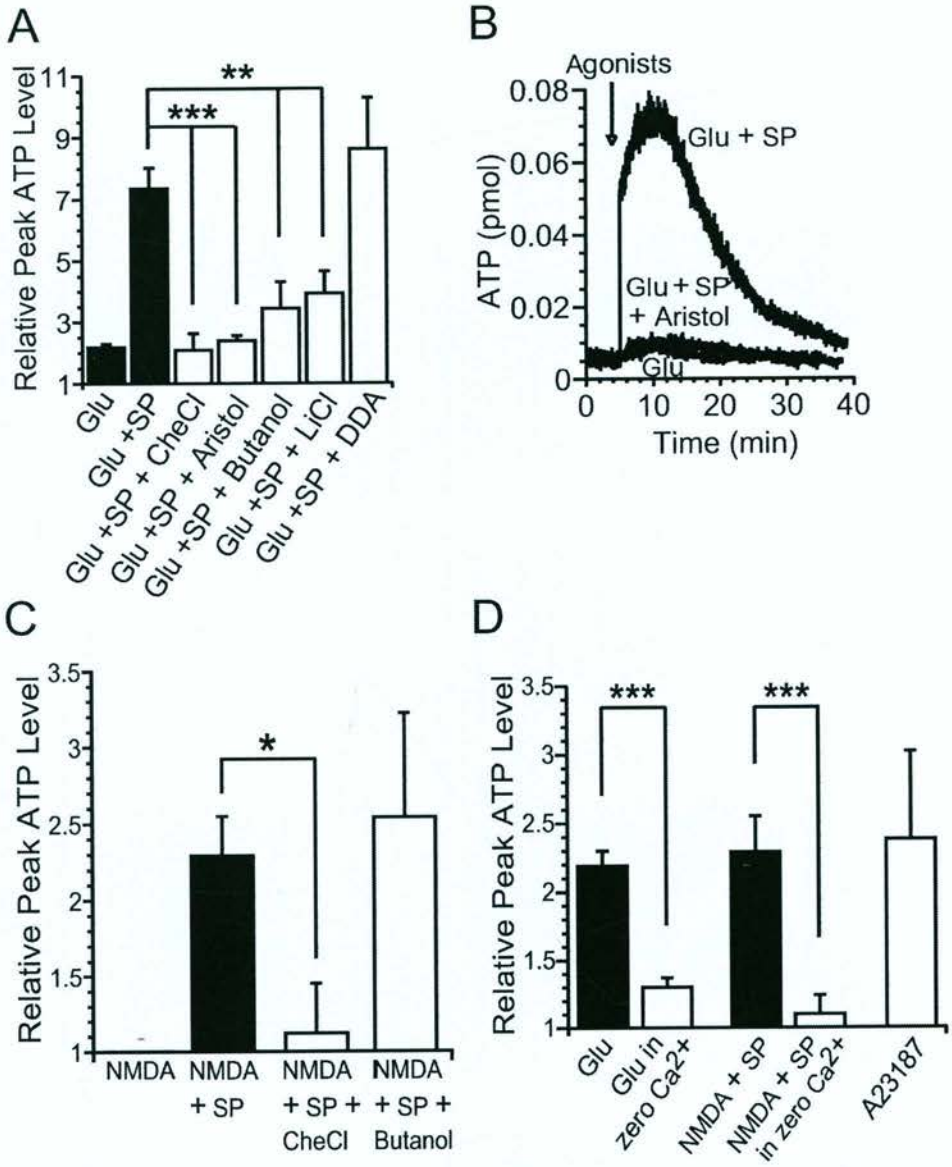


Figure 3.7 The modulation of glutamate-stimulated ATP release by SP requires Ca^{2+} influx and activation of multiple intracellular signaling pathways. A) In the presence of chelerythrine chloride (CheCl; PKC inhibitor; 20 μM; n = 6) or aristolochic acid (Aristol; PLA_2 inhibitor; 50 μM; n = 4) the potentiating effect of SP on glutamate-stimulated ATP release was blocked. In the presence of 1-butanol (PLD antagonist; 0.5%; n = 5) and lithium chloride (LiCl; inositol monophosphatase inhibitor; 1 mM; n = 8) the modulatory effects of SP were also significantly decreased. The potentiation was not significantly altered by DDA (cell-permeable AC inhibitor; 50 μM; n = 5). B) A typical time course of the effect of Aristol on the potentiation of glutamate-stimulated ATP release by SP. C) The potentiation of NMDA-stimulated ATP release by SP was significantly decreased by CheCl (n = 4), but not by 1-butanol (n = 4). D) In Ca^{2+} free bath with 5 mM EGTA (zero Ca^{2+}; n = 5), glutamate-stimulated ATP release was significantly reduced. Similarly, NMDA plus SP-stimulated ATP release was significantly reduced in these Ca^{2+}-free conditions (zero Ca^{2+}; n = 4). The Ca^{2+} ionophore A23187 (10 μM; n = 3) stimulated ATP release. *p<0.05, **p<0.01, ***p<0.001.

(1 mM; 2.40 ± 0.54; n = 3). Additionally, inhibitors to other intracellular signaling molecules known to be associated with glutamate receptors, such as the inhibitors chelerythrine chloride (20 μM; 1.84 ± 0.16; n = 4), aristolochic acid (50 μM; 2.38 ± 0.32; n = 4), 1-butanol (0.5% ; 2.31 ± 0.28; n = 5), LiCl (1 mM; 1.62 ± 0.22; n = 4), thapsigargin (1 μM; 1.84 ± 0.25; n = 4) and DDA (50 μM; 1.75 ± 0.11; n = 3), did not significantly alter glutamate-stimulated ATP release from spinal cord astrocytes.

3.4 DISCUSSION

In this chapter, the ability of glutamate and SP to stimulate ATP release from spinal cord astrocytes was investigated. It was found that glutamate elicited ATP release in a time- and concentration-dependent manner, as is seen with cortical astrocytes (Queiroz et al., 1997). On its own, SP did not elicit ATP release, but greatly potentiated glutamate-stimulated ATP release.

3.4.1 Receptors involved in glutamate-stimulated ATP release and its potentiation by SP

3.4.1.1 Glutamate receptor subtypes involved in glutamate-stimulated ATP release

The glutamate receptors involved in generating glutamate-invoked inward currents and ATP release primarily consist of the AMPA subtype as the effects of glutamate on ATP release were blocked by an AMPA receptor antagonist, but were not significantly blocked by antagonists to kainate, NMDA or mGluRs. Furthermore, only AMPA evoked inward currents and stimulated ATP release. In agreement with what has been reported on *in situ* spinal cord astrocytes (Brand-Schieber et al., 2004; Brand-Schieber and Werner, 2003), immunocytochemistry showed the existence of AMPA GluR2 and GluR3 subunits on the surface of these cultured spinal cord astrocytes. The GluR4 subunit of the AMPA receptor was not found on spinal cord astrocyte membranes. GluR4 subunits have been selectively identified on the end feet of astrocytes surrounding blood vessels (Brand-Schieber et al., 2004), which were absent from cultures in the present study.

Surprisingly ATP release was not elicited by kainate, which is known to activate AMPA receptors (Rodriguez et al., 2005). Kainate has been reported to cause small membrane potential changes in spinal cord astrocytes, although it has not been investigated whether these responses are due to the action on kainate or AMPA receptors (Ziak et al., 1998). Consequently, we hypothesise that in the present case, either kainate does not activate AMPA receptors on spinal cord astrocytes to the extent needed to produce ATP release or AMPA receptors on spinal cord astrocytes are insensitive to kainate, possibly due to the presence of a variant form of kainate receptor that has an altered primary structure at the key amino acid sites that regulate the kainate sensitivity of AMPA receptors (Rodriguez et al., 2005).

3.4.1.2 The effect of SP on glutamate receptor responses

Although NMDA and mGlu receptor agonists did not elicit ATP release when applied alone, when they were each co-applied with SP a release of ATP was observed. SP had no effect on the ATP release responses of spinal cord astrocytes to AMPA and kainate. These results suggest a model whereby glutamate stimulates ATP release through AMPA receptors, but in the presence of SP it can also stimulate ATP release through NMDA and mGlu receptors, leading to the observed potentiation of glutamate-stimulated ATP release. Further supporting this conclusion, simultaneous antagonism of NMDA and mGlu receptors completely abolished the potentiation of glutamate-stimulated ATP release by SP. Also, with regards to the concentration studies carried out, the E_{max} value of glutamate-stimulated ATP release in the presence of SP was much higher than in the absence of SP. This suggests that SP enhanced the number of ATP release-linked glutamate receptors in the membrane.

Immunocytochemistry identified NR1 subunits and mGluR1a and mGluR5 receptors expressed on the surface of a portion of spinal cord astrocytes, in accordance with previous reports (Aicher et al., 1997; Silva et al., 1999; Ziak et al., 1998), yet the fact that glutamate on its own does not stimulate ATP release through these receptors suggests that they are not functionally linked to the ATP release pathway. How SP may link these receptors to the ATP release pathway remains unclear. PKC is known to enhance the activity of NMDA receptors (Liao et al., 2001; Skeberdis et al., 2001) and arachidonic acid downstream of PLA_2 activation is known to enhance the activity of mGluRs (Collins et al., 1995; Sistiaga and Sanchez-Prieto, 2000). As both these signaling molecules are known to be activated on NK1 binding (Monastyrskaya et al., 2005; Rayner and Van Helden, 1997; Tanabe et al., 1996) and are central to the effects of SP in this study, SP may possibly potentiate the activity of these receptors to a degree where they can activate enough signaling molecules to release ATP. In addition, SP exposure may increase the density of NMDA receptors and mGluRs in spinal cord astrocyte membranes.

Although the precise mechanisms of action remain unknown, the interaction between SP and glutamate receptor subtypes described in this current research is similar to the interaction of SP with glutamate receptors on freshly isolated rat dorsal horn neurons. Exposure of these preparations to SP results in a potentiation of NMDA-mediated currents. This effect is specific for the NMDA receptor as AMPA and kainate mediated currents are not affected by SP (Randic et al., 1990). The effect of SP on neuronal mGluRs was not examined.

Surprisingly, the SP and NMDA co-stimulated responses occurred despite the presence of Mg^{2+} at a concentration known to inhibit neuronal NMDA receptor-mediated responses (Ziak et al., 1998). There are several examples of NMDA-mediated responses in glial cells that occur at a physiological level of Mg^{2+}, such as NMDA-stimulated ATP release from cortical astrocytes (Queiroz et al., 1997) and NMDA-induced inward currents in spinal cord astrocytes from rats younger than 14 days (Ziak et al., 1998). As suggested by Ziak et al (1998) and Kutsuwada et al. (1992) perhaps glial NMDA receptors contain Mg^{2+} resistant subunits, such as the NR2C (formerly ε3) subunit.

3.4.1.3 Neurokinin receptors involved in the potentiating effects of SP

To bring about potentiation of glutamate-stimulated ATP release, it is likely that SP acted synergistically on multiple neurokinin receptor subtypes. A NK1 receptor preferring antagonist abolished the effects of SP suggesting that action on the NK1 subtype was necessary for the effects of SP. However a selective NK1 receptor agonist was not sufficient to potentiate glutamate-stimulated ATP release. Similarly, selective stimulation of NK2 or NK3 was not sufficient to potentiate glutamate-stimulated ATP release. It was only when agonists to the neurokinin receptor subtypes were applied in combination that potentiation was observed. This suggests the NK1 receptor is necessary but not sufficient for the actions of SP on glutamate-stimulated ATP release and synergistic action on NK2 and/or NK3 is also necessary for the potentiation. This is consistent with reports of SP acting as an agonist at all three neurokinin receptor subtypes (Drew et al., 2005; Regoli et al., 1994) and with reports of functional NK1, NK2 and NK3 receptors on spinal cord astrocytes (Wienrich and Kettenmann, 1989; Zerari et al., 1998).

It was unlikely that the ATP found in solution after addition of agonists was due to release of adenosine and subsequent actions of adenosine kinase and adenylate kinase to produce ATP. Supporting this statement were the findings that antagonists to an adenosine transporter and adenosine kinase did not affect SP and glutamate-stimulated ATP release. Potential mechanisms of direct ATP release were not explored in this study, however previous studies have reported direct glutamate-stimulated ATP release from cortical astrocytes through exocytosis (Pangrsic et al., 2007; Zhang et al., 2007b).

3.4.2 Ca^{2+} influx and various intracellular signaling pathways participate in glutamate-stimulated ATP release and its potentiation by SP

3.4.2.1 Signaling mechanisms involved in glutamate-stimulated ATP release in the absence of SP

The PLC, PLA_2, AC and PLD signaling pathways did not play a role in glutamate-stimulated ATP release when SP was not present, since antagonists to these pathways had no significant affect. However, influx of Ca^{2+} was centrally involved in glutamate-stimulated ATP release since Ca^{2+} influx was shown to be sufficient to elicit ATP release and removing extracellular Ca^{2+} greatly attenuated glutamate-stimulated ATP release. This Ca^{2+} influx could be directly through non-GluR2 containing AMPA receptors (Hollmann et al., 1991; Verdoorn et al., 1991).

3.4.2.2 Signaling mechanisms involved in glutamate-stimulated ATP release in the presence of SP

Significant decreases in the glutamate-evoked release of ATP in the presence of SP were obtained on blocking PKC, PLA_2, PLD, IP_3 and DAG, but not AC. It is known that activation of NK1 receptors activates PKC in rheumatoid synovial cells (Tanabe et al., 1996) and HEK293 cells (Monastyrskaya et al., 2005) and PLA_2 is also elevated on activation of NK1 receptors on endothelial cells (Rayner and Van Helden, 1997). In murine astrocytes, activation of PKC leads to phosphorylation of PLA_2, necessary for its subsequent action in releasing arachidonic acid (Xu et al., 2002) and PLA_2 and arachidonic acid can activate and translocate PKC (Mau and Vilhardt, 1997; O'Flaherty et al., 2001), possibly explaining the result that SP's potentiating effects are completely removed when blocking PKC but leaving PLA_2 active and vice versa. It is known that SP elevates PLD, PLC and phosphatidylinositol hydrolysis in Chinese hamster cells and astrocytoma cells (Nakajima et al., 1992; Torrens et al., 1998), but that it does not elevate cAMP in cortical astrocytes (Rougon et al., 1983).

ATP release evoked by activation of NMDA glutamate receptors in the presence of SP is greatly reduced in Ca^{2+}-free external solution, indicating that an influx of Ca^{2+} is necessary for this effect. It is unlikely that this influx of Ca^{2+} ions is due to activation of neurokinin receptors as we were unable to detect any inward current in spinal cord astrocytes in response to applied SP. It is known that SP can directly enhance PLC and phophatidylinositol hydrolysis in membrane preparations independent of Ca^{2+} (Nakajima et al., 1992). We suggest that the dependence of the

effect of SP on NMDA receptor-activated release of ATP involves an influx of Ca^{2+} ions through the NMDA receptor channel.

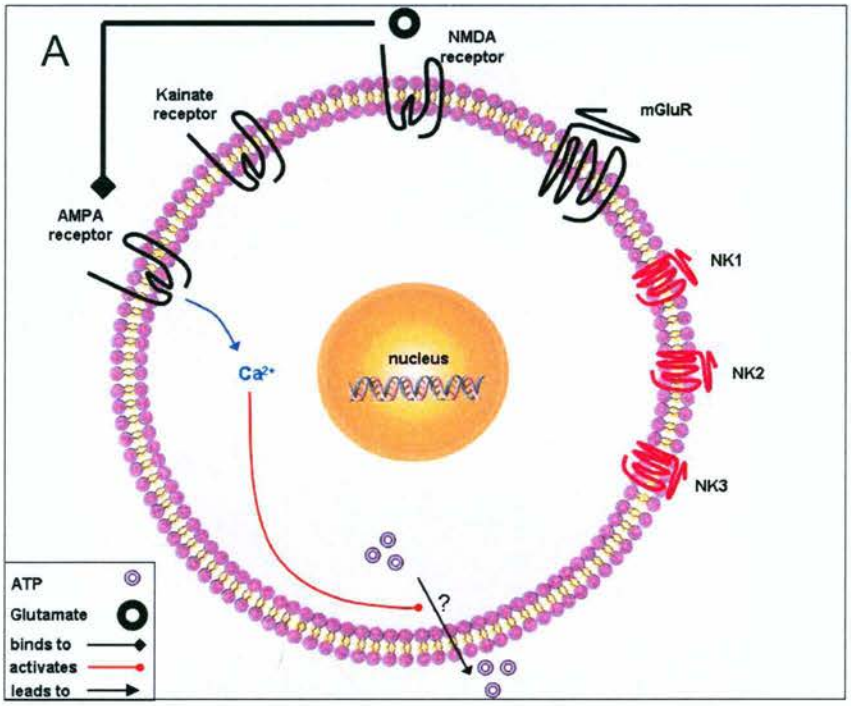
3.4.3 Specificity of the potentiation to spinal cord astrocytes

Interestingly, the potentiation of glutamate-stimulated ATP release by SP was not replicated with cortical astrocytes. The density of NK1 receptors on spinal cord astrocytes has been shown to be twelve times higher than that on cortical astrocytes in rat cultures (Beaujouan et al., 1990) and six times higher in human cultures (Palma et al., 1997). Consequently, in cortical astrocytes SP will be able to activate less NK1-mediated signaling molecules, which are necessary for the interaction to occur, resulting in no interaction. For example, the activation of PLC by NK1 receptors is involved in the potentiation of SP on glutamate-stimulated ATP release, as shown in this study. Phosophoinositide turnover, a consequence of PLC activation, occurs when spinal cord astrocytes are exposed to SP but not when cortical astrocytes are exposed (Cholewinski et al., 1988; Marriott et al., 1991).

Although modulation of glutamate-stimulated ATP release by SP did not occur in cortical astrocytes, it may be of interest to examine whether the modulation occurs with astrocytes from other areas of the CNS that have dense tachykinin receptor expression, such as the basal ganglia and amygdala (Dam et al., 1990; Humpel and Saria, 1993; McLean et al., 1985). Since the actions of SP in the basal ganglia are emerging as an effective target in Parkinson's disease (for a review see Chen et al. (2004)) and its actions in the amygdala are emerging as an effective target in depression treatment (for a review see Rupniak (2002a; 2002b)), the actions of SP on all cell types in these regions should be investigated.

3.4.4 A summary of the mechanisms of glutamate-stimulated ATP release from spinal cord astrocytes in the absence and presence of SP

These observations, of the receptors and signaling molecules important in the interaction between SP and glutamate, suggest that glutamate can elicit ATP release from spinal cord astrocytes via two pathways. The first is the action of glutamate on AMPA receptors resulting in influx of Ca^{2+}. An additional pathway is recruited in the presence of SP through its joint actions on NK1, NK2 and NK3 receptors. Binding of these receptors leads to activation of PKC, PLA_2, PLD and the formation of IP_3 and DAG. The result of this activation is the additional ability of glutamate to bring about ATP release by binding NMDA receptors and mGluRs. These pathways are summarised in Figure 3.8.


A
NMDA receptor
Kainate receptor
mGluR
AMPA receptor
NK1
NK2
NK3
nucleus
Ca2+
?
ATP
Glutamate
binds to
activates
leads to

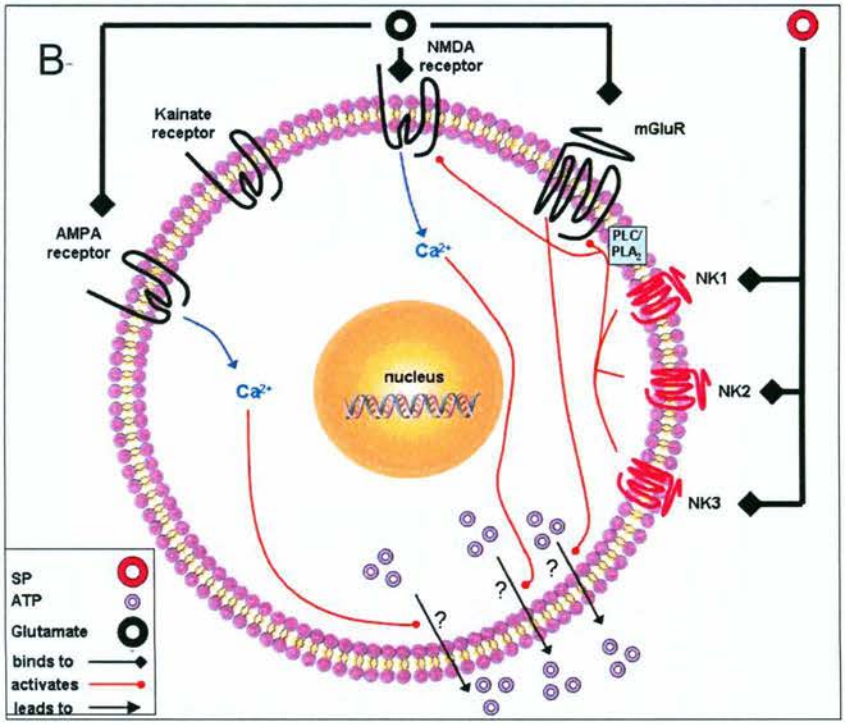

B
NMDA receptor
Kainate receptor
mGluR
AMPA receptor
PLC/PLA2
NK1
NK2
NK3
nucleus
Ca2+
?
SP
ATP
Glutamate
binds to
activates
leads to

Figure 3.8 A model of the mechanisms of glutamate-stimulated ATP release from spinal cord astrocytes and its potentiation by SP. A) In isolation, glutamate acts on the AMPA receptor to bring about an influx of Ca^{2+}. B) When SP is also present, it synergistically acts on the NK1, NK2 and NK3 receptors to activate the NMDA receptor and mGluR. ATP release occurs in a phospholipase C (PLC) and phospholipase A_2 (PLA_2)-dependent manner. The result of this is an increase in the level of glutamate-stimulated ATP release in the presence of SP. The mechanism of ATP release in these two situations is unknown.

CHAPTER 4

RELEASE OF IL-10 FROM SPINAL CORD ASTROCYTES AFTER ACTIVATION OF THE TLR4 AND THE EFFECT OF CONCOMITANT mGLUR ACTIVATION

Contents

4.1 INTRODUCTION

IL-10 is a member of the four α-helix bundle family of cytokines (Zdanov, 2004). It has an anti-inflammatory effect in the CNS, for example it has been reported to decrease the morphological signs of glial activation (Wirjatijasa et al., 2002; Woiciechowsky et al., 2004). Associated with this, IL-10 can decrease the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 from activated glia (Benveniste et al., 1995; Heyen et al., 2000; Kremlev and Palmer, 2005; Ledeboer et al., 2000; Ledeboer et al., 2002; Mizuno et al., 1994; Sawada et al., 1999). Glial activation and high levels of spinal pro-inflammatory cytokines contribute to numerous CNS pathologies, including inflammatory and neuropathic pain (Arruda et al., 2000; DeLeo et al., 1996; Milligan et al., 2003; Reeve et al., 2000; Sung et al., 2004; Watkins et al., 1997; Wieseler-Frank et al., 2005). Spinal application of IL-10 can be beneficial in such conditions, for example intrathecal IL-10 gene therapy abolishes neuropathic pain (Ledeboer et al., 2007; Milligan et al., 2006a; Milligan et al., 2006b). Moreover, patients with a painless neuropathy have on average double the blood IL-10 mRNA levels than those with a painful neuropathy (Uceyler et al., 2007).

Possible sources of IL-10 in the spinal cord during chronic pain have not been examined. Given that spinal cord IL-10 has important anti-nociceptive outcomes, it is of interest to identify possible sources of IL-10. Astrocytes are a source of IL-10 in the brain. Cultured rat and human astrocytes from the brain express an innate immune pattern recognition receptor called the TLR4 which is often experimentally

stimulated with the agonist LPS (Bowman et al., 2003; Bsibsi et al., 2002; Jou et al., 2006). Unstimulated cultured rat brain astrocytes express very low IL-10 mRNA levels and do not release IL-10. When these astrocytes are exposed to LPS, IL-10 mRNA levels are upregulated and IL-10 protein release is increased (Ledeboer et al., 2002). There is a functional regional heterogeneity amongst astrocytes from different regions of the brain, for example, vasopressin and oxytocin elicit phosphoinositide turnover from cortical but not spinal cord astrocytes (Cholewinski et al., 1988). Hence, even though cortical astrocytes show TLR4-stimulated IL-10 release it can not be assumed that spinal cord astrocytes will behave similarly. It is appealing, however, to hypothesise that TLR4 stimulation of astrocytes may be a source of IL-10 in the spinal cord as spinal TLR4s are activated in neuropathic pain (Tanga et al., 2005), cultured spinal cord astrocytes express functional TLR4s (Pehar et al., 2004) and also express a key protein involved in TLR4-stimulated IL-10 release, JNK (Zhuang et al., 2006).

In addition to TLR4-stimulated IL-10 release from spinal cord astrocytes, it is appealing to suggest the neurotransmitter glutamate may be involved in IL-10 release from astrocytes in the spinal cord. Glutamate is a neurotransmitter released in increased amounts in the dorsal horn of the spinal cord in chronic pain (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992). Astrocytes are exposed to glutamate released from neurons, and possibly neighbouring astrocytes and activated microglia (Chen et al., 2005; Lalo et al., 2006; Takeuchi et al., 2006). Moreover, spinal cord

astrocytes express all four subtypes of glutamate receptor, namely AMPA, KA, NMDA and mGluRs (see the results of section 3.3.4, and Aicher et al. (1997), Silva et al. (1999) and Brand-Schieber et al. (2004)). Exposure of astrocytes to glutamate often results in anti-inflammatory outcomes (Bruno et al., 1998; Murphy et al., 1995; Zhou et al., 2006) and also the activation of c/EBPβ, a key transcription factor involved in IL-10 release (Liu et al., 2003; Yano et al., 1996).

Considering no spinal sources of IL-10 have been identified despite the demonstration that spinal IL-10 levels are anti-nociceptive, the question will be addressed as to whether spinal cord astrocytic TLR4 and glutamate receptor stimulation can result in IL-10 release. We report here that TLR4-stimulated IL-10 release occurs from spinal cord astrocytes and glutamate co-application significantly increases TLR4-stimulated IL-10 release.

4.2 MATERIALS AND METHODS

4.2.1 Spinal cord astrocyte cell cultures

Detailed protocol for the preparation of astrocyte cultures is given in section 2.2. Briefly, spinal cords from 0 – 2 day old Sprague-Dawley rats were dissected out and the meninges and peripheral nerves were removed. The remaining spinal cord tissue was enzymatically and mechanically dissociated then washed with DMEM+. Dissociated cells were plated on a poly-D-lysine (20 μg/mL) coated culture flask for 4 hours in a 5% CO_2 incubator at 37°C, were washed and then maintained at 5% CO_2 and 37°C in DMEM+, replaced every 3 – 4 days. To purify astrocytes, 14 days after initial plating, flasks were shaken for 40 min at 400 rpm and 37°C and this was repeated 20 days after initial plating for 16 h. Additionally, 16 days after initial plating DMEM+ was replaced with DMEM supplemented by 10% horse serum, 1% penicillin/streptomycin/glutamine and 25 mM sorbitol. After 21 days cells were transferred and plated onto plastic 24-well plates for ELISA and LDH assay experiments or 13 mm diameter round glass coverslips for immunocytochemistry experiments at a density of 1×10^5 cells per well or coverslip. For RT-PCR experiments cells were plated on 35 mm diameter plastic Petri dishes at a density of 6.77×10^5 cells per dish. Cells were used for ELISA, immunocytochemistry or LDH assay experiments 1 to 2 days after transfer. The purity of cultures was verified by immunocytochemistry.

4.2.2 Sandwich ELISA

Detailed protocol for the measurement of IL-10 by ELISA is given in section 2.6. Briefly, after a 16 h incubation of astrocytes in agonists or antagonists and DMEM/2% BSA, medium was collected and centrifuged. Ninety-six well clear polystyrene microplates were coated in anti-rat IL-10 monoclonal antibody (1:250) at 4°C overnight. Wells were washed three times for 5 min each wash with 0.05 % Tween® 20 in PBS and incubated for 1 h in 1 % BSA in PBS. Wells were then washed three times for 5 min a wash with 0.05 % Tween® 20 in PBS. Immediately after centrifugation, medium samples were added to wells along with standard concentrations of IL-10 diluted in DMEM/2% BSA. After a 2 h incubation at room temperature wells were washed five times for 5 min each wash with 0.05 % Tween® 20 in PBS. Wells were incubated for 2 h in a biotinylated anti-rat IL-10 antibody (1:250) and wells were washed five times for 5 min each wash with 0.05 % Tween® 20 in PBS. A streptavidin-horseradish peroxidase conjugate (1:250) was added to wells for 20 min and wells were washed five times for 5 min each wash with 0.05 % Tween 20 in PBS. Wells were incubated in a 1:1 mix of hydrogen peroxide and TMB for 20 min and 1 M sulfuric acid was added to stop the reaction. Absorbance of plates at 450 nm was read within 20 min of sulfuric acid addition using a Fluostar Galaxy Multiplate reader and wavelength correction was performed at 550 nm. A standard curve was constructed for each plate and was used to convert absorbance measurements for each condition into IL-10 concentration values.

Each experimental condition was tested on at least three different culture batches and at least two repetitions of measurement per batch giving a minimum n = 6, apart from select conditions in the time- and dose-response curves where n = 4. Values in time- and concentration-experiments, agonist and antagonist experiments were compared to the average level of LPS-stimulated IL-10 release. Results were presented as mean ± SEM . Statistically significant differences between means were analysed with a one-tailed Student's two-sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

4.2.3 Lactate Dehydrogenase Toxicology Assay

Detailed protocol for the assessment of membrane integrity with an LDH assay is given in section 2.7. Briefly, cell toxicity was assessed with a Lactate Dehydrogenase (LDH) based *In Vitro* Toxicology Assay Kit as per the manufacturer's protocol. Absorbance was measured at 490 nm with a Fluostar Galaxy Multiplate reader and wavelength correction was performed at 650 nm. The absorbance reading corresponding to each extracellular LDH level was divided by the absorbance reading for each total intracellular LDH level to obtain a percentage of total LDH released per condition in accordance with Dringen and Hamprecht (1996). Statistically significant differences between means were analysed with a one-tailed Student's two-sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

4.2.4 Reverse Transcription-Polymerase Chain Reaction

Detailed protocol for RT-PCR is given in section 2.8. Briefly, total RNA was extracted from astrocytes using a GenElute™ Mammalian Total RNA Miniprep Kit, according to the manufacturer's protocol. The quantity of isolated RNA was determined spectroscopically using a NanoDrop ND-1000 Spectrophotometer. Extracted total RNA was reverse transcribed to cDNA using a SuperScript™ III First-Strand Synthesis System and oligo$(dT)_{20}$ primer according to the manufacturer's protocol. The potential for residual DNA contamination was examined by replacing the reverse transcriptase enzyme with RNAse free water. The cDNA was stored at -20°C until the polymerase chain reaction (PCR). Aliquots of first strand cDNA (40 ng) were amplified in 10 µL of Platinum SYBR-Green qPCR SuperMix-UDG with 0.8 µL of forward primer, 0.8 µL of reverse primer, made up to a total volume of 20 µL with diethylpyrocarbonate-treated H_2O. PCR was carried out using the Rotor-Gene 3000 system. Incubation conditions were as follows: 2 min at 50°C, 2 min at 95°C, then thermo-cycling for 40 cycles of 20 s at 95°C, 20 s at 60°C then 20 s at 72°C. The threshold cycle number and reaction efficiencies of each condition were identified with Rotor-Gene 3000 v3.1 Software. Relative quantification was carried out using the relative expression software tool (Pfaffl et al., 2002) whereby IL-10 mRNA expression was standardized by the house-keeping gene 18S rRNA expression. Mean relative mRNA levels were determined with at least three separate reactions per condition and mean levels were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Values are expressed as mean relative IL-10 mRNA expression ± SEM.

4.2.5 Fluorescence Immunocytochemistry

Detailed protocol for the detection of cellular proteins using fluorescent antibodies is given in section 2.5. Briefly, 24 h after transfer of coverslips described in section 4.2.1, a number of coverslips were incubated in 1 μg/ml LPS for 24 h, while others were left in DMEM/BSA. Subsequently coverslips were washed twice with HEPES buffered salt solution, fixed with 2% formaldehyde solution in PBS for 10 min, washed twice with PBS and then incubated in PBS containing 1% BSA and 0.05% saponin for 30 min. Cells on coverslips were then incubated for 2 h at room temperature with primary antibodies diluted in PBS containing 1% BSA and 0.05% saponin. Primary antibodies were washed out 3 times with PBS and cells were then incubated in secondary antibodies diluted in PBS containing 1% BSA and 0.05% saponin for 1 hour at room temperature. Secondary antibodies were washed out three times with PBS and coverslips were then mounted on rectangular glass coverslips using ProLong Gold anti-fade mounting media with DAPI. Slides were sealed with nail polish and subsequently stored at 4°C until viewing.

Fluorescence was visualised with an inverted Axiovert 200M inverted microscope and images were acquired with an AxioCam HR digital monochrome charge-coupled device camera using Axiovision 3.1 software. A constant exposure time was used to acquire images assessed for brightness of TLR4 staining. Using Image J a constant arbitrary brightness threshold was applied to all these images and the number of cells with regions of brightness above this threshold as a percentage of total cells was

counted per field of view. This percentage was averaged over a minimum of three different fields of view per condition and mean percentages were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Percentages were expressed as mean ± SEM.

4.3 RESULTS

4.3.1 The TLR4 is present on spinal cord astrocytes

Although there is functional evidence for the presence of the TLR4 on spinal cord astrocytes (Pehar et al., 2004), to date immunocytochemical studies have not been carried out to verify the presence of the TLR4. Immunocytochemistry revealed that cultured spinal cord astrocytes positively expressed the TLR4 (Figure 4.1). In unstimulated astrocytes, 88 ± 9% of cells (n = 169 cells; 3 batches) stained brightly for TLR4 (as determined by use of an arbitrary brightness threshold detailed in section 2.5). Exposure of astrocytes to LPS (1 μg/ml, 24 h) significantly increased to 100% the percentage of spinal cord astrocytes that were classified as bright (n = 139 cells; 3 batches; $p < 0.05$).

4.3.2 LPS stimulates IL-10 release from spinal cord astrocytes and this release is enhanced on co-exposure to glutamate

Determination was next made as to whether glutamate or the TLR4 agonist LPS stimulated IL-10 release from spinal cord astrocytes. Astrocytes were pre-exposed to 1 μg/ml LPS for 8 h. A time period of 8 h was chosen as this duration of LPS exposure did not cause significant IL-10 release from brain astrocytes (Ledeboer et al., 2002). After an 8 h pre-incubation in LPS, a further 16 h exposure to LPS stimulated 561 ± 45 pg/ml IL-10 release (n = 65). After an 8 h pre-incubation in LPS, a further 16 h exposure to LPS + 1 mM glutamate stimulated significantly more IL-10 release than that stimulated by LPS only (872 ± 62 pg/ml; n = 65; $p < 0.001$;

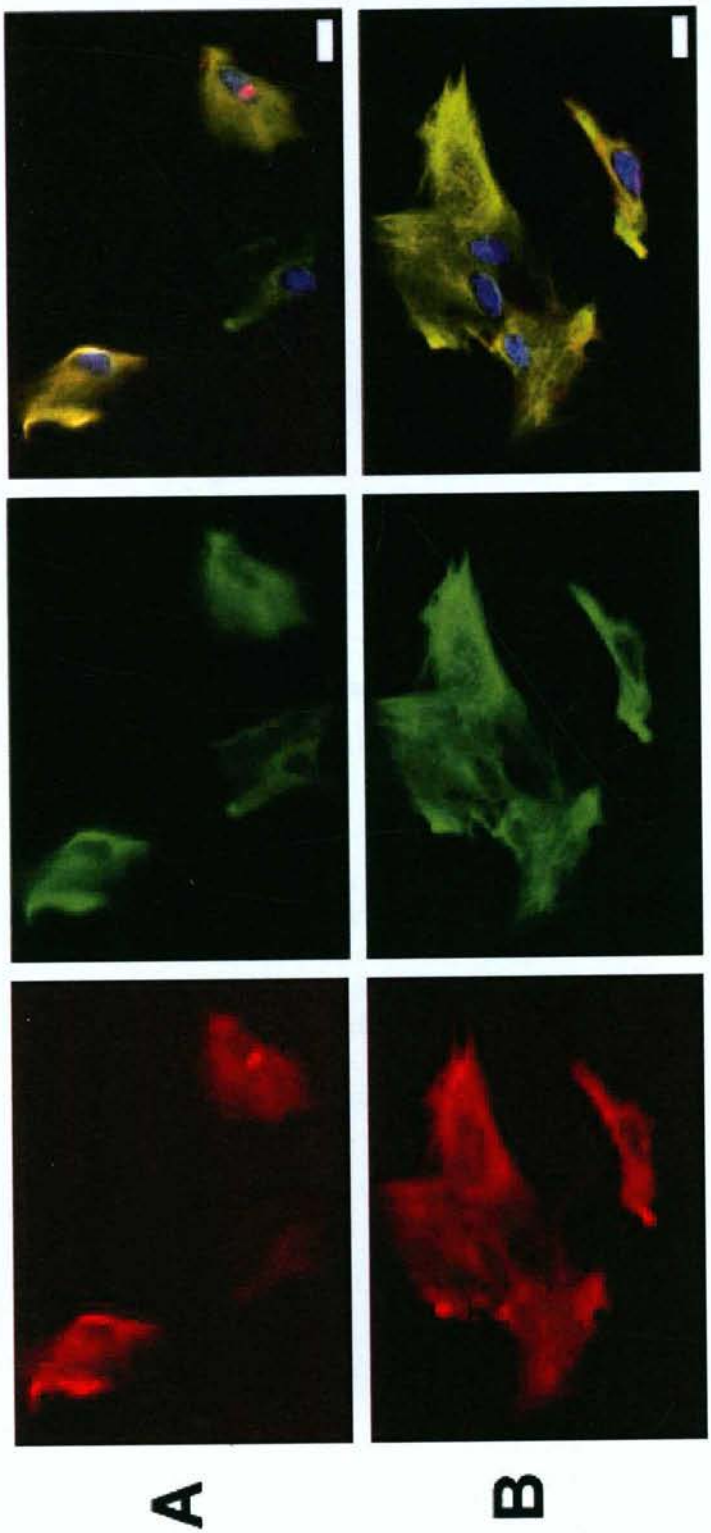


Figure 4.1 The TLR4 is expressed on spinal cord astrocytes. Representative images of A) unstimulated spina l cord astrocytes and B) spinal cord astrocytes exposed to 24 h LPS (1 μ g/ml). The left column represents TLR4 staining in red. The middle column represents staining of the astrocyte intermediate filament marker glial fibrillary acidic protein (GFAP) in green. The right column displays a merged image of the left and middle columns. In the merged image, blue represents DAPI-staining of cell nuclei. The scale bars represent 20μ m and hold for all images in the row.

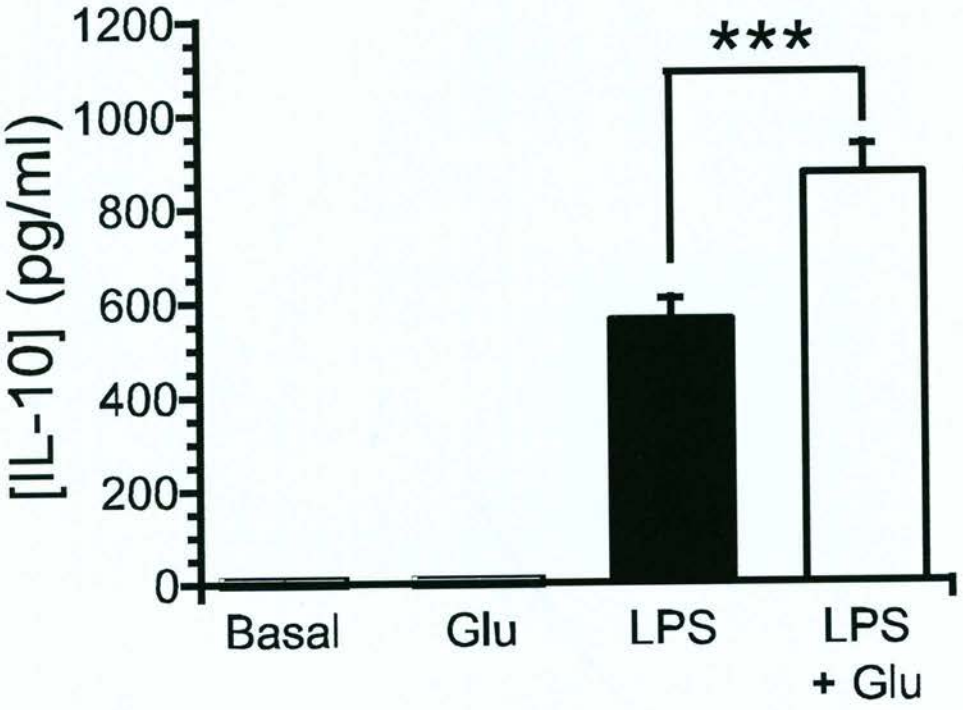


Figure 4.2 LPS stimulates IL-10 release from spinal cord astrocytes and this release is increased in the presence of glutamate. Treatments of astrocytes were as follows: basal – no addition of agonists (n = 57); Glu – no pre-incubation in LPS but a 16 h exposure to 1 mM glutamate (n = 10); LPS – 8 h pre-incubation in LPS (1 μg/ml) after which the media was removed and replaced with LPS for 16 h (n = 65); LPS + Glu – 8 h pre-incubation in LPS after which the media was removed and replaced with LPS + 1 mM glutamate for 16 h (n = 65). ***$p<0.001$.

Figure 4.2), whilst unstimulated astrocytes released 3.1 ± 3.3 pg/ml IL-10 (n = 57) and this value was not enhanced by the application of 1 mM glutamate for 16 h (3.4 ± 0.9 pg/ml; n = 10; Figure 4.2). The average percentage increase of LPS-stimulated IL-10 release in the presence of glutamate was 166 ± 6 % (n = 65).

To explore if an 8 h pre-incubation in LPS was necessary for LPS to stimulate IL-10 release or for the potentiating effect of glutamate co-application, agonists were applied with different pre-incubation conditions. Without a pre-incubation in LPS, it was found a 16 h application of 1 μg/ml LPS stimulated 211 ± 12 pg/ml IL-10 release (n = 8). When 1 mM glutamate was co-applied with LPS, this release significantly increased to 240 ± 8 pg/ml (n = 8; $p < 0.05$). If cells were subjected to an 8 h pre-incubation in 1 mM glutamate, the amount of IL-10 released from a subsequent 16 h exposure to LPS + glutamate was significantly higher than that seen if there was no pre-incubation (291 ± 18 pg/ml, n = 6, $p < 0.01$). As the highest levels of IL-10 release were seen with an 8 h pre-incubation in LPS, subsequent experiments were performed with an 8 h pre-incubation.

To verify the effect of LPS and glutamate on IL-10 release did not reflect compromised membrane integrity, an LDH assay was performed. The amount of LDH found in the extracellular medium as a percentage of total intracellular LDH did not significantly differ in unstimulated astrocytes over 16 h (3.78 ± 0.86 %; n = 4) as compared to astrocytes which were stimulated with LPS (1 μg/ml) and glutamate (1 mM) for 16 h after an 8 h LPS pre-incubation (4.42 ± 0.29 %; n = 4). In addition,

immunocytochemical analysis of spinal cord astrocyte cultures revealed only a small percentage of cells (8%, n = 284) were found to be positive for CD11b, an adhesion molecule found on microglia, and morphologically there appeared to be no oligodendrocytes present in the culture, suggesting that the IL-10 release observed was from astrocytes. The issue of culture purity is discussed further in section 6.1.7.

4.3.3 The enhancement of IL-10 release by glutamate is dependent on the concentration of glutamate

Glutamate potentiated LPS-stimulated IL-10 release in a dose-dependent manner with an EC_{50} of 400 μM (Figure 4.3). After pre-incubation in LPS, when glutamate was applied for 16 h with 1 μg/ml LPS, 1 μM glutamate elicited 554 ± 14 pg/ml IL-10 release (n = 4), 10 μM glutamate elicited 563 ± 7 pg/ml (n = 4) and 100 μM glutamate elicited 601 ± 18 pg/ml (n = 4). Compared to levels elicited without glutamate (561 ± 45 pg/ml; n = 65), 500 μM glutamate significantly increased IL-10 release to 782 ± 13 pg/ml (n = 4; $p < 0.001$), as did 1 mM glutamate (872 ± 62 pg/ml; n = 65) and 10 mM glutamate (928 ± 38 pg/ml; n = 4; $p < 0.001$). To ensure a concentration above EC_{50} was used, all subsequent experiments involving glutamate were carried out with a concentration of 1 mM. LPS was used at a concentration of 1 μg/ml in subsequent experiments as this concentration is commonly used in the previous literature (for example Beck et al. (2008), Noga et al. (2008) and Seo et al. (2004)).

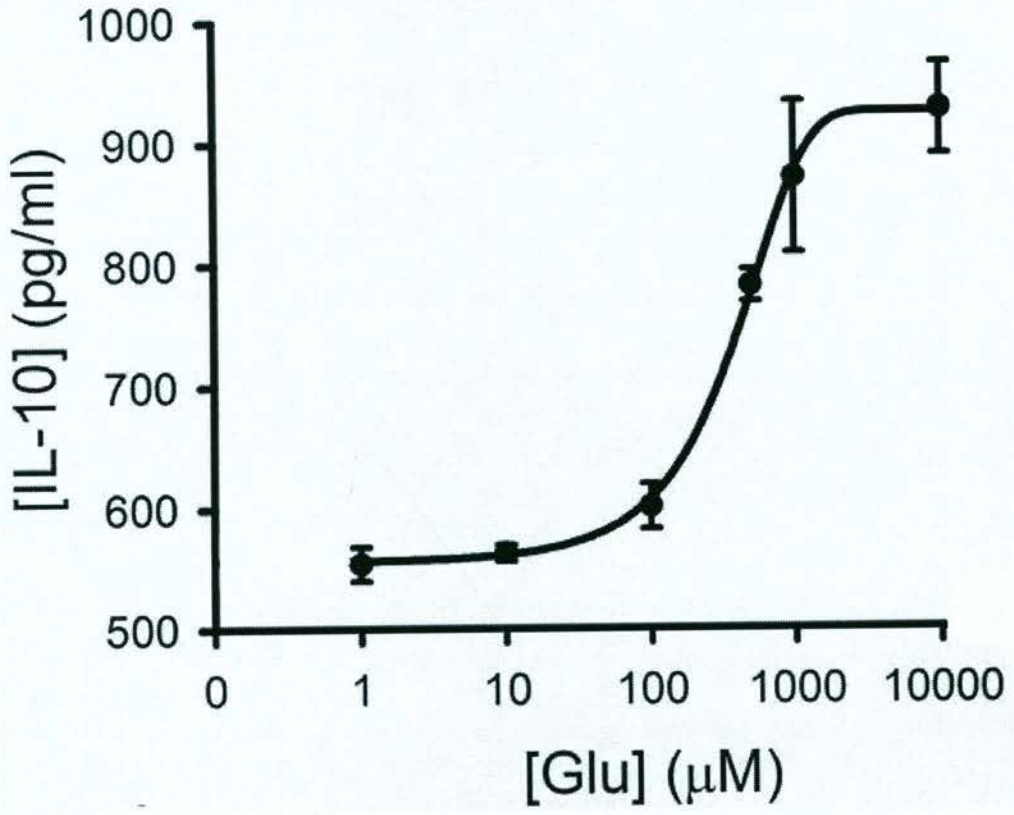


Figure 4.3 The modulation of LPS-stimulated IL-10 release by glutamate is dose-dependent. Astrocytes were pre-incubated in 1 μg/ml LPS for 8 h. Media was removed and LPS with various concentrations of glutamate (Glu) were added. EC_{50} was 400 μM. For each concentration of glutamate, n was at least 4.

4.3.4 LPS-stimulated IL-10 release from spinal cord astrocytes and enhancement in the presence of glutamate is time-dependent

To examine if LPS-stimulated IL-10 release was dependent on the duration of LPS exposure, LPS was added for various times after a constant 8 h pre-incubation in LPS. A subsequent 2 h exposure to LPS produced IL-10 levels of 147 ± 9 pg/ml (n = 4), a 4 h exposure produced 210 ± 24 pg/ml (n = 6), an 8 h exposure produced 323 ± 41 pg/ml (n = 6), a 16 h exposure produced 561 ± 45 pg/ml (n = 65), a 24 h exposure produced 1094 ± 124 pg/ml (n = 6) and a 48 h exposure produced 2551 ± 353 pg/ml IL-10 (n = 6; Figure 4.4A).

To examine if the increase in IL-10 release on co-exposure to glutamate and LPS was also dependent on the duration of agonist exposure, LPS and glutamate were added for various times after a constant 8 h pre-incubation in LPS. A further 2 h co-exposure to LPS and glutamate produced IL-10 levels of 169 ± 3 pg/ml (n = 4), a 4 h co-exposure produced 244 ± 21 pg/ml (n = 6), an 8 h co-exposure produced 391 ± 45 pg/ml (n = 6), a 16 h co-exposure produced 872 ± 62 pg/ml (n = 65), a 24 h co-exposure produced 1466 ± 120 pg/ml (n = 6) and a 48 h co-exposure produced 2921 ± 225 pg/ml IL-10 (n = 6; Figure 4.4A).

The level of IL-10 release measured on exposure to glutamate and LPS was compared to the level stimulated on exposure to LPS alone for the same amount of time

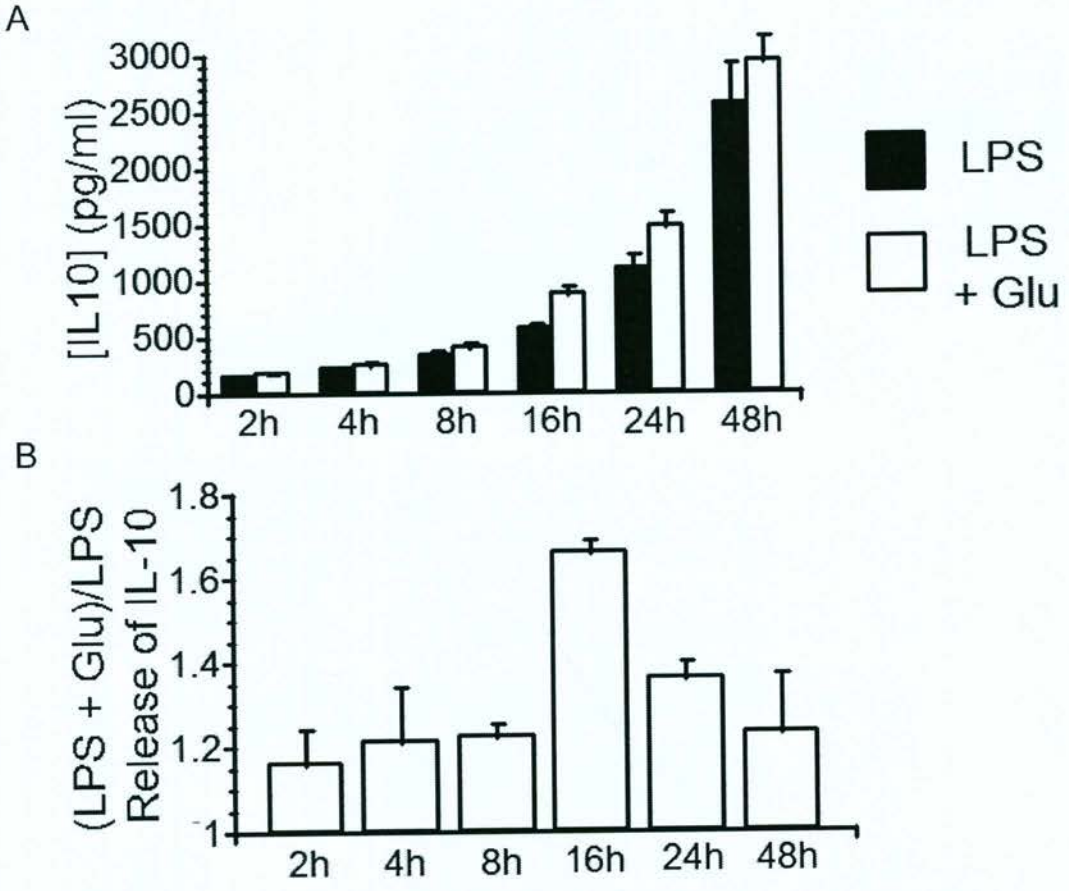


Figure 4.4 The modulation of LPS-stimulated IL-10 release by glutamate is time-dependent. Astrocytes were pre-incubated in 1 μg/ml LPS for 8 h. Media was removed and either LPS (1 μg/ml) or LPS + glutamate (1 mM) was added for various durations. A) Summary histogram of the amount of LPS (1 μg/ml; black column) and LPS+Glu (1 mM; white column)-stimulated IL-10 release over the differing time periods. B) Summary histogram of how the ratio of LPS+Glu to LPS-stimulated IL-10 release varied over the differing time periods. Values are expressed as a mean ratio of LPS + Glu release to LPS release ± SEM. The peak modulatory effect occurred when glutamate was applied with LPS for 16 h. For each condition, n was at least 4.

(Figure 4.4B). The relative increase of LPS + glutamate-stimulated IL-10 release beyond that stimulated by LPS alone was largest when agonists were added for 16 h (1.66 ± 0.06 fold higher; n = 65). A 2 h exposure to LPS + glutamate elicited a 1.16 ± 0.08 fold higher IL-10 release than a 2 h exposure to LPS alone (n = 4). A 4 h exposure to agonists elicited a 1.21 ± 0.13 fold higher release (n = 6). An 8 h exposure elicited a 1.22 ± 0.03 fold higher release (n = 6). A 24 h exposure to agonists elicited a 1.36 ± 0.04 fold higher release (n = 6) and a 48 h exposure elicited a 1.23 ± 0.14 fold higher response (n = 6). Consequently, agonists were applied for 16 h in subsequent experiments to ensure maximal interaction between glutamate and LPS.

4.3.5 Transcription is key to the potentiating effect of glutamate exposure

In spinal cord astrocytes, glutamate is known to activate c/EBPβ which is a transcription factor that can stimulate increased IL-10 production (Liu et al., 2003; Yano et al., 1996). Additionally, the 16 h optimal time of exposure to glutamate described above falls within IL-10 mRNA production time lengths (Ledeboer et al., 2002). An investigation was therefore made into whether transcription and subsequent translation were involved in the potentiating effect of glutamate presence.

Blocking mRNA transcription with actinomycin D (1 μg/ml) did not significantly decrease LPS-stimulated IL-10 release (483 ± 53 pg/ml; n = 14) but significantly decreased LPS + glutamate-stimulated IL-10 release (428 ± 37 pg/ml; n = 14;

$p < 0.001$). Figure 4.5A illustrates that glutamate had no potentiating effect in the presence of actinomycin D. Blocking translation with cycloheximide (1 µg/ml) ablated both LPS-stimulated IL-10 release (13 ± 5 pg/ml; $n = 10$; $p < 0.001$) and LPS + glutamate-stimulated IL-10 release (11 ± 7 pg/ml; $n = 10$; $p < 0.001$; Figure 4.5A).

Incubation in actinomycin D and cycloheximide caused astrocytic processes to hypertrophy. An LDH assay was performed to verify that this did not reflect compromised membrane integrity or cell death. The amount of LDH found in the extracellular medium as a percentage of total intracellular LDH did not significantly differ in astrocytes incubated in actinomycin D with LPS + glutamate (4.51 ± 0.33 %; $n = 4$) compared to the percentage found in the medium of unstimulated astrocytes or astrocytes incubated in LPS + glutamate (as reported in section 4.3.2). Similarly, incubation in cycloheximide with LPS + glutamate did not generate significantly different amounts of released LDH (4.35 ± 0.06 %; $n = 4$) compared to unstimulated or LPS + glutamate-stimulated astrocytes.

The effect of glutamate on IL-10 mRNA expression in spinal cord astrocytes was investigated using RT-PCR to further explore the role of transcription. Glutamate on its own stimulated a 1.3 ± 1.6 ($n = 3$) fold increase in mRNA levels over those found in unstimulated astrocytes. LPS stimulated a 6.2 ± 2.7 ($n = 3$) fold increase whilst LPS + glutamate significantly increased expression levels beyond this LPS-stimulated level to 30.3 ± 7.9 fold above basal expression ($n = 3$; $p < 0.05$; Figure 4.5B).

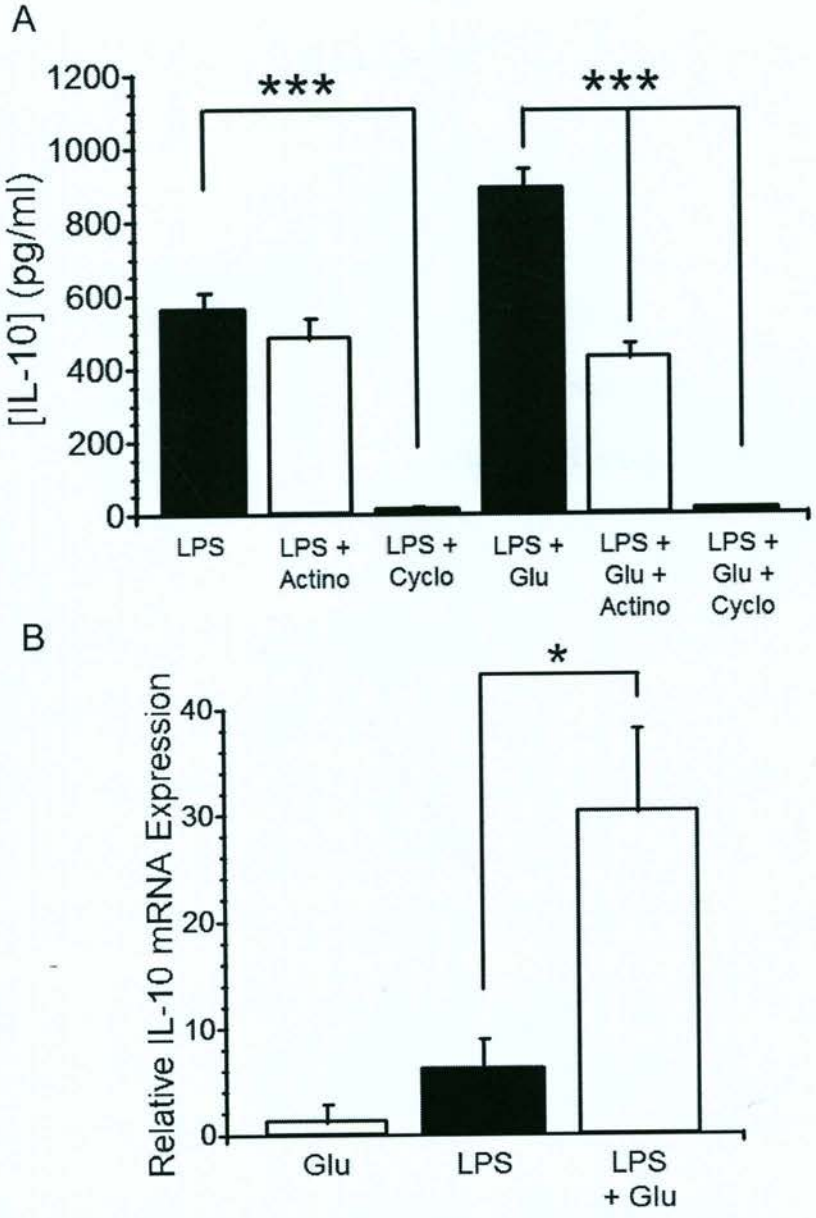


Figure 4.5 Transcription is involved in the potentiating effect of glutamate. A) The effect of incubation in actinomycin D (Actino; transcription inhibitor; 1 μg/ml) and cycloheximide (Cyclo; translation inhibitor; 1 μg/ml) on LPS (1 μg/ml) and LPS plus glutamate (Glu; 1 mM)-stimulated IL-10 release. Whilst actinomycin D did not significantly effect LPS-stimulated IL-10 release (n = 14), cycloheximide ablated it (n = 10). Conversely, both actinomycin D (n = 14) and cycloheximide (n = 10) removed the potentiating effect of glutamate. B) IL-10 mRNA expression after a 24 h incubation in Glu (n = 3), LPS (n = 3) or LPS + Glu (n = 3) normalised to 18S rRNA expression and relative to basal IL-10 expression *$p<0.05$, ***$p<0.001$.

4.3.6 Glutamate acts on multiple mGluR groups to bring about IL-10 release in the presence of LPS

Agonists and antagonists to glutamate receptor subtypes were used to examine the role of AMPA, kainate, NMDA and mGluRs in the potentiation of IL-10 release seen in the presence of glutamate and LPS. tACPD, an mGluRI and II receptor agonist, increased LPS-stimulated IL-10 release across a range of concentrations. The ratio of IL-10 release in the presence of 10 μM tACPD + LPS compared to LPS alone was 1.25 ± 0.08 (n = 6). This ratio for 100 μM tACPD was 1.58 ± 0.06 (n = 6; Figure 5.6A) and for 1 mM tACPD was 1.84 ± 0.03 (n = 6). Surprisingly, application of agonists for individual mGluR groups showed no potentiation of LPS-stimulated IL-10 release. The ratio of IL-10 release in the presence of 500 μM DHPG (mGluRI agonist) + LPS compared to LPS alone was 1.05 ± 0.02 (n = 6; Figure 5.6A). The ratio for the mGluRII agonist APDC was 1.00 ± 0.02 (5 μM; n = 6; Figure 5.6A) and for the mGluRIII agonist L-AP4 was 1.13 ± 0.03 (1 mM; n = 6; Figure 5.6A). Kainate (100 μM; 1.06 ± 0.01; n = 6), AMPA (100 μM; 1.10 ± 0.03; n = 6) and NMDA (500 μM, 1.10 ± 0.02; n = 6) also had little impact on LPS-stimulated IL-10 release (Figure 5.6A).

The ratio of IL-10 release in the presence of LPS + glutamate + 1 μM MPEP (mGluR5 antagonist) to IL-10 release in the presence of LPS + 1 μM MPEP was 1.64 ± 0.06 (n = 8; Figure 5.6B). This ratio for 75 μM CPCCOEt (mGluR1 antagonist) was 1.85 ± 0.09 (n = 13; Figure 5.6B) and for 500 μM EGLU (mGluRII antagonist) was 1.63 ± 0.09 (n = 9; Figure 5.6B). None of these ratios were

significantly different from the ratio of IL-10 release stimulated by LPS + glutamate to that stimulated by LPS alone (1.66 ± 0.06; n = 65; Figure 5.6B). However, mGluR antagonists that concurrently block multiple receptor groups significantly decreased the potentiation of glutamate on LPS-stimulated IL-10 release levels. The ratio of IL-10 release in the presence of LPS + glutamate + 1 mM MSPG (mGluRII and III antagonist) to IL-10 release in the presence of LPS + 1 mM MSPG was 1.40 ± 0.09 (n = 11; p <0.01; Figure 5.6B). The potentiation was further decreased by co-exposure to 1 μM MPEP and 500 μM EGLU, which produced a ratio of 1.19 ± 0.13 (n = 6; p < 0.01; Figure 5.6B) and co-exposure to 1 μM MPEP, 500 μM EGLU and 75 μM CPCCOEt which produced a ratio of 1.13 ± 0.13 (n = 6, p < 0.01; Figure 5.6B).

Ionotropic glutamate receptor antagonists had no significant effect on the increase in IL-10 release seen on co-application of glutamate and LPS. The ratio of IL-10 release stimulated by LPS + glutamate + 200 μM AP5 (NMDA receptor antagonist) to IL-10 release stimulated by LPS + AP5 was 1.51 ± 0.09 (n = 6; Figure 5.6B) and the ratio was 1.72 ± 0.03 (n = 6; Figure 5.6B) for the AMPA and kainate receptor antagonist 20 μM CNQX.

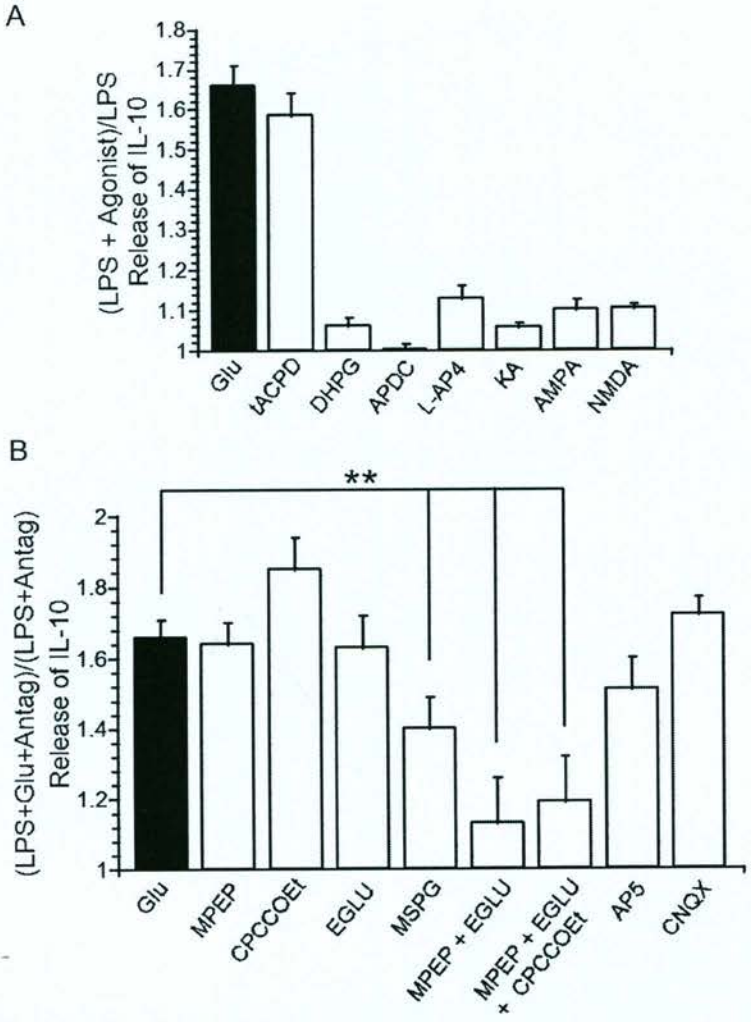


Figure 4.6 Glutamate potentiates LPS-stimulated IL-10 release by co-activating mGluR groups. A) The effect of glutamate receptor agonists glutamate (Glu; 1 mM; n = 65), tACPD (mGluRI and II agonist, 100μM; n = 6), DHPG (mGluRI agonist, 500μM; n = 6), APDC (mGluRII agonist, 5 μM; n = 6), L-AP4 (mGluRIII agonist, 1 mM; n = 6), KA (100 μM; n = 6), AMPA (100 μM; n = 6) and NMDA (500 μM; n = 6) on LPS (1 μg/ml)-stimulated IL-10 release. Values are expressed as mean ratio of LPS + agonist-stimulated release to LPS-stimulated release ± SEM. B) The effect of glutamate receptor antagonists MPEP (mGluR5 antagonist, 1 μM; n = 8), CPCCOEt (mGluR1 antagonist, 75 μM; n = 13), EGLU (mGluRII antagonist, 500 μM; n = 9), MSPG (mGluRII and III antagonist, 1 mM; n = 11), MPEP (1 μM) + EGLU (500 μM; n = 6), MPEP (1 μM) + EGLU (500 μM) + CPCCOEt (75 μM; n = 6), AP5 (NMDA receptor antagonist, 200 μM; n = 6) and CNQX (AMPA and kainate receptor antagonist, 20 μM; n = 6) on glutamate + LPS-stimulated IL-10 release. Values are expressed as mean ratio of LPS + Glu + antagonist to LPS + antagonist ± SEM. **$p<0.01$.

4.4 DISCUSSION

Unstimulated rat spinal cord astrocytes release very little IL-10 but increase the amount of IL10 release on exposure to LPS, reflecting what has been reported with rat forebrain astrocytes (Ledeboer et al., 2002). LPS-stimulated IL-10 release is significantly increased in the presence of glutamate in a dose and time-dependent manner, whilst glutamate itself lacked an effect on IL-10 release levels.

4.4.1 The interaction between glutamate and LPS involves upregulation of IL-10 mRNA

The increase in release of IL-10 in the presence of LPS and glutamate compared to that in the sole presence of LPS appears to require upregulation of IL-10 mRNA expression. In comparison to the level of IL-10 mRNA stimulated by LPS, co-exposure to glutamate greatly increased IL-10 mRNA expression. Additionally, maximum potentiation of IL-10 release occurred after 16 h, which is within the time required for IL-10 mRNA production (Ledeboer et al., 2002). Glutamate had no effect on LPS-stimulated IL-10 release when transcription was prevented.

It is intriguing that glutamate alone does not substantially increase IL-10 mRNA levels, despite the large enhancement seen on co-exposure with LPS. It has recently been discovered that in order for transcription factors to effectively access IL-10 promoter sites, there must be covalent modification of histones at the IL-10 locus (Lucas et al., 2005). In macrophages, LPS alone does not stimulate such modifications and so only produces moderate IL-10 mRNA and protein increases

despite activating a range of transcription factors (Lucas et al., 2005). Extracellular signal-related kinase (ERK) is a MAPK, the activation of which brings about the needed covalent modifications to render the IL-10 promoter accessible to relevant transcription factors. Activation of ERK during LPS exposure greatly increases the ability of LPS to enhance IL-10 mRNA and protein levels (Lucas et al., 2005). Moreover, it has been shown that activation of ERK on its own does not increase IL-10 release, indicating that ERK activation is necessary but not sufficient to potentiate IL-10 release (Lucas et al., 2005). In astrocytes, stimulation of mGluRI and II receptors results in phosphorylation and increased activity of ERK (D'Onofrio et al., 2001; Peavy et al., 2001; Schinkmann et al., 2000). Hence, although the exact mechanism of IL-10 mRNA increase is still to be determined, these factors suggest the hypothesis that glutamate may increase LPS-stimulated IL-10 mRNA levels by activation of ERK, resulting in no increases in IL-10 levels by itself but allowing LPS-induced transcription factors to more readily access IL-10 promoter sites. ERK inhibitors could be used to investigate this hypothesis.

It should be noted, however, that without such experiments, it can not be ruled out that TLR4 stimulation may be facilitating the ability of glutamate to bring about IL-10 release. As mentioned, glutamate is known to elicit c/EBPβ activation in astrocytes (Peavy et al., 2001) and activation of this transcription factor can result in increased IL-10 production (Liu et al., 2003). An alternative hypothesis to explain the data may be that there is a threshold as to the level of c/EBPβ activation required to affect IL-10 production and glutamate on its own does not increase activation of

c/EBPβ above this threshold. When glutamate is co-applied with LPS, however, the amount of c/EBPβ stimulated by LPS on its own in combination with that elicited by glutamate is enough to result in the observed enhancement of IL-10 mRNA and protein levels.

4.4.2 Transcriptional activation by LPS does not contribute to LPS-stimulated IL-10 levels

IL-10 release resulting from TLR4 stimulation of spinal cord astrocytes does not require the process of transcription. Spinal cord astrocytes display low constitutive IL-10 mRNA levels which are upregulated by LPS, in correspondence with previous RT-PCR studies on rat and mouse cortical astrocytes (Ledeboer et al., 2002; Mizuno et al., 1994). Even though LPS upregulates IL-10 mRNA levels, this upregulation did not contribute to the increased IL-10 release as inhibition of transcription did not significantly reduce LPS-stimulated IL-10 release. Rather, release was ablated by blockade of translation. This result suggests that the LPS-induced upregulation of IL-10 mRNA was not large enough to result in a measurable difference in IL-10 release and that LPS must exert its enhancing effects on IL-10 release in spinal cord astrocytes by a post-transcriptional mechanism. Post-transcriptional modifications in the process of IL-10 release have been documented. The 3' end of IL-10 mRNA contains an untranslated region with a number of mRNA destabilising sequences. IL-10 mRNA constructed to lack this region has a much longer half life than that which contains it (Nemeth et al., 2005). In macrophages, adenosine potentiates LPS-stimulated IL-10 release by inducing cytoplasmic proteins to bind to the destabilising

regions of the 3' untranslated end of IL-10 mRNA, relieving the associated transcriptional repression (Nemeth et al., 2005). The possibility that LPS may have affected the 3' untranslated end of IL-10 mRNA to result in the increased IL-10 release observed in the present work could be tested in further studies by using luciferase constructs with the sequence of the 3' untranslated end of IL-10 mRNA, as in the study by Nemeth et al. (2005).

4.4.3 Co-ligation of group I and II mGluRs is necessary for the increase in LPS-stimulated IL-10 release in the presence of glutamate

The increase in LPS-stimulated IL-10 release in the presence of glutamate exclusively required group I, II and possibly III mGluRs. Ionotropic glutamate receptor agonists did not enhance LPS-stimulated IL-10 release and antagonists to ionotropic receptors did not decrease the potentiating effect of glutamate. Large increases in LPS-stimulated IL-10 release were only seen when both mGluRI and II were stimulated together and the potentiating effect of glutamate presence was only reversed when antagonists to mGluR5, 2 and 3 were co-applied. When agonists and antagonists to mGluR I or II receptors were added separately, no significant effects were found suggesting co-activation of mGluR2, 3 and 5 is necessary. A small contribution of mGluRIII cannot be ruled out due to the small increase in IL-10 release elicited by an mGluRIII agonist and residual IL-10 release remaining after LPS and glutamate-stimulated IL-10 release was antagonized with mGluR2, 3 and 5 antagonists. This is congruent with reports of the expression of mGluRI subunits (mGluR 1 and 5) and

mGluRII subunits (mGluR 2 and 3) on spinal cord astrocytes by Silva et al (1999) and in this thesis in section 3.3.4. Although no reports exist of the mGluRIII on unperturbed spinal cord astrocytes, expression has been identified in astrocytes in pathological conditions such as in multiple sclerosis (Geurts et al., 2005). It is unknown if exposure to a chronic pain-inducing pathology results in upregulation of the mGluRIII.

It remains a matter of speculation why co-activation of both mGluRI and II are necessary for the effects of glutamate on LPS-stimulated IL-10 release. Stimulation of the mGluRII class of metabotropic receptor has been shown to potentiate mGluRI-stimulated IP_3 accumulation in hippocampal and cerebral slices without itself affecting IP_3 accumulation (Mistry et al., 1998). This may be relevant to the current study if ERK activation is involved in the potentiation as postulated above. In astrocytes, one pathway of phosphorylation of ERK after stimulation of mGluR5 and mGluRII involves stimulation of intracellular Ca^{2+} increases and PKC activation subsequent to PLC-β activation (Schinkmann et al., 2000) and presumably IP_3 production. Perhaps stimulation of either mGluR class on its own does not enhance ERK activation via IP_3 generation sufficiently and the reported synergy in mGluR5 and mGluR II-mediated IP_3 production is necessary to bring about the required levels of ERK activation to cause increased IL-10 release.

Alternatively, it could be conceptualised that co-activation of both mGluRI and II may be necessary if the astrocytic densities of each of these subtypes were low.

Although the relative densities of mGluRI and II on spinal cord astrocytes are unknown, it may be possible that activation of each individual subtype could produce a response below the threshold of IL-10 detection if their densities were low. Furthermore, if co-stimulated, this may be enough to generate IL-10 release levels above the detectable threshold level.

Although the reason remains unclear as to why co-activation of mGluRI and II is necessary in this present study, another report of this phenomenon exists in the literature. Co-activation of mGluRI and mGluRII is necessary for the induction of long term depression in the perirhinal cortex at resting potentials (Cho et al., 2002). This present study provides further evidence for a functional interaction between these two groups of mGluR.

4.4.4 Spinal cord astrocytes express TLR4 and this expression is increased by LPS exposure

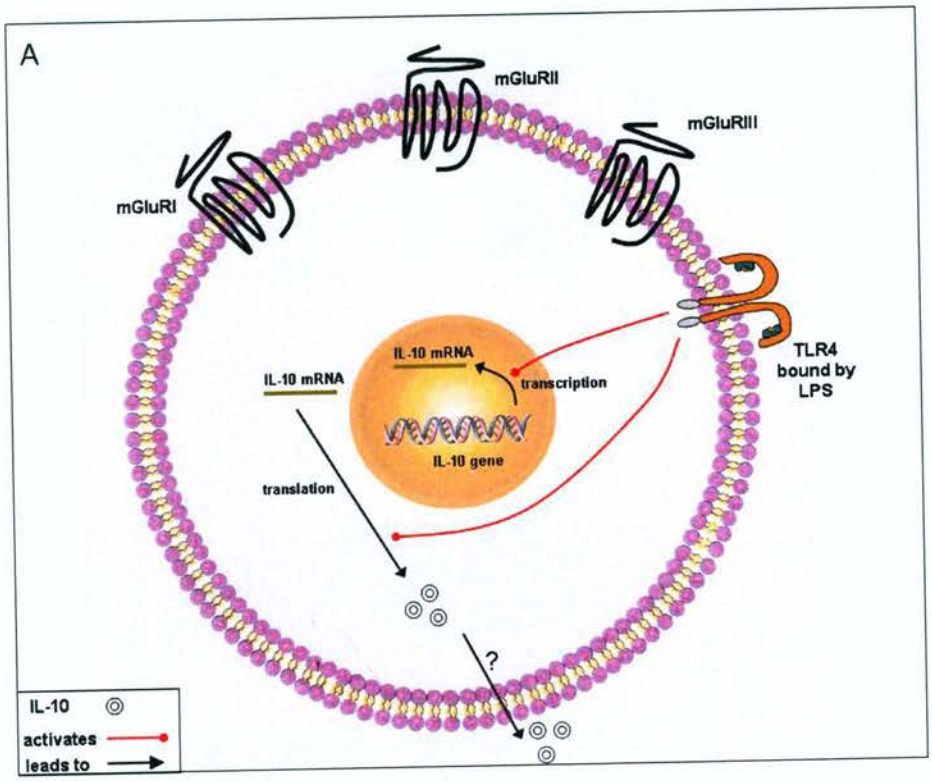
The TLR4 has been identified on the surface of astrocytes in the brain (Bowman et al., 2003; Bsibsi et al., 2002; Jou et al., 2006). In the existing literature there is functional evidence for the presence of the TLR4 on spinal cord astrocytes whereby it was demonstrated that LPS could stimulate nerve growth factor release from these cells (Pehar et al., 2004). In the current work the presence of the TLR4 on cultured spinal cord astrocytes was further confirmed immunocytochemically.

Exposure to LPS led to a significant increase in the number of highly expressing-TLR4 spinal cord astrocytes. In other areas of the nervous system such as the cortex and brain, exposure of astrocytes to TLR4 agonists has been reported to result in a down-regulation of TLR4 mRNA and protein in some studies (Bowman et al., 2003; Jou et al., 2006) and has been reported to not affect TLR4 mRNA in other studies (Laflamme and Rivest, 2001; Lehnardt et al., 2002). It is unclear why TLR4s on spinal cord astrocytes respond to LPS differently to astrocytes from other areas of the brain, however reports exist of a regional heterogeneity in the response of spinal cord and cortical astrocytes to transmitters (Cholewinski et al., 1988; Marriott et al., 1991), as detailed more fully in section 1.4.1.3. In the present study, a higher level of surface TLR4 after LPS pre-incubation may be one reason why pre-incubation in LPS produced a higher level of LPS-stimulated IL-10 release to that seen without LPS pre-incubation.

4.4.5 Summary of mechanisms

In summary, it can be seen that TLR4 stimulation of spinal cord astrocytes results in an increase in IL-10 mRNA expression and IL-10 release. The increase in IL-10 release is not dependent on the increase in IL-10 mRNA expression, presumably suggesting TLR4 stimulation brings about increased IL-10 release via post-transcriptional mechanisms. This increase in IL-10 release on LPS application is greatly enhanced in the presence of glutamate, whilst glutamate itself does not affect IL-10 protein levels. This potentiation in the presence of glutamate requires co-activation of mGluRI and II (and possibly III) receptors, resulting in a synergistic

increase in the expression of IL-10 mRNA in the presence of LPS and glutamate and subsequently the observed increase in IL-10 release. These pathways are summarised in Figure 4.7.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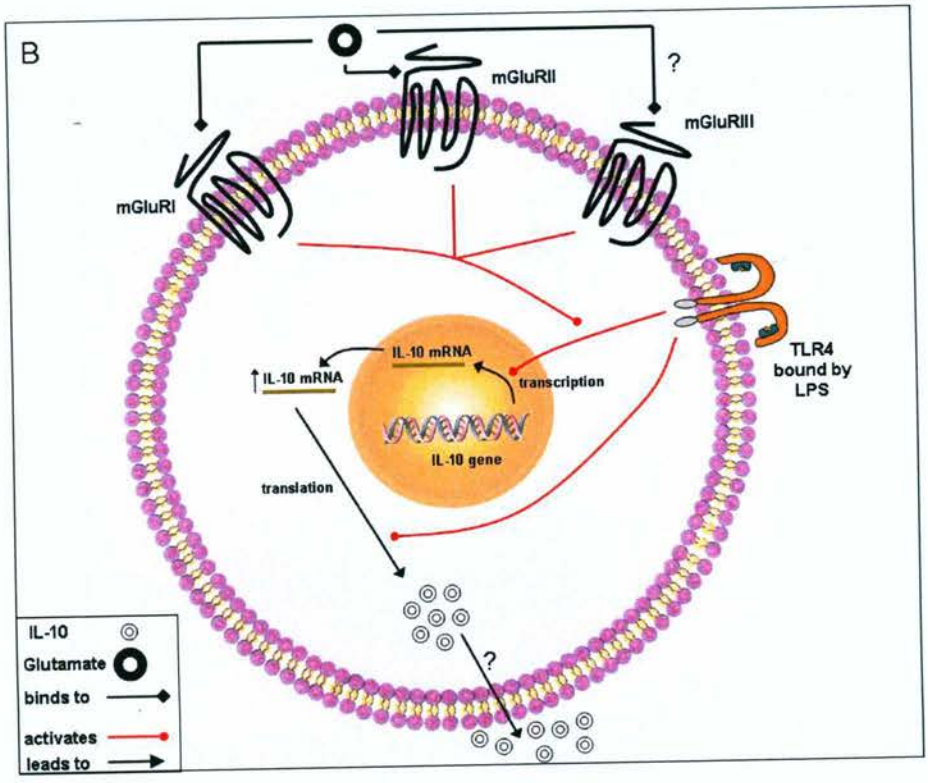
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Figure 4.7 A model of the mechanisms of LPS-stimulated IL-10 release in the absence and presence of glutamate from spinal cord astrocytes. A) Binding of the TLR4 by LPS results in an increase in IL-10 release, presumably by a post-transcriptional mechanism. TLR4 activation also results in activation of transcription but this does not contribute to the increase in IL-10 release. B) Co-activation of mGluRI and II and possibly III receptors at the same time as TLR4 activation results in an increase in IL-10 mRNA levels. One explanation may be that this occurs through mGluR-mediated enhancement of LPS-stimulated IL-10 transcription. The result is an increase in IL-10 release. Glutamate itself does not directly affect IL-10 mRNA levels. IL-10 release mechanisms are not clear.

CHAPTER 5

SPINAL MICROGLIAL IL-10 RELEASE ON ACTIVATION OF THE TLR4 AND THE EFFECT OF CONCOMITANT GLUTAMATE RECEPTOR ACTIVATION

Contents

5.1 INTRODUCTION

As detailed in sections 1.3.2 and 4.1, IL-10 is an anti-inflammatory cytokine with important anti-nociceptive effects. To date, the demonstration in Chapter 4 that stimulation of spinal cord astrocyte TLR4s results in release of IL-10 is the only documented report of spinal IL-10 release. It is of interest to identify further possible sources of IL-10 to get a more complete picture of processes that occur during pain and to identify other novel targets for intervention in chronic pain treatment.

It is possible that TLR4-stimulated IL-10 release described from spinal cord astrocytes may also occur from spinal cord microglia. Cultured spinal cord microglia have functional TLR4s (Ogata et al., 2003) and also p38, a key MAPK involved in IL-10 transcription activation (Svensson et al., 2003). In addition to TLR4-stimulated IL-10 release from spinal cord microglia, it is appealing to suggest that the neurotransmitter glutamate may be involved in IL-10 release from microglia in the spinal cord, as it is with astrocytes, as described in Chapter 4. In inflammatory conditions such as chronic pain, microglia are exposed to increased levels of glutamate (Juranek and Lembeck, 1997; Kawamata and Omote, 1996; Sasaki et al., 1998; Somers and Clemente, 2002; Sorkin et al., 1992) and spinal cord microglia express AMPA, NMDA and mGlu receptors (Liu et al., 2006), allowing them to respond to these increased levels of glutamate. When glutamate binds receptors on activated microglia anti-inflammatory effects such as a decrease in microglial activation and a decrease in microglial-induced neurotoxicity have been described (Taylor et al., 2003). In addition to these anti-inflammatory effects, exposure of

spinal cord microglia to glutamate results in activation of p38 (Ejarque-Ortiz et al., 2007; Tikka and Koistinaho, 2001), suggesting a possible effect of glutamate on IL-10 levels.

Considering this evidence, this present study will examine if spinal cord microglial TLR4 and glutamate receptor stimulation produce IL-10 release. Results show that glutamate significantly increased TLR4-stimulated IL-10 release from microglia, dependent upon an upregulation of IL-10 mRNA.

5.2 MATERIALS AND METHODS

5.2.1 Spinal cord microglial cell cultures

Detailed protocol for the preparation of spinal cord microglial cultures is given in section 2.2. Briefly, spinal cords from 0 – 2 day old Sprague-Dawley rats were dissected out and the meninges and peripheral nerves were removed. The remaining spinal cord tissue was enzymatically and mechanically dissociated then washed with DMEM+. Dissociated cells were plated on a poly-D-lysine (20 μg/mL) coated culture flask for 4 h in a 5% CO_2 incubator at 37°C, were washed and then maintained at 5% CO_2 and 37°C in DMEM+, replaced every 3 – 4 days. To purify microglia, 14 days after initial plating, flasks were shaken for 40 min at 400 rpm and 37°C. Media containing detached glial cells were removed and centrifuged at 1500 rpm for 5 min. The glial conditioned medium supernatant was kept at 37°C and the pellet washed twice with 1 ml DMEM+ and then plated onto plastic 24-well plates for ELISA and LDH assay experiments, 13 mm diameter round glass coverslips for immunocytochemistry experiments or 25 mm diameter plastic Petri dishes. Cells were plated at a density of 1 x 10^5 cells per well or coverslip or 1 x 10^6 cells per dish. All cell-adherent surfaces were pre-coated in 20 μg/ml poly-D-lysine. After 15 min, cells were washed and a 1:1 mix of DMEM+ and glial conditioned medium was added. Cells were used 1 to 2 days after this for experiments. The purity of cultures was verified by immunocytochemistry.

5.2.2 Sandwich ELISA

Detailed protocol for the measurement of IL-10 with ELISA is given in section 2.6. Briefly, after a 16 h incubation of microglia in agonists or antagonists and DMEM/2% BSA, medium was collected and centrifuged. Ninety-six well clear polystyrene microplates were coated in anti-rat IL-10 monoclonal antibody (1:250) at 4°C overnight. Wells were washed three times for 5 min each wash with 0.05 % Tween® 20 in PBS and incubated for 1 h in 1 % BSA in PBS. Wells were then washed three times for 5 min a wash with 0.05 % Tween® 20 in PBS. Immediately after centrifugation, medium samples were added to wells along with standard concentrations of IL-10 diluted in DMEM/2% BSA. After a 2 h incubation at room temperature wells were washed five times for 5 min each wash with 0.05 % Tween® 20 in PBS. Wells were incubated for 2 h in a biotinylated anti-rat IL-10 antibody (1:250) and wells were washed 5 times for 5 min each wash with 0.05 % Tween® 20 in PBS. A streptavidin-horseradish peroxidase conjugate (1:250) was added to wells for 20 min and wells were washed 5 times for 5 min each wash with 0.05 % Tween 20 in PBS. Wells were incubated in a 1:1 mix of hydrogen peroxide and tetramethylbenzidine for 20 min and 1 M sulfuric acid was added to stop the reaction. Absorbance of plates at 450 nm was read within 20 min of sulfuric acid addition using a Fluostar Galaxy Multiplate reader and wavelength correction was performed at 550 nm. A standard curve was constructed for each plate and was used to convert absorbance measurements for each condition into IL-10 concentration values.

Each experimental condition was tested on at least three different culture batches and at least two repetitions of measurement per batch giving a minimum $n = 6$, apart from select conditions in the time- and dose-response curves where $n = 4$. Values in time- and concentration-experiments, agonist and antagonist experiments were normalised to the average level of LPS-stimulated IL-10 release. Results were presented as mean ± SEM . Statistically significant differences between means were analysed with a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

5.2.3 Lactate Dehydrogenase Toxicology Assay

Detailed protocol for the assessment of membrane integrity with an LDH assay is given in section 2.7. Briefly, cell toxicity was assessed with a Lactate Dehydrogenase (LDH) based *In Vitro* Toxicology Assay Kit as per the manufacturer's protocol. Absorbance was measured at 490 nm with a Fluostar Galaxy Multiplate reader and wavelength correction was performed at 650 nm. The absorbance reading corresponding to each extracellular LDH level was divided by the absorbance reading for each total intracellular LDH level to obtain a percentage of total LDH released per condition in accordance with Dringen and Hamprecht (1996). Statistically significant differences between means were analysed with a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant.

5.2.4 Reverse Transcription-Polymerase Chain Reaction

Detailed protocol for RT-PCR is given in section 2.8. Briefly, total RNA was extracted from microglia using a GenElute™ Mammalian Total RNA Miniprep Kit, according to the manufacturer's protocol. The quantity of isolated RNA was determined spectroscopically using a NanoDrop ND-1000 Spectrophotometer. Extracted total RNA was reverse transcribed to cDNA using a SuperScript™ III First-Strand Synthesis System and oligo$(dT)_{20}$ primer according to the manufacturer's protocol. The possibility of residual DNA contamination was tested for by replacing the reverse transcriptase enzyme with RNAse free water. The cDNA was stored at -20°C until the polymerase chain reaction (PCR). Aliquots of first strand cDNA (40 ng) were amplified in 10 µL of Platinum SYBR-Green qPCR SuperMix-UDG with 0.8 µL of forward primer, 0.8 µL of reverse primer, made up to a total volume of 20 µL with diethylpyrocarbonate-treated H_2O. PCR was carried out using the Rotor-Gene 3000 system. Incubation conditions were as follows: 2 min at 50°C, 2 min at 95°C, then thermo-cycling for 40 cycles of 20 s at 95°C, 20 s at 60°C then 20 s at 72°C. The threshold cycle number and reaction efficiencies of each condition were identified with Rotor-Gene 3000 v3.1 software. Relative quantification was carried out using the relative expression software tool (Pfaffl et al., 2002) whereby IL-10 mRNA expression was standardized by the house-keeping gene 18S rRNA expression. Mean relative mRNA levels were determined with at least 3 separate reactions per condition and mean levels were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Values are expressed as mean relative IL-10 mRNA expression ± SEM.

5.2.5 Fluorescence immunocytochemistry

Detailed protocol for the detection of cellular proteins using fluorescent antibodies is given in section 2.5. Briefly, 24 h after transfer of coverslips prepared in section 4.2.1, a number of coverslips were incubated in 1 μg/ml LPS for 24 h, while others were left in DMEM/BSA. Subsequently coverslips were washed twice for 5 min each wash HEPES buffered salt solution. Cells on coverslips were then fixed with 2% paraformaldehyde solution in PBS for 10 min. Coverslips were washed twice for 5 min each with PBS and then incubated in PBS containing 1% BSA and 0.05% saponin for 30 min. Cells on coverslips were then incubated for 2 h at room temperature with primary antibodies diluted in PBS containing 1% BSA and 0.05% saponin. Primary antibodies were washed out three times for 10 min each with PBS and then cells were incubated in secondary antibodies diluted in PBS containing 1% BSA and 0.05% saponin for 1 hour at room temperature. Secondary antibodies were washed out three times with PBS for 10 min each wash. Excess moisture was removed from the back of coverslips and they were mounted on rectangular glass coverslips using ProLong Gold anti-fade mounting media with DAPI. Slides were sealed with nail polish and subsequently stored at 4°C until viewing. Fluorescence was visualised with an inverted Axiovert 200M inverted microscope and images were acquired with an AxioCam HR digital monochrome charge-coupled device camera at a constant exposure time using Axiovision 3.1 software. Using Image J a constant arbitrary brightness threshold was applied to all these images and the number of cells with regions of brightness above this threshold as a percentage of total cells was counted per field of view. This percentage was averaged over a minimum of three

different fields of view per condition and mean percentages were compared using a one-tailed Student's two sample t-test assuming unequal variance, with $p < 0.05$ considered as statistically significant. Percentages were expressed as mean ± SEM.

5.3 RESULTS

5.3.1 The TLR4 is present on spinal cord microglia

There is functional evidence for the presence of the TLR4 on spinal cord microglia (Ogata et al., 2003). However the presence of the TLR4 protein on the surface of spinal cord microglia is yet to be demonstrated by immunocytochemistry. In the present study, 85 ± 3% of microglial cells examined (n = 157 cells; 3 batches) stained brightly for TLR4 (as determined by use of an arbitrary brightness threshold; Figure 5.1). Pre-incubation in 1 μg/ml LPS for 24 h did not significantly alter the percentage of brightly stained cells (83 ± 5%; n = 158 cells; 3 batches).

5.3.2 LPS stimulates IL-10 release from spinal cord microglia which is enhanced on co-exposure to glutamate

The ability of glutamate and the TLR4 agonist LPS to stimulate IL-10 release from activated spinal cord microglia was explored. Microglia were pre-incubated in 1 μg/ml LPS for 8 h as it takes at least 4 h for microglia to display an activated morphology on exposure to LPS (Sugaya et al., 1998). After this pre-incubation, a further 16 h exposure to LPS stimulated release of 174 ± 17 pg/ml IL-10 (n = 59) while a 16 h exposure to LPS + 1 mM glutamate significantly enhanced IL-10 release as compared to release elicited by LPS alone (266 ± 22 pg/ml; n = 59; $p < 0.001$; Figure 5.2). Conversely, unstimulated microglia released 31 ± 9 pg/ml IL-10 over 16 h (n = 53) and this value was not enhanced by the application of 1 mM glutamate for 16 h (26 ± 8; n = 7; Figure 5.2). The average percentage increase of IL-10 release

Figure 5.1 The TLR4 is expressed on spinal cord microglia. Representative images of unstimulated spinal cord microglia. The left panel displays TLR4 staining in red. The middle panel represents staining in green of CD11b, an adhesion molecule found on microglia. The right column displays a merged image of the left and middle columns. In the merge d image blue represents DAPI-staining of cell nuclei. The scale bar represents 20μ m and holds for all images in the row.

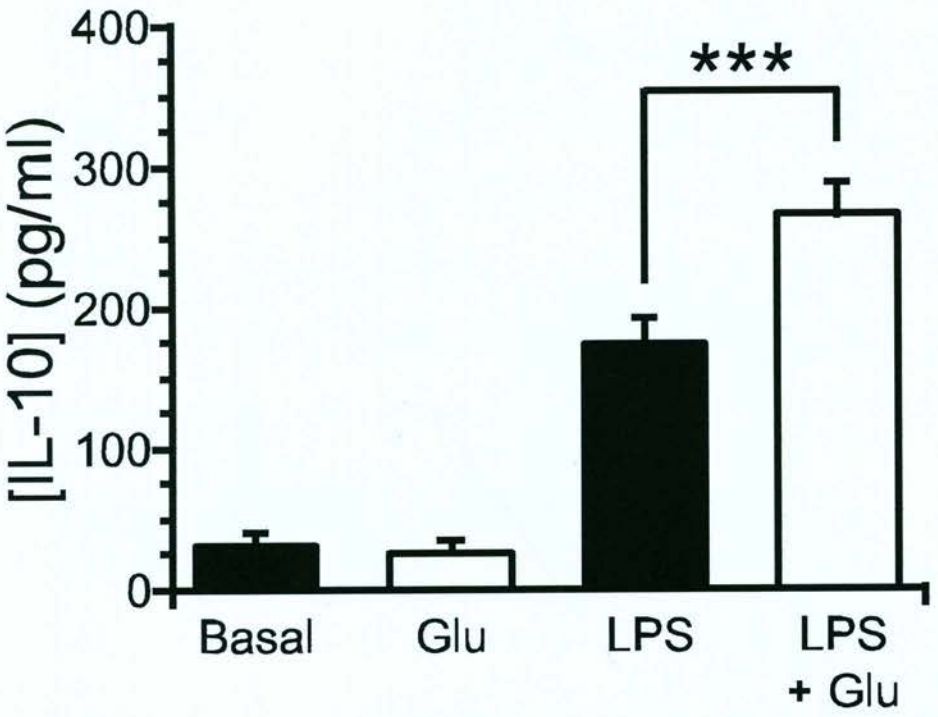


Figure 5.2 Glutamate significantly potentiates LPS-stimulated IL-10 release from spinal cord microglia. Treatments of microglia were as follows: basal – no addition of agonists (n = 53); Glu – a 16 h exposure to 1 mM glutamate (n = 7); LPS – 8 h pre-incubation in LPS (1 μg/ml) after which the media was removed and replaced with LPS for 16 h (n = 59); LPS + Glu – 8 h pre-incubation in LPS after which the media was removed and replaced with LPS + 1 mM glutamate for 16 h (n = 59). ***$p<0.001$.

in the presence of glutamate was 172 ± 7 % (n = 59). Immunocytochemistry revealed there were no astrocytes present in cultures displaying enhanced IL-10 release (n = 234) and no cells displayed the morphology of oligodendrocytes. All cells examined were CD11b positive (n = 234), and although this does not rule out the possibility that blood-born macrophages may have been in cultures, the morphology of cells resembled that of microglia as opposed to blood-born macrophages. Additionally, it has been reported that > 99 % of mononuclear cells isolated from murine central nervous system tissue are microglia (Giulian et al., 1995), strongly suggesting that the cell type which displayed LPS and glutamate-stimulated IL-10 release in these experiments was microglia.

Agonists were applied with different pre-incubation conditions in order to explore if an 8 h pre-incubation in LPS was necessary for LPS-stimulated IL-10 release and the potentiating effect of glutamate presence. Without prior exposure to LPS, a 16 h application of 1 μg/ml LPS stimulated 12 ± 7 pg/ml IL-10 release (n = 4). This release significantly increased to 48 ± 2 pg/ml (n = 4; $p < 0.01$) when 1 mM glutamate was co-applied with LPS. If cells were exposed to an 8 h pre-incubation in 1 mM glutamate, then the amount of IL-10 released from a subsequent 16 h exposure to LPS + glutamate was significantly higher than that seen if there was no pre-incubation (39 ± 5 pg/ml, n = 6, $p < 0.01$). Ensuing experiments were performed with an 8 h pre-incubation in LPS as the highest levels of IL-10 release were seen with this time of pre-incubation.

To verify that release of IL-10 on exposure to LPS and glutamate was not a result of an increase in cell death, an LDH assay was conducted. The amount of LDH accumulated in the extracellular medium of unstimulated cells over 16 h as a percentage of the total intracellular level of LDH (13.30 ± 2.52 %; n = 6) did not significantly differ from the percentage for cells exposed to LPS (1 μg/ml) and glutamate (1 mM) for 16 h (7.85 ± 1.77 % ; n = 6).

5.3.3 The enhancement of IL-10 release by glutamate is dependent on the concentration of glutamate

LPS-stimulated IL-10 release was potentiated by glutamate in a dose-dependent manner with an EC_{50} of 103 μM (Figure 5.3). After pre-incubation in LPS, when glutamate was applied for 16 h with 1 μg/ml LPS, 1 μM glutamate elicited 189 ± 12 pg/ml IL-10 release (n = 4), 10 μM elicited 154 ± 13 pg/ml (n = 4), 100 μM elicited 217 ± 22 pg/ml (n = 4) and 500 μM elicited 245 ± 39 pg/ml IL-10 release (n = 4). Glutamate at 1 mM enhanced LPS-stimulated release of IL-10 to 266 ± 22 pg/ml (n = 59) which is significantly greater than IL-10 release stimulated by LPS alone (174 ± 17 pg/ml; $p < 0.05$) and at 10 mM glutamate enhanced release to 264 ± 26 pg/ml (n = 4; $p < 0.05$). To ensure a concentration above EC_{50} was used, all proceeding experiments involving glutamate were carried out with a glutamate concentration of 1 mM. As mentioned in section 4.3.3, LPS was used at a concentration of 1 μg/ml in subsequent experiments as this concentration is commonly used in the previous literature (for example Beck et al. (2008), Noga et al. (2008) and Seo et al. (2004)).

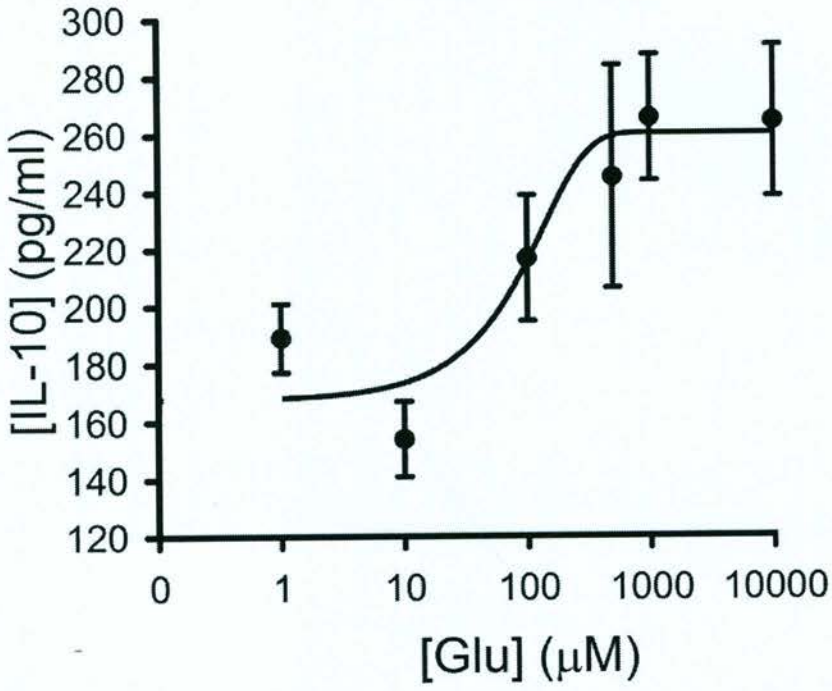


Figure 5.3 The modulation of LPS-stimulated IL-10 release by glutamate is dose-dependent. Microglia were pre-incubated in 1 μg/ml LPS for 8 h. Media was removed and LPS with various concentrations of glutamate (Glu) was added. EC_{50} was 103 μM. For all concentrations, n was at least 4.

5.3.4 LPS-stimulated IL-10 release from spinal cord microglia and enhancement in the presence of glutamate is time-dependent

In section 4.3.4 it was seen that LPS-stimulated IL-10 release from spinal cord astrocytes was time-dependent. It was examined whether LPS-stimulated IL-10 release from spinal cord microglia was also dependent on the duration of LPS application. After an 8 h pre-incubation in LPS, a subsequent 2 h exposure to LPS produced IL-10

levels of 48 ± 7 pg/ml (n = 6), a 4 h exposure produced 92 ± 19 pg/ml (n = 6), an 8 h exposure produced 132 ± 21 pg/ml (n = 6), a 16 h exposure produced 174 ± 17 pg/ml (n = 65), a 24 h exposure produced 387 ± 30 pg/ml (n = 6) and a 48 h exposure produced 690 ± 16 pg/ml IL-10 (n = 6; Figure 5.4A).

To determine if the enhancement of IL-10 levels in the presence of glutamate and LPS were similarly time-dependent, LPS and glutamate were added for various times after an 8 h pre-incubation in LPS. Further co-exposure to LPS and glutamate for 2 h produced IL-10 levels of 46 ± 7 pg/ml (n = 6), a 4 h co-exposure produced 132 ± 28 pg/ml, an 8 h co-exposure produced 167 ± 32 pg/ml (n = 6), a 16 h co-exposure produced 266 ± 22 pg/ml (n = 65), a 24 h co-exposure produced 540 ± 85 pg/ml (n = 6) and a 48 h co-exposure produced 733 ± 88 pg/ml IL-10 (n = 6; Figure 5.4A).

The level of IL-10 release elicited by co-exposure to LPS and glutamate was compared to the level stimulated on exposure to LPS alone for the same amount of

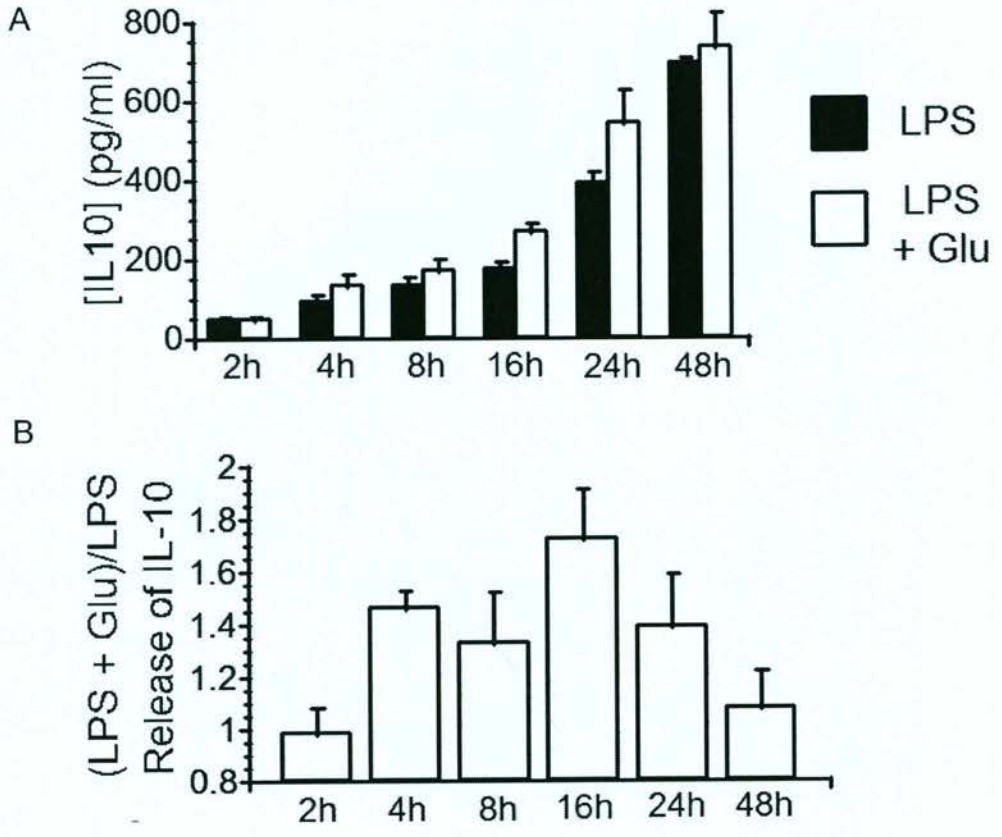


Figure 5.4 LPS-stimulated IL-10 release and the potentiating effect of glutamate is time-dependent. Microglia were pre-incubated in 1 μg/ml LPS for 8 h. Media was removed and either LPS (1 μg/ml) or LPS + glutamate (1 mM) were added for various durations. A) Summary histogram of the effect of various exposure times to LPS alone (1 μg/ml; black columns) or LPS + Glu (1 mM; white columns). B) Summary histogram of the ratio of LPS-stimulated IL-10 release in the absence and presence of glutamate. The peak modulatory effect occurred when glutamate was applied with LPS for 16 h. Values are expressed as a mean ratio of LPS + Glu release to LPS release ± SEM. For all time-points, n was at least 4.

time (Figure 5.4B). The relative increase of LPS + glutamate-stimulated IL-10 release beyond that stimulated by LPS alone was largest when agonists were added for 16 h (1.72 ± 0.07 fold higher; n = 59; Figure 5.4B). A 2 h exposure to LPS + glutamate elicited a 0.98 ± 0.10 fold change in IL-10 release compared to a 2 h exposure to only LPS (n = 6). A 4 h exposure to agonists elicited a 1.46 ± 0.07 fold higher release (n = 6). An 8 h exposure elicited a 1.33 ± 0.19 fold higher release (n = 6). A 24 h exposure to agonists elicited a 1.39 ± 0.20 fold higher release (n = 6) and a 48 h exposure elicited a 1.08 ± 0.14 fold higher response (n = 6). Consequently, agonists were applied for 16 h in subsequent experiments to ensure maximal IL-10 release.

5.3.5 LPS-stimulated IL-10 release and the potentiating effect of glutamate exposure involves transcription

In spinal cord microglia glutamate is known to activate p38, a MAPK involved in IL-10 mRNA production (Ejarque-Ortiz et al., 2007; Tikka and Koistinaho, 2001). Furthermore, the 16 h optimal time of exposure to glutamate reported in section 5.3.4 is within the time documented for IL-10 mRNA production (Ledeboer et al., 2002). Given this, it was investigated if transcription and translation were involved in the potentiating effect of glutamate.

LPS-stimulated IL-10 release (72 ± 4 pg/ml; n = 8; $p < 0.001$) and LPS + glutamate-stimulated IL-10 release (62 ± 5; n = 8; $p < 0.001$) were significantly decreased by preventing mRNA transcription with actinomycin D (1 μg/ml). Glutamate had no

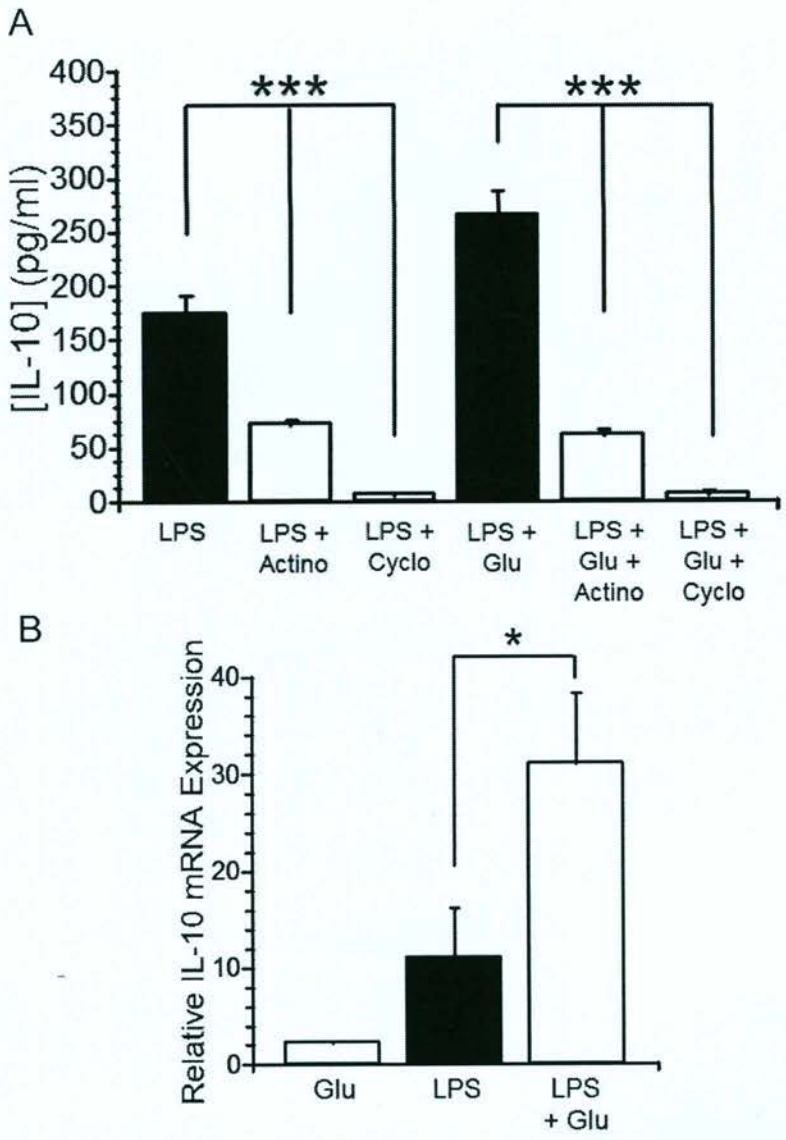


Figure 5.5 The potentiating effect of glutamate involves transcription. A) The effect of incubation in actinomycin D (Actino; transcription inhibitor; 1 μg/ml) and cycloheximide (Cyclo; translation inhibitor; 1 μg/ml) on LPS (1 μg/ml) and LPS plus glutamate (Glu; 1 mM)-stimulated IL-10 release. Both actinomycin D (n = 8) and cycloheximide (n = 8) significantly decreased LPS-stimulated IL-10 release. Similarly, both actinomycin D (n = 8) and cycloheximide (n = 8) significantly decreased the effects of glutamate. B) IL-10 mRNA expression after a 24 h incubation in Glu (n = 4), LPS (n = 3) or LPS + Glu (n = 3), normalised to 18S rRNA expression relative to unstimulated IL-10 mRNA expression. *$p<0.05$, ***$p<0.001$.

potentiating effect in the presence of actinomycin D, as shown in Figure 5.5A. Also, both LPS-stimulated IL-10 release (6.62 ± 1.63 pg/ml; n = 8; $p < 0.001$) and LPS + glutamate-stimulated IL-10 release (7.06 ± 2.38 pg/ml; n = 8; $p < 0.001$; Figure 5.5A) were ablated by preventing translation with cycloheximide (1 μg/ml).

Incubation in actinomycin D and cycloheximide caused the number of coverslip-attached microglial cells to decrease. To ensure incubation in these inhibitors did not lead to increased cell death, an LDH assay was performed. The amount of LDH found in the extracellular medium as a percentage of total intracellular LDH was not significantly larger in microglia incubated in actinomycin D with LPS + glutamate (13.91 ± 1.96 %; n = 4) from the percentage found in medium of unstimulated microglia (13.30 ± 2.52 %; n = 6). Similarly, incubation in cycloheximide with LPS + glutamate did not generate significantly larger amounts of released LDH (8.36 ± 0.91 %; n =4) compared to unstimulated microglia.

RT-PCR was used to investigate the influence of glutamate on IL-10 mRNA expression in spinal cord microglia. Glutamate elicited a 2.42 ± 0.21 (n = 4) fold increase in mRNA levels over those found in unstimulated microglia. An 11.06 ± 5.21 (n = 3) fold increase was elicited by LPS. LPS + glutamate significantly increased expression levels beyond this LPS-stimulated level to 31.09 ± 7.26 fold above basal expression (n = 3; $p < 0.05$; Figure 5.5B).

5.3.6 Glutamate acts on multiple glutamate receptor subtypes to bring about increased IL-10 release in the presence of LPS

Agonists and antagonists were used to examine the role of AMPA, kainate, NMDA and mGluRs in the increase of IL-10 release seen on co-exposure to glutamate and LPS. A range of agonists to glutamate receptor subtypes potentiated LPS-stimulated IL-10 release. tACPD, a mGluRI and II agonist, increased LPS-stimulated IL-10 release across a variety of concentrations. The ratio of IL-10 release in the presence of 10 μM tACPD + LPS compared to LPS alone was 1.36 ± 0.07 (n = 11). This ratio for 100 μM tACPD was 1.61 ± 0.07 (n = 8; Figure 5.6A) and for 1 mM tACPD was 1.70 ± 0.10 (n = 6). The ratio of IL-10 release in the presence of 500 μM DHPG (mGluRI agonist) + LPS compared to LPS alone was 1.50 ± 0.11 (n = 6; Figure 5.6A). The ratio for the mGluRII agonist APDC was 1.59 ± 0.24 (5 μM; n = 6; Figure 5A). Contrastingly, for the mGluRIII agonist L-AP4 this ratio was 0.89 ± 0.08 (1 mM; n = 11; Figure 5.6A). Additionally, kainate (100 μM; 1.22 ± 0.05; n = 11), AMPA (100 μM; 1.28 ± 0.05; n = 7) and NMDA (500 μM, 1.71 ± 0.17; n = 7) also showed increased levels of IL-10 release compared to the level seen on sole application of LPS (Figure 5.6A).

The potentiation of IL-10 release in the presence of LPS and glutamate was significantly decreased by mGluR antagonists. The ratio of IL-10 release in the presence of LPS + glutamate + 1 mM MSPG (mGluRII and III antagonist) to IL-10 release in the presence of LPS + 1 mM MSPG was 1.27 ± 0.10 (n = 11; $p < 0.001$ compared to LPS + glutamate/LPS; Figure 5.6B). The potentiation was further

decreased by co-exposure to 1 μM MPEP (mGluR 5 antagonist) and 75 μM CPCCOEt (mGlu receptor 1 antagonist), producing a ratio of 1.06 ± 0.16 ($n = 6$; $p < 0.01$; Figure 5.6B) and by co-exposure to 1 μM MPEP, 75 μM CPCCOEt and 500 μM EGLU (mGluR group II antagonist), producing a ratio of 1.13 ± 0.09 ($n = 6$; $p < 0.001$; Figure 5.6B).

Ionotropic glutamate receptor antagonists also significantly decreased the potentiation by glutamate. The ratio of IL-10 release stimulated by LPS + glutamate + 40 μM MK-801 (NMDA receptor antagonist) to IL-10 release stimulated by LPS + MK-801 was 1.31 ± 0.11 ($n = 11$; $p < 0.01$; Figure 5.6B) and this ratio was 1.11 ± 0.06 ($n = 11$; $p < 0.001$; Figure 5.6B) for the AMPA and kainate receptor antagonist 20 μM CNQX.

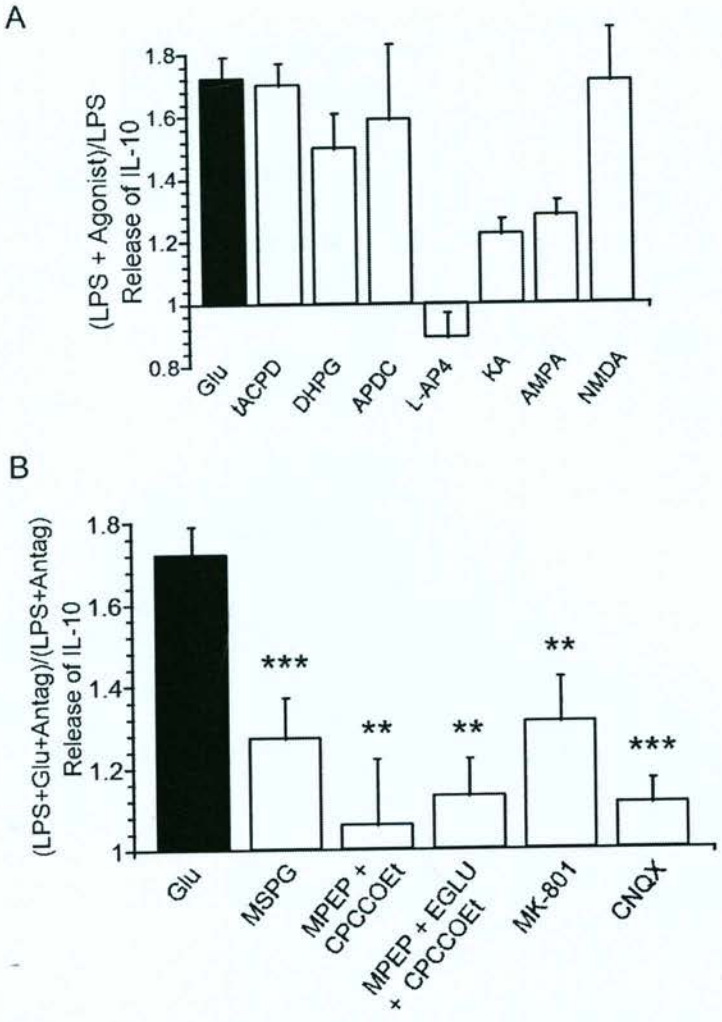


Figure 5.6 Glutamate potentiates LPS-stimulated IL-10 release by activating a range of glutamate receptor subtypes. A) The effect of glutamate receptor agonists glutamate (Glu; 1 mM ; n = 59), tACPD (mGluRI and II agonist; 100μM ; n = 8), DHPG (mGluRI agonist; 500μM ; n = 6), APDC (mGluRII agonist; 5 μM ; n = 6), L-AP4 (mGluRIII agonist; 1 mM; n = 11), KA (100 μM ; n = 11), AMPA (100 μM ; n = 7) and NMDA (500 μM ; n = 7) on LPS (1 μg/ml)-stimulated IL-10 release. Values are expressed as mean ratio of LPS + agonist release to LPS release ± SEM. B) The effect of glutamate receptor antagonists M SPG (mGluRII and III antagonist; 1 mM ; n = 11), MPEP (mGluR5 antagonist; 1 μM) + CPCCOEt (mGluR1 antagonist; 75 μM ; n = 6), MPEP + CPCCOEt + EGLU (mGluRII antagonist, 500 μM ; n = 6), MK-801 (NMDA receptor antagonist, 40 μM ; n = 11) and CNQX (AMPA and kainate receptor antagonist, 20 μM ; n = 11) on glutamate + LPS-stimulated IL-10 release. Values are expressed as mean ratio of LPS + Glu + antagonist to LPS + antagonist ± SEM. **p<0.01, ***p<0.001.

5.4 DISCUSSION

In accordance with previous research on mouse, human and rat microglia from the brain, the present study shows unstimulated cultured rat spinal cord microglia release a small amount of IL-10 (Ledeboer et al., 2002; Mizuno et al., 1994; Williams et al., 1996). LPS stimulates IL-10 release from spinal cord microglia, as has been demonstrated with brain microglia (Ledeboer et al., 2002; Mizuno et al., 1994; Williams et al., 1996). In a dose- and time-dependent manner, the presence of glutamate significantly enhances the release of IL-10 on LPS exposure, without itself stimulating IL-10 release.

5.4.1 The interaction between LPS and glutamate involves IL-10 mRNA upregulation

Unstimulated spinal cord microglia displayed low constitutive IL-10 mRNA levels which were upregulated by LPS as previously reported for brain microglia (Ledeboer et al., 2002; Mizuno et al., 1994). LPS increased IL-10 release by working at a transcriptional level as the prevention of transcription greatly decreased the ability of LPS to stimulate IL-10 release. Prevention of transcription, however, did not completely ablate LPS-stimulated IL-10 release. This only occurred on blockade of translation, suggesting LPS also has post-transcriptional effects as discussed for astrocytes in section 4.4.2.

The interaction between glutamate and LPS resulting in enhanced IL-10 release appears to require the upregulation of LPS-induced IL-10 mRNA. IL-10 mRNA

levels were vastly increased by glutamate in the presence of LPS and glutamate had no effect on LPS-stimulated IL-10 release when transcription was prevented. Maximal potentiation in this present study occurred at 16 h after exposure to glutamate, within IL-10 mRNA production times (Ledeboer et al., 2002).

Despite the evidence that glutamate exposure led to increased IL-10 release via an enhancement of transcription, glutamate did not have a large impact on IL-10 mRNA levels on its own. This situation was similar to that described in section 4.4.1 with spinal cord astrocytes. As mentioned in section 4.4.1, ERK can enhance LPS-stimulated IL-10 mRNA levels and release, without alone increasing IL-10 release through covalent modification of histones at the IL-10 promoter locus (Lucas et al., 2005). It has not been examined whether glutamate activates ERK in spinal cord microglia, however given that glutamate activates ERK in spinal cord astrocytes (D'Onofrio et al., 2001; Peavy et al., 2001) and increases LPS-stimulated IL-10 mRNA without itself substantially increasing IL-10 mRNA levels in these cells, we hypothesise that glutamate also brings about an increase in LPS-stimulated IL-10 mRNA and release by activation of ERK. This could be tested in further experiments through use of ERK inhibitors.

Alternatively, the possibility exists that TLR4 stimulation may be enhancing the ability of glutamate to bring about IL-10 release. As mentioned in sections 1.4.2.4 and 5.1, glutamate activates p38, a MAPK which can lead to increased IL-10 production (Ejarque-Ortiz et al., 2007; Tikka and Koistinaho, 2001). Similar to what

was stated in section 4.4.1, perhaps there is a threshold as to the level of p38 activation required to affect IL-10 production and glutamate on its own does not increase activation of p38 above this threshold. When glutamate is co-applied with LPS, however, the amount of p38 stimulated by LPS on its own in combination with that elicited by glutamate may be enough to result in the observed increase of IL-10.

5.4.2 Glutamate activates AMPA, NMDA, mGlu and possibly KA receptors to interact with LPS and enhance IL-10 release

All glutamate receptor subtypes (apart from mGluRIII and possibly KA) contributed to the interaction with LPS. Antagonists to all subtypes significantly blocked the interaction and agonists to all subtypes (apart from mGluRIII) elicited IL-10 release in the presence of LPS. Spinal cord microglia have been shown to express AMPA, NMDA and mGlu receptors and although the presence of kainate receptors on spinal cord microglia has not been examined, microglia from the cortex and hippocampus have been shown to express kainate receptors (Christensen et al., 2006; Liu et al., 2006; Yamada et al., 2006). The functional significance of these kainate receptors is uncertain, however, as kainate-induced currents are completely ablated by a specific AMPA receptor blocker in cortical microglia after cell quiescence (Hagino et al., 2004; Yamada et al., 2006). In light of this, in the present study it can not be ruled out that the increase in IL-10 release seen on co-addition of kainate with LPS may be a result of kainate acting on AMPA receptors.

Although the effect of glutamate on microglia is not extensively investigated in the existing literature, there is another report of more than one subtype of glutamate receptor being involved in an effect of glutamate on microglia. All four receptor subtypes have been linked to the activation of the transcription factors c-fos and c-jun in microglia by glutamate (Eun et al., 2004). This present study is another example of the pantheic action of glutamate on its receptor subtypes in microglia.

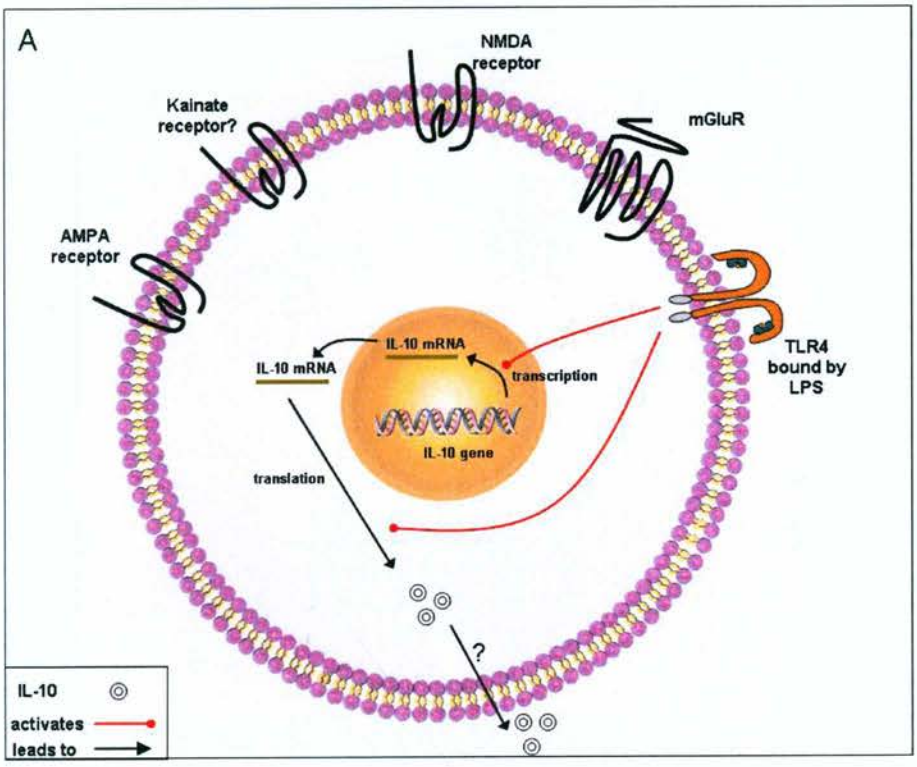
5.4.3 Presence of the TLR4 on spinal cord microglia

The immunocytochemical identification of TLR4s on the surface of cultured spinal cord microglia in this study adds to the functional evidence that the TLR4 is present on the surface of spinal cord microglia (Ogata et al., 2003). In addition to the surface localization of the TLR4, a cytoplasmic localization was also apparent. LPS exposure could not redirect cytoplasmic TLR4 to the membrane, nor could it increase intensity of TLR4 staining. This is consistent with demonstrations that LPS exposure does not alter TLR4 mRNA expression in cortical microglia (Laflamme and Rivest, 2001; Lehnardt et al., 2002).

5.4.4 Summary of the mechanisms of IL-10 release from spinal cord microglia on exposure to LPS and glutamate

In conclusion, activation of TLR4s on spinal cord microglia results in an increase in IL-10 mRNA expression and subsequent increase in IL-10 release. This increase in IL-10 mRNA levels and IL-10 protein levels on LPS application is greatly enhanced in the presence of glutamate, whilst glutamate itself does not substantially affect

IL-10 mRNA expression and IL-10 protein levels. Glutamate acts on NMDA, AMPA and mGlu receptors to contribute to this increase, with a possible action on kainate receptors. These steps are summarized in Figure 5.7.


A
NMDA receptor
Kainate receptor?
mGluR
AMPA receptor
TLR4 bound by LPS
IL-10 mRNA
IL-10 mRNA
transcription
IL-10 gene
translation
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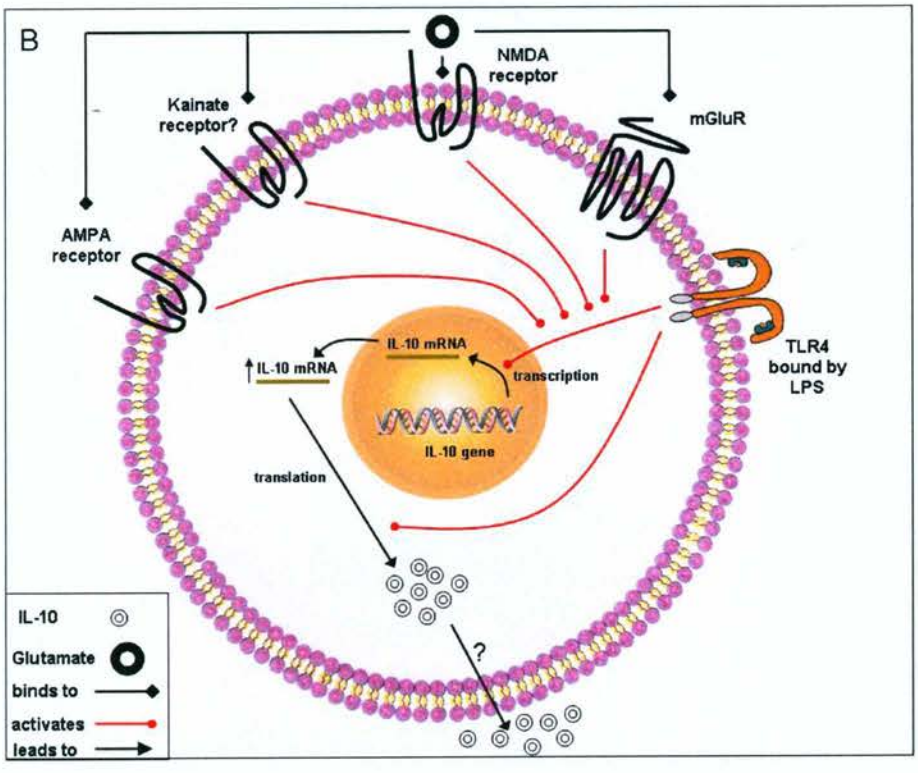
IL-10
activates
leads to


B
NMDA receptor
Kainate receptor?
mGluR
AMPA receptor
TLR4 bound by LPS
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IL-10 mRNA
transcription
IL-10 gene
translation
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IL-10
Glutamate
binds to
activates
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Figure 5.7 A model of the mechanisms of LPS-stimulated IL-10 release in the absence and presence of glutamate from spinal cord microglia. A) TLR4 activation enhances IL-10 transcription and translation, resulting in IL-10 release. B) Co-activation of AMPA, NMDA, mGlu and possibly KA receptors at the same time as TLR4 activation results in an increase in IL-10 mRNA levels. One explanation may be that this occurs through glutamate receptor-mediated enhancement of LPS-stimulated IL-10 transcription. The result is an increase in IL-10 release. Glutamate itself does not directly have a substantial effect on IL-10 mRNA levels. IL-10 release mechanisms are not clear.

CHAPTER 6

GENERAL DISCUSSION

Contents

6.1 IMPLICATIONS OF FINDINGS

In Chapter 3 it is shown that spinal cord astrocytes release ATP in response to glutamate. This release is greatly potentiated in the presence of SP. In Chapters 4 and 5 it is shown that spinal cord microglia and astrocytes release IL-10 in response to TLR4 stimulation and this release is potentiated in the presence of glutamate. These findings may be applied to extend the current knowledge of spinal chronic pain mechanisms, general spinal processing and glial biology.

6.1.1 Potential implications of glutamate and SP-stimulated ATP release for the understanding of chronic pain mechanisms

By binding spinal $P2X_4$, $P2X_3$ and potentially $P2X_7$ receptors, ATP contributes to the production of the chronic pain symptoms hyperalgesia and allodynia (Chessell et al., 2005; Dorn et al., 2004; Tsuda et al., 2003). To date, however, the only confirmed source of ATP in the spinal cord in response to non-purinergic nociceptive substances was the report that microglia release ATP in response to glutamate (Liu et al., 2006). Results from the current research have identified further possible sources of spinal ATP in pain. SP and glutamate are released from nociceptive nerve terminals in the dorsal horn by relatively high frequencies of nerve stimulation (Allen et al., 1999; Honore et al., 2000; Sasaki et al., 1998), and their concomitant release enhances pain transmission in the dorsal horn (Ishizaki et al., 1999; Mjellem-Joly et al., 1991). Results from Chapter 3 suggest that one major source of spinal ATP release in pain may be from spinal cord astrocytes in response to the concomitant release of SP and glutamate during heightened activity of nociceptive peripheral afferent terminals.

Furthermore, the potentiation of glutamate-stimulated ATP release from spinal cord astrocytes may allow ATP levels in the spinal cord to be dependent on neuronal firing patterns. Generally, classical neurotransmitters such as glutamate are packaged into small synaptic vesicles for exocytotic release from neurons whilst peptides are released from large dense core vesicles (Morris et al., 2005). Whilst small synaptic vesicles can be released from neurons after a short burst of low frequency stimulation, large dense core vesicles containing peptides such as SP require either a prolonged low frequency stimulation or a short burst of high frequency stimulation (Whim and Lloyd, 1989). This allows a dissociation between the release of classical transmitters and peptides, for example, when *Aplysia* motor neurons are briefly stimulated at a low frequency they release only acetylcholine, whereas when they are stimulated at a high frequency they release both acetycholine and peptides (Whim and Lloyd, 1989). As such, peripheral afferent nociceptive fibres do not show a significantly measurable increase in SP release when stimulated with a short electrical pulse of 1 or 10 Hz (Marvizon et al., 1997). Rather, release of SP from these fibres is seen with a prolonged (8 min) stimulation at 1 Hz or on brief high frequency stimulation (Lever et al., 2001). In inflammatory hyperalgesia, peripheral afferents demonstrate a continuous low frequency firing of about 1 Hz. Prior to the induction of chronic pain these fibres do not display continuous discharges (Schaible and Schmidt, 1988). A similar pattern of low frequency continuous firing is seen in lesioned peripheral nerves. In addition, these fibres show a more prolonged after-discharge following noxious stimulation compared to that displayed in non-lesioned controls (Keller et al., 2007). Hence, it could be predicted that large neuroactive amounts of SP are only

released into the dorsal horn during a severe or prolonged noxious stimulation, such as in cases of inflammatory or neuropathic pain and not during acute pain. This hypothesis is supported by a study demonstrating that a low concentration of the C fibre activator capsaicin only caused a measurable release of SP if it was applied to neurons after inflammatory pain induction (Warsame Afrah et al., 2004). Consequently, it is likely in acute pain that astrocytes surrounding nociceptive nerve terminals may only be exposed to glutamate, whereas in neuropathic or inflammatory pain they may be exposed to both glutamate and substance P, leading to a much larger amount of ATP released in neuropathic and inflammatory pain as opposed to acute pain. This hypothesis of a 'switch like' mechanism of ATP release, in combination with other mechanisms (Herrero et al., 2000), may help explain the different outcomes of acute pain-producing versus chronic pain-producing stimuli.

The identification of SP and glutamate-mediated ATP release from spinal cord astrocytes as a potential major source of spinal ATP suggests this process may be a possible target in the quest for novel chronic pain treatments. Perhaps one way this process might be beneficially perturbed is by upregulating ecto-ATPases on spinal cord astrocyte membranes or intrathecally injecting ATP metabolizing compounds. This may be beneficial on two levels. Firstly the released ATP may be broken down before it can reach $P2X_4$ and $P2X_7$ receptors on microglia and $P2X_3$ receptors on neurons. Additionally, increasing the breakdown of ATP will increase the levels of the anti-nociceptive adenosine in the spinal cord (for a review of the anti-nociceptive actions of adenosine, see Li and Perl (1994)).

6.1.2 Potential implications of TLR4 and glutamate-stimulated IL-10 release for the understanding of chronic pain mechanisms

Chronic pain symptoms are abrogated by introduction of IL-10 into the spinal cord and high levels of IL-10 are common in people with a painless neuropathy whereas low levels are common in people with a painful neuropathy (Milligan et al., 2006a; Milligan et al., 2006b; Uceyler et al., 2007). Despite this, to date, endogenous sources of spinal IL-10 have not been identified. In neuropathic pain the TLR4 is activated. Results from Chapters 4 and 5 suggest that TLR4 activation of spinal cord astrocytes and microglia are potential sources of IL-10 in the spinal cord. Moreover, when TLR4 activation occurs in conjunction with glutamate receptor activation on these cells, the amount of IL-10 release is significantly increased. This suggests that the excitatory nervous system and immune system of the spinal cord can synergistically interact to form an endogenous protection mechanism against the deleterious inflammatory events that occur in the spinal cord during chronic pain. The present results also suggest that enhancing the interaction between these two systems may be a potential novel target in the search for a chronic pain treatment.

This demonstration of the anti-nociceptive effects of TLR4 stimulation should be considered alongside the pro-nociceptive effects of glial TLR4 stimulation. TLR4 stimulation elicits TNF-α, IL-1β and IL-6 release from spinal cord glia (Benveniste et al., 1995; Heyen et al., 2000; Kremlev and Palmer, 2005; Ledeboer et al., 2000; Mizuno et al., 1994; Sawada et al., 1999). Release of such pro-inflammatory cytokines occurs soon after TLR4 stimulation. For example, TNF-α mRNA

expression increases significantly within 30 min of microglial TLR4 activation (Kremlev and Palmer, 2005) and significant increases in IL-1β release are seen within 1 h of microglial TLR4 activation (Sanz and Di Virgilio, 2000). Moreover, in animal models of pain increases in IL-1β and its pro-inflammatory effects in the spinal cord are seen within 1 h of inflammatory pain induction (Sweitzer et al., 1999; Watkins et al., 1997) and the inflammatory effects of TNF-α in the spinal cord can be observed within 1 h of neuropathic pain induction (Milligan et al., 2003). Additionally, IL-6 release and IL-1β release subsequent to TLR4 stimulation on microglia peaks at 6 h after TLR4 stimulation (Ledeboer et al., 2002; Sanz and Di Virgilio, 2000).

The timecourse of IL-10 release after TLR4 activation (and concomitant glutamate receptor stimulation) from glial cells is quite different to the timecourse of pro-inflammatory cytokine release. In the present study, it was not until 4 h and 8 h after TLR4 stimulation that substantial (> 150 pg/ml) amounts of IL-10 were released from astrocytes and microglia respectively. Levels of IL-10 continued to exponentially rise over the 48 h timecourse examined. This is in accordance with the timecourse of TLR4-mediated IL-10 release from cortical microglia (Ledeboer et al., 2002).

Considering the differences in timecourse between the pro- and anti-nociceptive actions of TLR4 stimulation, it could be hypothesised that after the induction of chronic pain TLR4 activation initially results in pro-nociceptive effects through the release of pro-inflammatory cytokines. As IL-10 release starts to increase in a delayed timeframe, it is hypothesised that the anti-inflammatory and anti-nociceptive

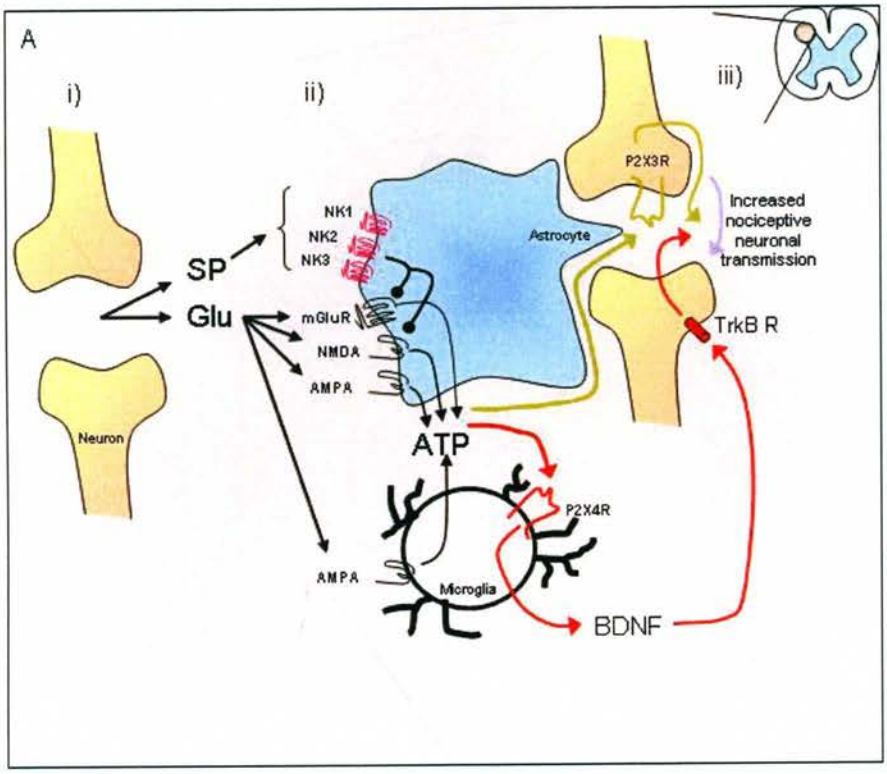
effects of TLR4 stimulation start to hinder the deleterious effects of the pro-inflammatory cytokines. In support of this, the peak in IL-1β and IL-6 release at 6 h after TLR4 stimulation occurs at the time when IL-10 release is starting to become appreciable as demonstrated by the present study and that by Ledeboer et al. (2002), opening the possibility that the decrease in pro-inflammatory release could be a result of the inhibitory actions of IL-10. This hypothesis could be subsequently tested by measuring the timecourse of IL-6 and IL-1β release in the presence of an anti-IL-10 antibody or IL-10R antagonist.

6.1.3 A proposed model of spinal events following noxious stimulation

Considering the discussions presented in sections 6.1.1 and 6.1.2 and what is known in the existing literature, a three-phase sequence of events in the spinal cord during chronic pain can be hypothesised. The release of glutamate and SP into the dorsal horn occurs immediately at the induction of chronic pain, when peripheral afferents are initially stimulated (Millan, 1999). This study suggests that immediately on exposure to glutamate and SP, spinal cord astrocytes release ATP. The same immediate timeframe is seen with release of ATP by spinal cord microglia on exposure to glutamate as reported by Liu et al (2006). This immediate release of ATP both from astrocytes in this study and microglia in the study by Liu et al (2006) peaks rapidly, within 15 min. Once released, ATP may then be able to bind $P2X_4$ receptors on microglia, $P2X_3$ receptors on neurons and possibly $P2X_7$ receptors on microglia to contribute to allodynia and hyperalgesia. These events are hypothesized to be the

first phase in spinal processing after noxious stimulation. Glutamate and SP are elevated in the dorsal horn weeks after injury (Allen et al., 1999; Kawamata and Omote, 1996). As the present study only examined the affects of acute exposure to SP and glutamate, it is not known if chronic exposure to these transmitters results in prolonged elevation of ATP.

It is not clear whether TLR4 agonists are present in the spinal cord as soon after noxious damage as SP and glutamate are. It is known that the TLR4 is activated one day after damage as anti-sense TLR4 oligodeoxynucleotide injection into the spinal cord one day after neuropathic damage effects pain behaviours (Tanga et al., 2005) but the effect of interfering with TLR4 expression less than 24 h after the induction of pain has not been examined. That being said, the activation of the TLR4 on spinal cord microglia and astrocytes within one day of induction of chronic pain is predicted to result in pro-inflammatory cytokine release (phase 2) followed by IL-10 release (phase 3), as discussed in section 6.1.2. In this way, it is predicted astrocytes and microglia are involved in bringing about both pro-nociceptive and delayed anti-nociceptive effects in response to noxious stimulation. Furthermore, it is predicted glutamate has both pro-nociceptive and delayed anti-nociceptive effects in the spinal cord in response to noxious stimulation. This hypothesis is illustrated in Figure 6.1.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NK2
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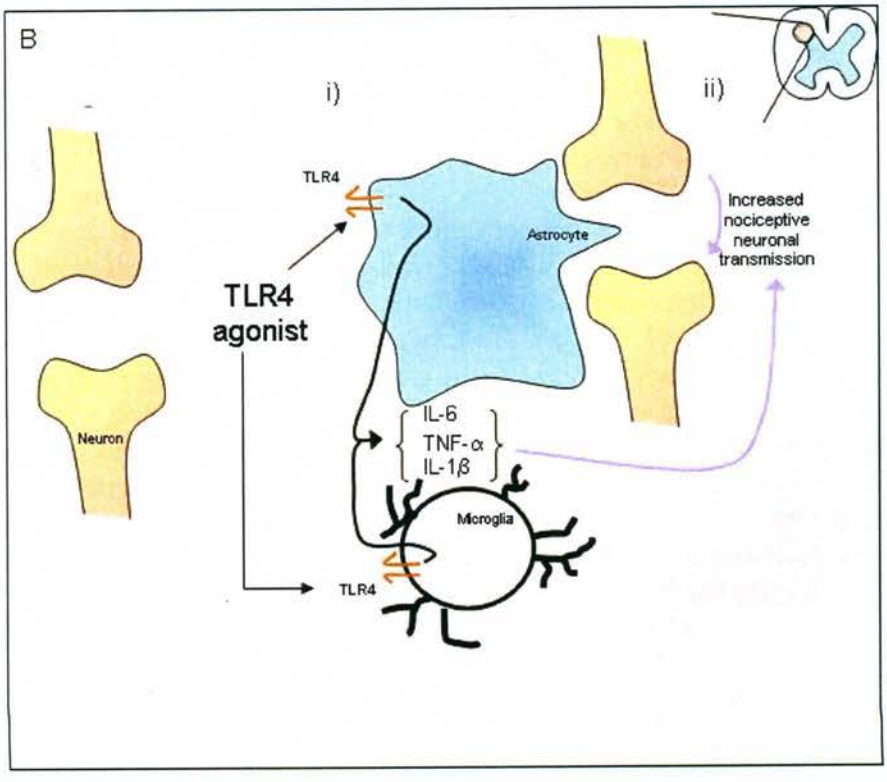
TrkB R
ATP
P2X4R
AMPA
Microglia
BDNF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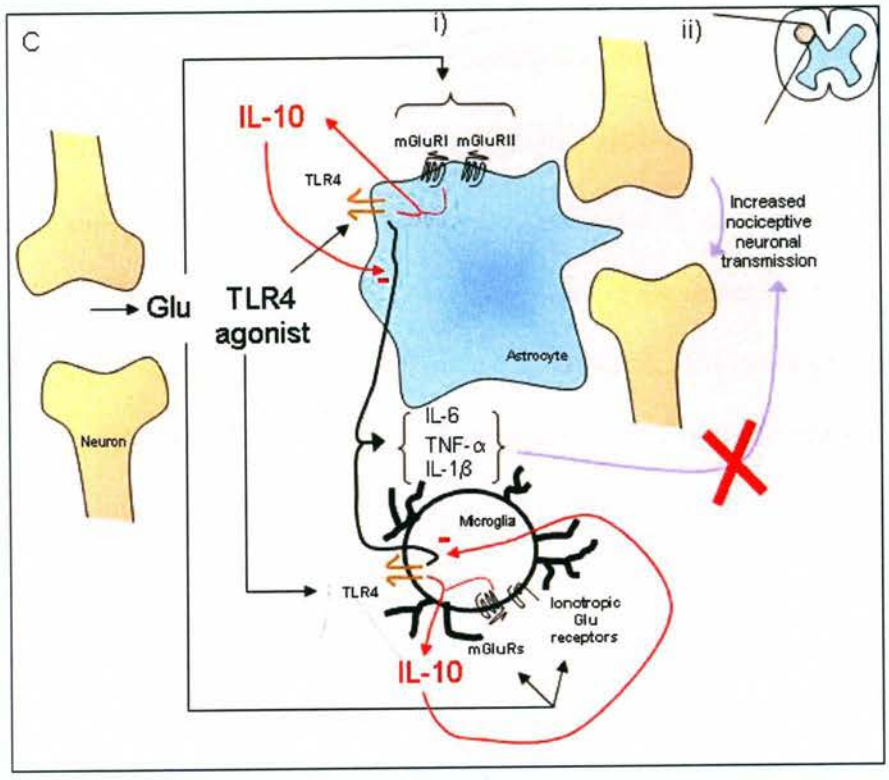

Figure 6.1 A three stage hypothesis of spinal processing after noxious stimulation in chronic pain. A) Phase 1 i) Substance P (SP) and glutamate (Glu) are released from dorsal horn neurons immediately after noxious stimulation. ii) On activation of NK receptors, mGluRs, AMPA receptors and NMDA receptors, ATP is released from astrocytes and microglia. iii) Spinal ATP can act on $P2X_4$ and $P2X_3$ receptors to increase nociceptive transmission for example by stimulating release of brain-derived neurotrophic factor (BDNF) and its actions on the receptor TrkB. The result is allodynia and hyperalgesia. Although not on this diagram, if TLR4 activation occurs at the same time as ATP release, ATP may also act on $P2X_7$ receptors on microglia to bring about IL-1β release. Lines with circular endings denote receptor potentiation. B) Phase 2 i) Within a day of noxious stimulation, activation of TLR4s by an unknown agonist results in pro-inflammatory cytokine release from astrocytes and microglia, ii) which can increase nociceptive neuronal processing. C) Phase 3. i) After an initial pro-inflammatory cytokine release, TLR4 stimulation coupled with glutamate receptor stimulation produces a delayed release of IL-10 from microglia and astrocytes which can inhibit pro-inflammatory cytokine release, ii) decreasing nociceptive neuronal processing.

6.1.4 Potential implications of glutamate and SP-stimulated ATP release for the understanding of non-pain related spinal processing

SP and glutamate-containing nerve terminals are found in descending bulbospinal neurons in the ventral horn (Hokfelt et al., 2000) and in the intermediolateral column (Helke et al., 1982; Kapoor et al., 1992) of the spinal cord. The results of this present study suggest a consequence of SP and glutamate release in these areas may be an increased release of ATP from astrocytes. As these areas are rich in purinergic receptors (Deng and Fyffe, 2004; Deuchars et al., 2001; Miles et al., 2002) this may lead to an increase in purinergic transmission in these areas, with possible functional consequences such as increased phrenic motor innervation of the diaphragm (Miles et al., 2002) and increased motor neuron firing (Deng and Fyffe, 2004).

In addition, glutamate-stimulated ATP release from hippocampal astrocytes has been shown to be involved in a negative feedback effect on neuronal propagation (Zhang et al., 2003) and the spreading of intracellular Ca^{2+} concentration increases from astrocyte to astrocyte (Cornell-Bell et al., 1990; Guthrie et al., 1999; Wang et al., 2000). Termed an intercellular Ca^{2+} wave, this propagation has been implicated in the spreading of astrocytic-ATP mediated neuronal inhibition from the original site of stimulation (Koizumi et al., 2003), modulating changes in cerebral blood flow (Filosa and Blanco, 2007; Gordon et al., 2007) and uptake of glucose from the blood by astrocytes in response to neuronal activity (Bernardinelli et al., 2004). As glutamate is found in most synapses and astrocytes enwrap these synapses, the

demonstration of glutamate-stimulated ATP release from spinal cord astrocytes opens the possibility that astrocytes surrounding glutamatergic synapses in the spinal cord may affect neuronal propagation, astrocytic intercellular Ca^{2+} wave propagation, cerebral blood flow and uptake of glucose in response to neuronal activity.

6.1.5 Potential implications of TLR4 and glutamate-stimulated ATP release for the understanding of non-pain related spinal processing

Glutamate is likely to potentiate IL-10 release induced by TLR4 activation from astrocytes and microglia in inflammatory conditions other than neuropathic pain, such as gram-negative bacterial infection and inflammatory neuronal damage. There are a number of reasons for this. Firstly, in such conditions TLR4 agonists are available. Gram-negative bacteria is a TLR4 agonist (Beutler, 2000) and neuronal damage and toxicity can cause membrane disruption and subsequent release of gangliosides, the TLR4-stimulating components of mammalian cell membranes (Jou et al., 2006). Secondly, levels of extracellular glutamate are elevated in such inflammatory conditions (Benveniste et al., 1984; Bezzi et al., 2001; Ko et al., 2003; Rego et al., 1996; Takeuchi et al., 2006) allowing the possibility that glutamate may rise to concentrations that potentiate LPS-induced IL-10 release from microglia and astrocytes.

In conditions of increased glutamate release and TLR4 activation, exposure to IL-10 has been shown to be beneficial. For example, dopaminergic neuronal degeneration resulting from exposure to gram-negative bacteria and pre-natal white matter damage

from maternal *Escherecia Coli* exposure can be decreased when infectious agents are co-applied with IL-10 (Mesples et al., 2003; Qian et al., 2006a; Qian et al., 2006b). IL-10 application at concentrations similar to that found released in the present study can decrease the amount of neuronal death when neuronal cultures are exposed to high levels of NMDA (Bachis et al., 2001). This suggests that one potential role of glutamate in such inflammatory conditions may be to restrict damage via potentiation of IL-10 levels. Indeed, the amount of neuronal death when neurons are exposed to LPS-activated astrocytes is decreased when these neurons are exposed to astrocytes which have had their mGluRs activated (Zhou et al., 2006). Similar neuroprotective effects are seen after microglial glutamate stimulation, for example, the level of neurotoxicity induced by LPS-activated microglia is greatly reduced when these microglia are stimulated with an mGluR agonist (Taylor et al., 2003). Results from this thesis may help explain part of the neuroprotective actions of glutamate in these studies. Consequently, artificial enhancement of the ability of glutamate to potentiate LPS-stimulated IL-10 release may be a therapeutic target in conditions such as infection and inflammatory neuronal damage.

6.1.6 A regional heterogeneity amongst astroctyes from the spinal cord and cortex

There is evidence in the literature for a functional difference between spinal cord and cortical astrocytes. For example SP can stimulate phosphoinositide turnover and prostaglandin release in spinal cord astrocytes but not in cortical astrocytes (Cholewinski et al., 1988; Marriott et al., 1991). Conversely, the neuropeptides

vasopressin and oxytocin can stimulate phosphoinositide turnover in cortical but not spinal cord astrocytes, even though both neuropeptides are found in the spinal cord and cortex and receptors to these neuropeptides have been identified on spinal cord astrocytes (Cholewinski et al., 1988; Condes-Lara et al., 2003; Hashimoto et al., 1985; Hosli and Hosli, 1992; Hosli et al., 1991). Additionally, a β-adrenergic agonist can increase cAMP in spinal cord and cortical astrocytes, however the hormone somatostatin blocks this response from cortical astrocytes but does not block it from spinal cord astrocytes (Cholewinski and Wilkin, 1988). It is unclear whether results presented in Chapter 4 support this idea of regional heterogeneity. In Chapter 4 it was found that LPS elicits IL-10 release from spinal cord astrocytes by acting through post-transcriptional mechanisms and not through transcription. In cortical astrocytes, LPS induces an increase in IL-10 mRNA expression and an activation of transcription factors in cortical astrocytes suggesting an action of LPS through transcription (Cao et al., 2006; Ejarque-Ortiz et al., 2007; Kelicen and Tindberg, 2004; Ledeboer et al., 2002; Ma et al., 2001; Mizuno et al., 1994; Seo et al., 2004; Williams et al., 1996). Nevertheless results in Chapter 4 show an increased mRNA expression does not necessitate increased IL-10 release, thereby clouding conclusions about the similarities of mechanism between cortical and spinal cord astrocytes.

On the other hand, the identification of AMPA as the sole receptor through which glutamate works to release ATP from spinal cord astrocytes described in Chapter 3 is in contrast with work done on cortical astrocytes by Queiroz et al (1997; 1999) as detailed in section 1.4.1.2. They found evidence that all glutamate receptor subtypes

were involved in glutamate-stimulated ATP release from cortical astrocytes. Additionally, AMPA-stimulated ATP release from spinal cord astrocytes required an influx of extracellular Ca^{2+} and was PLC-independent whereas AMPA-stimulated ATP release from cortical astrocytes was independent of Ca^{2+} influx and involved PLC (Queiroz et al., 1999). Furthermore, as explored in section 3.4.3, SP potentiates glutamate-stimulated ATP release from spinal cord but not cortical astroctyes. Hence this thesis provides support and further examples of the regional heterogeneity between spinal cord and cortical astroctyes.

6.1.7 A mechanistic heterogeneity amongst spinal cord astrocytes and microglia

In addition to the evidence for heterogeneity between spinal cord and cortical astrocytes, this thesis provides evidence for a heterogeneity in mechanisms between spinal cord astrocytes and microglia. Although spinal cord microglia and astrocytes both demonstrate glutamate-stimulated ATP release, LPS-stimulated IL-10 release, and show an enhancement of this release in the presence of glutamate, the mechanisms of each of these processes are different. Whereas spinal cord astrocytes require an influx of extracellular Ca^{2+} for glutamate-stimulated ATP release this is not involved in glutamate-stimulated ATP release from spinal cord microglia. Rather, this process requires a rise in intracellular Ca^{2+} levels and activation of PKC (Liu et al., 2006). LPS works at a transcriptional level to increase IL-10 release in spinal cord microglia but exerts its action post-transcriptionally in spinal cord astrocytes. Additionally, whereas glutamate works on multiple subtypes of microglial

glutamate receptors to enhance LPS-stimulated IL-10 release, only stimulation of mGluRs results in the effect in astrocytes.

Two points can be derived from this heterogeneity. Firstly, it provides a reassurance that observed protein release in astrocyte cultures was not due to contamination with microglia. For example, spinal cord astrocyte cultures were found to contain a small amount of microglia. The question may arise as to whether LPS-stimulated IL-10 release seen in astrocyte cultures was actually due to release from microglia. As microglial LPS-stimulated IL-10 release involved an enhancement of transcription but release from astrocyte cultures did not, this suggests that contaminating microglia did not contribute to observed IL-10 release from astrocyte cultures. Secondly, this heterogeneity suggests that it may be difficult to use the same treatment to alter the processes of glutamate-stimulated ATP release and glutamate and LPS-stimulated IL-10 release in both microglia and astrocytes. This needs to be kept in mind if these processes are to be novel targets in chronic pain treatment.

6.2 FURTHER QUESTIONS AND RESEARCH

6.2.1 Relevance to intact spinal cord function

Several hypotheses about the physiological and pathophysiological relevance of the results of this thesis have been raised in section 6.1 of this discussion. It must be remembered, when hypothesising about *in vivo* situations from the *in vitro* data of this research, that the properties and environment of cells in culture at room temperature may be different from those in the intact CNS. Amongst other differences, the

concentration of agonists *in vivo* may not be the same as those used in these experiments. It has been estimated that the concentration of synaptically released glutamate that reaches synapse-surrounding glia *in vivo* in the cerebellum is 190 μM (Dzubay and Jahr, 1999). In the present research, the EC_{50} of glutamate-stimulated ATP release from spinal cord astrocytes was 240 μM, which is in the vicinity of that reported by Dzubay and Jahr (1999). The EC_{50} of glutamate in its interaction with LPS-stimulated IL-10 release from spinal cord microglia was about 100 μM, well below the level reported by Dzubay and Jahr (1999), although microglia do not wrap synapses as tightly as cerebellar glia and hence may not be exposed to as high a level of synaptic glutamate. The EC_{50} of glutamate in its interaction with SP and with LPS on astrocytes was 347 μM and 400 μM respectively, which are values higher than the concentration reaching cerebellar glia on synaptic stimulation. In chronic pain and conditions of inflammation, extracellular levels of glutamate are increased. Additionally, synaptic glutamate is not the only source of endogenous extracellular glutamate with observations of release from astrocytes and microglia also reported (Chen et al., 2005; Takeuchi et al., 2006). Whether pooled levels of extracellular glutamate in pain and inflammation increase to those that allow potentiation of the effects of SP and LPS *in vivo* will need further investigation. Additionally, the concentration of SP and TLR4 agonists to reach glial membranes *in vivo* is unknown, so whether ATP and IL-10 releasing effects occur at *in vivo* concentrations will need further investigation.

Other possible differences between the *in vivo* and *in vitro* situations may be differences in density and expression of receptor subtypes. The expression of the TLR4, glutamate receptor subtypes, and neurokinin receptor subtypes, have been confirmed *in vitro* in this thesis and in work by Liu et al. (2005), Palma et al. (1997), Silva et al. (1999), Wienrich and Kettenmann (1989), Yashpal et al. (1990), Zaratin et al. (2000) and Zerari et al. (1997; 1998). Correspondingly, expression of glutamate receptor subtypes and the NK2 receptor on spinal cord astrocytes has been confirmed *in situ* (Aicher et al., 1997; Brand-Schieber and Werner, 2003; Zerari et al., 1998). On the other hand, it has not been investigated whether NK1 and NK3 receptors and the TLR4 are expressed on *in vivo* spinal cord astrocytes, and if glutamate receptor subtypes and the TLR4 are expressed on *in vivo* spinal cord microglia. Although beyond the scope of the present research, the expression of these receptors could be investigated in further studies by use of *in situ* immunohistochemistry.

As the similarity between the *in vitro* conditions of this present work and *in vivo* conditions is not known, the next step based on this research would be to investigate whether the processes shown in the current research occur in more physiological conditions. An exploration of whether SP potentiates glutamate-stimulated ATP release could be conducted *in vivo* by genetically engineering an animal to produce luciferase on the surface of its astrocytes, then focally injecting the CNS area of interest with luciferin. The fluorescence of the luciferase acting on ATP in the presence of luciferin could then be measured on stimulation of neurons that co-release SP and glutamate. Similarly, to explore whether the activation of TLR4s and

glutamate receptors on *in vivo* astrocytes and microglia results in IL-10 release, the cerebral spinal fluid IL-10 content of animals subject to intrathecal LPS and glutamate administration may be analysed with ELISA, as may the fluid of animals in conditions where there is increased glutamate and TLR4 activation such as neuropathic pain.

6.2.2 Effects of stimulation by and release of other pain-related substances

In chronic pain, glutamate is co-released with many different neurotransmitters, such as ATP, neurokinin-A, neurokinin-B and calcitonin gene related peptide (Millan, 1999; Sulzer et al., 1998). It would be interesting to investigate whether these neurotransmitters also modulate glutamate-stimulated ATP release and LPS-stimulated IL-10 release, as the possibility exists that a number of different neurotransmitters can synergistically interact to contribute to the spinal pools of ATP and IL-10, providing further possible targets for chronic pain intervention. To investigate this, the experiments presented in this thesis could be repeated with these substances.

Chapters 4 and 5 of this thesis focused on the anti-inflammatory effect of glutamate on glia in pain. Whilst glutamate is well known as a CNS excitotoxin at high concentrations (Matute et al., 2006), has been reported to be able to stimulate IL-1β release from astrocytes (Aronica et al., 2005) and has pro-inflammatory actions on resting microglia (Noda et al., 2000; Taylor et al., 2005), the exposure of astrocytes

and activated microglia to glutamate commonly results in neuroprotective or anti-inflammatory outcomes (Bruno et al., 1998; Taylor et al., 2003; Venero et al., 2002; Zhou et al., 2006), as backed up by the results of Chapters 4 and 5 of this thesis. A further exploration of the anti-inflammatory capacities of glutamate could be conducted by exploring whether glutamate itself or in combination with LPS decreases pro-inflammatory cytokine secretion from astrocytes and activated microglia. Again, to investigate this, the experiments presented in Chapters 4 and 5 of this thesis could be repeated with measurement of pro-inflammatory cytokines.

6.2.3 Extension of findings to other cell types

It would be useful to see if the interaction between SP and glutamate occurred in other cell types, especially Schwann cells of the peripheral nervous system. Schwann cells have been shown to release ATP when stimulated with glutamate. This release may possibly affect the excitability of neurons (Liu and Bennett, 2003). When the periphery is subject to a noxious stimulus, glutamate and SP are released (Millan, 1999) and this release is in excess in chronic pain, allowing the possibility that if the potentiation of glutamate-stimulated ATP release by SP occurs in Schwann cells, excess glutamate and substance P released in chronic pain may lead to vast increases in ATP release from Schwann cells. As Schwann cells express $P2X_7$ receptors, exposure of these receptors to an excess of ATP may contribute to $P2X_7$-mediated hyperalgesia (Chessell et al., 2005).

Additionally, it would be useful to see if the interaction between glutamate and the TLR4 occurred in other cell types. Again, one key cell type to investigate may be Schwann cells. Recently, functional TLR4s have been demonstrated on cultured Schwann cells which elicit TNF-α release on activation (Cheng et al., 2007). TNF-α has multiple roles after peripheral nerve injury. For example, it assists in macrophage recruitment, a key process in removing myelin debris after injury (Liefner et al., 2000) but also decreases Schwann cell viability in cultures (Myers et al., 2003). Although TNF-α may induce useful processes after axonal injury, the observation that TNF-α knockout mice show a higher number of preserved axons after the first 6 days of injury suggests that overall, TNF-α, has deleterious consequences after nerve injury within the first week after injury. Using immunohistochemistry to index the number of positive cells, it has been demonstrated that after a crush injury of the sciatic nerve there is an initial increase in cells expressing TNF-α which is reversed between 3 and 7 days after injury, the exact time where there is an increase in the number of cells expressing IL-10 (Sawada et al., 2007). If it can be shown that glutamate increases LPS-stimulated IL-10 release from Schwann cells, this may have implications for the process of axonal degeneration, repair and regeneration after peripheral nerve injury.

6.3 CONCLUSION

This thesis has provided evidence that spinal cord glia release both pro-nociceptive and anti-nociceptive compounds. Evidence was found that spinal cord astrocytes may contribute to the pool of the pro-nociceptive spinal ATP. By acting on AMPA receptors, glutamate was shown to stimulate a two-fold increase in ATP release from spinal cord astrocytes which was greatly potentiated in the presence of SP. SP brought about this potentiation by acting on all subtypes of neurokinin receptor, resulting in the ability of NMDA and mGlu receptor activation to result in ATP release. Additionally, this thesis has revealed that spinal cord microglia and astrocytes release the anti-nociceptive IL-10 on TLR4 activation and this release is enhanced in the presence of glutamate through an upregulation of IL-10 mRNA. These results can be integrated into a three-phase model of spinal chronic pain mechanisms which can be used as a basis for future research.

CHAPTER 7

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CHAPTER 8

APPENDIX

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Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P

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Abstract

ATP has recently emerged as a key molecule mediating pathological pain. The aim of this study was to examine whether spinal cord astrocytes could be a source of ATP in response to the nociceptive neurotransmitters glutamate and substance P. Glutamate stimulated ATP release from these astrocytes and this release was greatly potentiated by substance P, even though substance P alone did not elicit ATP release. Substance P also potentiated glutamate-induced inward currents, but did not cause such currents alone. When glutamate was applied alone it acted exclusively through α-amino-3-hydroxy-5-methylisoxazole-4-proprionate receptors to stimulate Ca^{2+} influx-dependent ATP release. However, when substance P was co-applied with glutamate, ATP release could be elicited by activation of NMDA and metabotropic glutamate receptors. Activation of neurokinin receptor subtypes, protein kinase C and phospholipases A_2, C and D were needed for substance P to bring about its effects. These results suggest that astrocytes may be a major source of ATP in the spinal cord on activation of nerve fibres that release substance P and glutamate.

Keywords: astrocytes, ATP, glutamate, substance P.

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ATP is a key spinal cord neurotransmitter. The ventral horn and intermediolateral column both contain P2X2 and P2X7 receptors (Kanjhan *et al.* 1999; Deuchars *et al.* 2001; Deng and Fyffe 2004), and ATP application to phrenic motor neurons in the ventral horn leads to increased firing of these diaphragm-innervating neurons (Miles *et al.* 2002). In addition, it has recently been discovered that inflammatory and neuropathic pain are completely removed in mice lacking the ATP P2X7 receptor, which is found on microglial cells among others (Chessell *et al.* 2005; Xiang and Burnstock 2005). Furthermore, formalin-induced hyperalgesia behaviour is significantly reduced in mice lacking the ATP P2X3 receptor, and activation of P2X3 receptors on nociceptor nerve terminals in the spinal cord enhances transmission of chronic inflammatory and neuropathic pain (Kennedy *et al.* 2003). In addition, nerve injury-induced mechanical allodynia involves up-regulation of ATP P2X4 receptors on microglia as a required molecular mediator (Tsuda *et al.* 2003). It appears that the level of ATP in the vicinity of nociceptor synapses in the dorsal horn is likely to be an important regulator of inflammatory and neuropathic pain. The question arises as to the source of this ATP in these areas of the spinal cord.

Astrocytes in the spinal cord are a major source of ATP, which they use to transmit propagating waves of intracellular Ca^{2+} increases (Salter and Hicks 1994). Studies suggest that astrocytes can release much greater amounts of ATP than neurons (Queiroz *et al.* 1999). ATP released from astrocytes in response to neurotransmitters can modulate neuronal

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Abbreviations used: A23187, calcimycin A23187; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-proprionate; Aristol, aristolochic acid; β-Ala, [β-Ala^8]-neurokinin A fragment 4–10; BSA, bovine serum albumin; CCS, cosmic calf serum; CheCl, chelerythrinechloride; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DDA, 2′,5′dideoxyadenosine; DMEM, Dulbecco's modified Eagle's medium; GAMS, γ-D-glutamyl-amino methylsulfonic acid; GFAP, glial fibrillary acidic protein; GYKI, GYKI52466; H 33342, bisBenzimide H 33342 trihydrochloride; IP_3, inositol triphosphate; KA, kainate; L703606, *cis*-2-(diphenylmethyl)-*N*-[2-iodophenylmethyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate salt; MCPG, (+)-α-methyl-4-carboxyphenylglycine; mGlu, metabotropic glutamate; MK-801, (+)-MK-801 hydrogen maleate; NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine; NeuN, Neuron-specific Nuclear protein; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol-4,5-bis-phosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; Sar, sarcosine; SarMet, [Sar^9, Met$(O_2)^{11}$]-substance P; Senk, senktide; SP, substance P; tACPD, *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid.

transmission (Zhang *et al.* 2003) and microglial function (Verderio and Matteoli 2001). Astrocytes are in intimate contact with nerve terminals in the spinal cord (Zerari *et al.* 1998) as they are at all central synapses and are exposed to spinal neurotransmitters. Two neurotransmitters released in the dorsal horn, ventral horn and intermediolateral column of the spinal cord are glutamate and substance P (SP) (Helke *et al.* 1982; Kapoor *et al.* 1992; Sasaki *et al.* 1998; Allen *et al.* 1999; Hokfelt *et al.* 2000) and spinal cord astrocytes possess receptors to both transmitters (Wienrich and Kettenmann 1989; Beaujouan *et al.* 1990; Agrawal and Fehlings 1997; Zerari *et al.* 1998).

Given this background, it is natural to examine whether spinal cord astrocytes, excited by the concomitant application of glutamate and SP, release ATP in relatively large amounts which could excite the range of ionotropic purinergic receptors in the spinal cord that have been implicated in events such as respiration and inflammatory pain. We report here that glutamate can stimulate ATP release from spinal cord astrocytes and that this release is greatly enhanced when SP is present. Mechanisms involved in this phenomenon are reported. Preliminary accounts of this work have appeared (Werry *et al.* 2005).

Materials and methods

Spinal cord and cortical astrocyte cultures

Astrocytes were cultured according to Cole and deVellis (1997) with modifications. The University of Sydney Animal Ethics Committee approved the procedures, and procedures were in accordance with the Australian Government National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes. Briefly, whole spinal cords from 0–2-day-old neonatal rats were dissected out, and the meninges and peripheral nerves removed. The remaining spinal cord tissue was incubated in 0.125% porcine trypsin (Sigma-Aldrich, St Louis, MO, USA) dissolved in Hanks balanced salt solution (136.89 mM NaCl, 5.37 mM KCl, 4.17 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.55 mM D-glucose, pH 7.4) for 20 min at 37°C and then washed with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% New Zealand cosmic calf serum (CCS; Hyclone Logan, UT, USA) and 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA, USA) and triturated. Dissociated cells were plated on a culture flask coated with poly-D-lysine (20 μg/mL; Sigma) for 4 h in a 5% CO_2 incubator at 37°C. Non-attached cells were then removed and cultures were maintained at 5% CO_2 and 37°C. The culture medium was replaced with fresh supplemented DMEM every 3–4 days. Twice during *in vitro* days 14–20 cultures were shaken on an IKA-VIBRAX-VXR shaker (Janke & Kunkel, Staufen, Germany) at 7 *g* and 37°C for 4 h in a Certomat chamber (B. Braun, Bethlehem, PA, USA). Detached cells were predominantly microglia and oligodendrocytes (Cole and deVellis 1997), and were removed from the culture by replacement of the culture medium. After *in vitro* day 16 the numbers of remaining microglia and oligodendrocytes were further reduced by replacing DMEM supplemented with CCS and penicillin/streptomycin/glutamine with sorbitol medium [DMEM without glucose (Sigma), 10% horse serum (Trace Bioscientific, Castle Hill, NSW, Australia), 1% penicillin/streptomycin/glutamine and 25 mM sorbitol (Sigma)] (Wiesinger *et al.* 1991). Oligodendrocytes and microglia do not have aldose reductase and sorbitol dehydrogenase, and consequently cannot use sorbitol for energy. Thus if sorbitol is substituted for glucose in the culture medium, oligodendrocytes and microglia die off, leaving astrocytes which have the enzymes necessary to use sorbitol for energy (Wiesinger *et al.* 1991). After 21 days, the age at which there becomes only one predominant (> 90%) subtype of spinal cord astrocyte (Black *et al.* 1993), cells were transferred to 13-mm glass coverslips and were used for ATP release, electrophysiology and immunocytochemistry experiments 1–2 days after transfer.

Online bioluminescence

ATP release was measured by online bioluminescence as detailed elsewhere (Taylor *et al.* 1998; Liu *et al.* 2005). A luciferin–luciferase enzymatic ATP assay mix (Sigma) was used to measure ATP release; for every molecule of ATP that is evolved in solution, a photon of light is produced (Taylor *et al.* 1998). Consequently, the amount of ATP released by cells can be indirectly calculated by measuring in real time the number of photons produced by cells incubated in the luciferin–luciferase assay with a photomultiplier.

Coverslips were washed in HEPES-buffered salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) for 5 min. Excess solution was removed from cell-free sides of the coverslip and it was placed on an 18-mm round plastic photomultiplier stage. Eighty microlitres of ATP assay mix (1 mg/mL; Sigma) was immediately added to the coverslip. The photomultiplier stage was placed inside a light-impermeable chamber and photons were counted with a P30CWAD5-45 photodetector package (Electron Tubes, Ruislip, UK) and displayed using an RS232 photon counting program (Electron Tubes). Photons were collected every second and experiments were performed at room temperature (22–24°C).

The initial photon count was high as there was an imbalance between the basal amount of ATP produced and accumulated by cells and the amount of ATP consumed by the ATP assay mix and the ecto-ATPases. Some 60–80 min later the amount of ATP produced by cells and the amount of ATP consumed by the assay and ecto-ATPases reached equilibrium, illustrated by the photon count readings not varying by more than 10%. When this stable photon count extended for 10 min, the average photon count over this period of time was recorded as the baseline level. After the baseline had been reached, recording of photon counts was stopped and agonists of interest were applied at 10% of the volume of liquid on the coverslip. Recording was immediately recommenced (within 20 s) after addition of the chemicals. Chemicals were not washed out during experimentation. At the end of each experiment, 1% Triton X-100 (Bio-Rad, Hercules, CA, USA) was added to lyse cells, allowing an approximation of the number of cells on the coverslip. For each batch of ATP assay mix used for experiments, a standard curve was computed using known concentrations of ATP, allowing conversion of the photon count data to ATP concentration values.

ATP release data were analysed to determine the peak increase in ATP release relative to baseline (referred to as relative peak ATP

level). Experiments were repeated at least three times and results were expressed as mean ± SEM relative peak ATP level. Means were analysed for statistically significant differences with an unpaired Student's two-sample two-tailed *t*-test. $p < 0.05$ was considered statistically significant.

All inhibitors were applied 1 h before addition of agonists. All agonists and antagonists used in bioluminescence studies were purchased from Sigma-Aldrich, except thapsigargin epoxide from Alomone Laboratories (Jerusalem, Israel) and iodotubercidin from EMD Biosciences (San Diego, CA, USA). All chemicals were dissolved in HEPES-buffered salt solution except the following: thapsigargin, thapsigargin epoxide, aristolochic acid, 2′,5′dideoxyadenosine (DDA), A23187, 7-chlorokynurenic acid, *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR) and iodotubercidin were dissolved in dimethyl sulfoxide (maximum final concentration 0.17%); (+)-α-methyl-4-carboxyphenylglycine (MCPG) was dissolved in 0.1 M NaOH (maximum final concentration 0.7%); GYKI52466 was dissolved in 2.5 mM HCl and [β-Ala^8]-neurokinin A fragment 4–10 was dissolved in 20% acetonitrile and 0.01% trifluoroacetic acid. None of the solvents at the concentrations used affected the luciferase activity, induced ATP release from the astrocytes by themselves or affected glutamate-stimulated ATP release. All antagonists used in experiments involving co-application of SP and glutamate were examined for an effect on glutamate-stimulated ATP release, and no significant effects of the antagonists were found, apart from 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX).

Patch-clamp recording

Perforated patch-clamp was used to record whole-cell currents in astrocytes using a Multiclamp 700A amplifier (Axon Instruments, Sunnyvale, CA, USA) at room temperature (22–24°C). Astrocytes were identified according to the morphological features of type I astrocytes, which possess a large flat cell body in comparison to microglia and oligodendrocytes. The morphology of these astrocytes corresponded with the morphology of cells that stained positive for glial fibrillary acidic protein (GFAP) in immunocytochemistry experiments and differed from the smaller CDIIb-positive microglia. Resistances of pipettes were 4–8 MΩ when a patch pipette was filled with pipette solution containing 145 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES and 250 μg/mL nystatin (pH 7.3); the external bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Mg^{2+} was omitted from the bath solution when NMDA was used as an agonist. Whole-cell currents were recorded in voltage-clamp mode at a holding membrane potential of – 60 mV. The currents were sampled on-line using a Digidata 1322A interface and pClamp8 program (Axon Instruments). Glutamate, its analogs and SP were applied by pressure ejection via a picosprizer (General Valve, Fairfield, NJ, USA).

Immunocytochemistry

Cells grown on coverslips from the same culture batches used in bioluminescence experiments were washed twice for 5 min each with the HEPES-buffered salt solution used in bioluminescence studies. Cells on coverslips were then fixed with 2% formaldehyde solution (BDH chemicals, Kilsyth, VA, Australia) in phosphate-buffered saline (PBS; 136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) for 10 min. Coverslips were washed twice for 5 min each with PBS and then incubated in PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.05% saponin (Sigma-Aldrich) for 30 min. Cells on coverslips were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 1% BSA and 0.05% saponin. Primary antibodies were washed out three times for 10 min each with PBS and then cells were incubated in secondary antibodies diluted in PBS containing 1% BSA and 0.05% saponin for 1 h at room temperature (22–24°C).

The coverslips were washed twice for 10 min each with Ca^{2+}/Mg^{2+}-free HEPES-buffered salt solution and incubated in the DNA marker bisBenzimide H 33342 trihydrochloride (H 33342; 5 μg/mL; Sigma) for 15 min. Following this, coverslips were washed twice for 10 min each with Ca^{2+}/Mg^{2+}-free HEPES-buffered salt solution and then mounted on glass slides using 20% glycerol (Sigma-Aldrich) in PBS and sealed with nail polish.

Fluorescence was visualized with an Axioplan2 upright microscope (Zeiss, Jena, Germany) and images were acquired with an AxioCam HR digital monochrome charge-coupled device camera (Zeiss) using Axiovision 4.0 software (Zeiss). Experiments with each antibody were performed on at least three different batches of cultures and were repeated at least twice on each of the batches. For each coverslip at least three fields of view were imaged. No autofluorescence of primary antibodies or non-specific binding of secondary antibodies was observed.

To verify the results obtained using the above method, experiments probing the NMDA receptor 1 and glutamate receptor 4 subunits were repeated with one of the following changes per experiment: omission of incubation of cells in H 33342; substitution of the step in which cells were incubated in saponin and BSA with three steps in which cells were incubated in 1% Triton X-100 which was washed out three times with PBS followed by incubation for 1 h in 1% BSA; use of a different rabbit anti-rat NMDA receptor 1 antibody (1 : 50–1 : 200; Chemicon, Temecula, CA, USA); and incubation of cells in rabbit anti-rat glutamate receptor antibodies before fixation of cells with formaldehyde solution. Results using these different methods were consistent.

Materials

Agonists used in bioluminescence experiments were: neurotransmitters glutamate (30 μM – 10 mM) and SP (3–300 μM); tachykinin receptor agonists [Sar^9, Met(O$_2$)^{11}]-SP (100 μM), [β-Ala^8]-neurokinin A fragment 4–10 (64 μM) and succinyl-[Asp^6, *N*-Me-Phe^8]-SP fragment 6–11 (senktide; 100 μM); glutamate receptor subtype agonists kainate (5 mM), α-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA; 50 μM), NMDA (500 μM to 5 mM), *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid (tACPD; 1 mM) and Ca^{2+} ionophore calcinycin A23187 (10 μM). Inhibitors used in the bioluminescence experiments were: NMDA receptor antagonist (+)-MK-801 hydrogen maleate (MK-801; 40 μM); kainate receptor antagonist γ-D-glutamylamino methylsulfonic acid (GAMS; 30 μM); metabotropic glutamate (mGlu) receptor antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG; 500 μM); AMPA receptor antagonist GYKI52466 hydrochloride (50 μM); kainate and AMPA receptor antagonist CNQX (20 μM); phosphatidylinositol-4,5-bisphosphate (PIP_2) synthesis inhibitor LiCl (1 mM); endoplasmic reticulum Ca^{2+}-dependent ATPase inhibitor thapsigargin (1 μM); the inactive form of thapsigargin, called thapsigargin epoxide (1 μM); protein kinase C (PKC) inhibitor chelerythrine chloride (20 μM); voltage-dependent

Ca^{2+} channel inhibitor $CdCl_2$ (1 mM); Ca^{2+} chelator EGTA (5 mM); phospholipase D (PLD) inhibitor 1-butanol (0.5%); cell-permeable adenylyl cyclase inhibitor DDA (50 μM); neurokinin-1 and neurokinin-2 receptor antagonist spantide I (3 μM); neurokinin-1 antagonist *cis*-2-(diphenylmethyl)-*N*-[2-iodophenylmethyl]-1-azabicyclo[2.2.2] octan-3-amine oxalate salt (L703606; 10 μM); phospholipase A_2 (PLA_2) antagonist aristolochic acid (50 μM); NMDA receptor glycine co-binding-site antagonist 7-chlorokynurenic acid (5 μM); nucleoside transporter 1 inhibitor NBMPR (10 μM); adenosine kinase inhibitor iodotubercidin (10 μM); and ecto-ATPase inhibitor ARL 67156 trisodium salt (100 μM).

Primary monoclonal antibodies used were the astrocyte-specific marker mouse anti-rat GFAP (1 : 800; Sigma), microglia- and macrophage-specific mouse anti-rat CD11b (1 : 100; Chemicon) and neuron-specific antibody anti-Neuron-specific Nuclear protein (NeuN) (1 : 150; Chemicon). Primary polyclonal antibodies used were the oligodendrocyte marker rabbit anti-rat galactocerebroside (1 : 50–1 : 200; Chemicon), rabbit anti-rat glutamate receptor 2/3 (1 : 50–1 : 200; Sigma-Aldrich), rabbit anti-rat glutamate receptor 4 (1 : 50–1 : 200; Tocris, Bristol, UK), rabbit anti-rat mGlu receptor 2/3 (1 : 50–1 : 200; Sigma-Aldrich), rabbit anti-rat mGlu 1α/5 receptor (1 : 250; Chemicon) and rabbit anti-rat NMDA receptor 1 (1 : 50–1 : 200; Sigma-Aldrich). Secondary antibodies were goat anti-mouse IgG conjugated to Alexa Fluor 488 (1 : 100), used to detect primary monoclonal antibodies, and goat anti-rabbit IgG conjugated to Alexa Fluor 594 (1 : 100), used to detect primary polyclonal antibodies. Both secondary antibodies were from Molecular Probes (Eugene, OR, USA).

Results

SP enhances glutamate-stimulated ATP release from spinal cord astrocytes

We initially determined the ability of SP and glutamate to stimulate ATP release from spinal cord astrocytes when applied separately and together using online bioluminescence. Glutamate (1 mM) elicited approximately a 2-fold increase in ATP release levels at the peak of its release compared with the baseline level (2.19 ± 0.11; $n = 67$) (Figs 1a and b). This measure is referred to as the relative peak ATP level. Peak ATP release was reached 5–7 min after the application of glutamate and ATP release levels returned to baseline within 30 min after application (Fig. 1b). When applied alone at concentrations of 100 μM or less, SP did not change ATP release levels from baseline level (1 μM, $n = 3$; 10 μM, $n = 12$; 30 μM, $n = 3$; 100 μM, $n = 15$) (Figs 1a and b). However, when 100 μM SP and 1 mM glutamate were co-applied, ATP release levels significantly increased to approximately 7-fold above the baseline level (7.35 ± 0.68; $n = 38$; $p < 0.001$) (Figs 1a and b). This equated to a 336% potentiation of glutamate-stimulated ATP release by SP compared with the effect of glutamate alone. SP did not alter the time course of glutamate-stimulated ATP release (Fig. 1b).

To verify the identity of the cells acted upon by SP and glutamate to bring about the modulation of ATP release, immunocytochemistry was carried out. Over 90% of cells were positive for the astrocyte-specific marker GFAP, whereas less than 10% were positive for the microglial and macrophage marker CD11b. Anti-galactocerebroside non-specifically labelled all cells. Consequently the presence of oligodendrocytes was identified on the basis of morphological characteristics and oligodendrocytes were found to comprise < 1% of total cells. There were no neurons present in cultures. To examine a possible contribution of microglia and oligodendrocytes to the modulation of glutamate-stimulated ATP release by SP, the effect of SP on glutamate-stimulated ATP release from coverslips with total microglia and oligodendrocyte populations of ≤4% were compared with that from coverslips with the same number of astrocytes but with microglia and oligodendrocytes added until the total microglial and oligodendrocyte population reached 30%. In cells with ≤4% microglia and oligodendrocytes, SP (100 μM) increased glutamate (1 mM)-stimulated ATP release by 412%, with an ATP release of 1.64 ± 0.16 ($n = 3$) times above basal ATP release values being elicited by glutamate only and 6.78 ± 1.32 ($n = 4$) times by co-application of SP and glutamate. Similarly, on cells with 30% microglia and oligodendrocytes, SP (100 μM) increased glutamate (1 mM)-stimulated ATP release by 379%, with an ATP release of 1.61 ± 0.15 ($n = 3$) times above basal ATP release values being elicited by glutamate only and 6.10 ± 2.98 ($n = 3$) times by co-application of SP and glutamate. These results suggest that microglia and oligodendrocytes do not contribute to the interaction of SP with glutamate-stimulated ATP release, and that the potentiation results from SP and glutamate acting on astrocytes.

It was possible that ATP measured on application of agonists could be a consequence of adenosine release through the equilibrative nucleoside transporter 1 and subsequent anabolism by adenosine kinase to ATP. Neither the equilibrative nucleoside transporter 1 antagonist NBMPR (10 μM; 7.06 ± 1.78; $n = 6$) nor the adenosine kinase antagonist iodotubercidin (10 μM; 7.92 ± 1.01; $n = 3$) significantly changed SP and glutamate-stimulated ATP release. Similarly the ecto-ATPase inhibitor ARL 67156 did not have a significant effect on SP and glutamate-stimulated ATP release (100 μM; 6.27 ± 1.10; $n = 4$).

To examine whether SP modulated glutamate-induced currents in spinal cord astrocytes, glutamate-induced whole-cell currents were recorded and the effects of SP were determined. Glutamate (1 mM) caused slow inward currents in 17 of 19 cells from which recordings were made, which took approximately 10 s to peak. The average peak amplitude of the inward currents was 49 ± 9 pA. SP (100 μM) did not cause inward currents in any cells examined ($n = 4$). However, on co-application of glutamate and SP, inward currents were significantly larger than those recorded from astrocytes exposed to glutamate alone (150 ± 29 pA; $n = 3$; $p < 0.05$) (Fig. 1c).

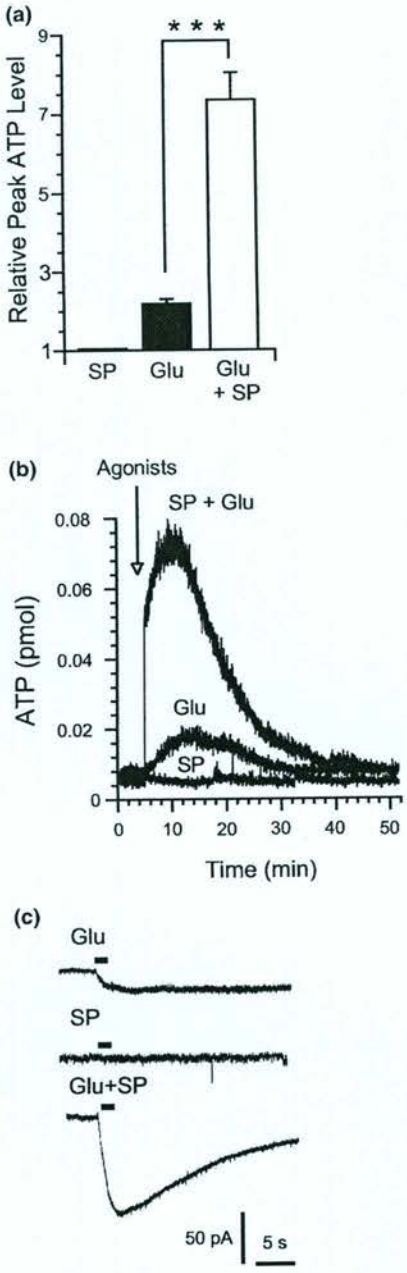

Fig. 1 SP significantly potentiates glutamate-stimulated ATP release from cultured spinal cord astrocytes. (a) Summary histogram of the effect of application of 100 μM SP, 1 mM glutamate (Glu) and co-application of 1 mM Glu and 100 μM SP (Glu + SP) on ATP release relative to the basal release level from cultured spinal cord astrocytes. In this figure and histograms in following figures, relative peak ATP level is defined as the peak ATP release divided by the baseline ATP value. $p < 0.001$ for ATP release stimulated by glutamate plus SP versus that stimulated by glutamate. Values are mean ± SEM. ***$p < 0.001$ (Student's two-sample two-tailed *t*-test) (b) Representative time courses of ATP release induced by these agonists from spinal cord astrocytes. The arrow represents time of addition of agonists to the bath. (c) Representative trace of a glutamate (1 mM)-induced inward current from a spinal cord astrocyte that was dramatically enhanced by 100 μM SP, wheras SP itself did not induce currents. The black bars above the current traces represent the duration of agonist application.

The potentiation of glutamate-stimulated ATP release by SP is concentration and time dependent

To examine whether the interaction between SP and glutamate is dependent on the concentration of glutamate, ATP release was measured on co-application of increasing concentrations of glutamate with a constant 100 μM SP, and compared with the level of ATP release stimulated by the corresponding concentration of glutamate alone. In the absence of SP, 30 μM glutamate elicited ATP release (1.51 ± 0.25; $n = 4$) and increasing concentrations increased ATP release in a dose-dependent manner (100 μM, 1.66 ± 0.24, $n = 4$; 300 μM, 1.78 ± 0.29, $n = 3$; 1 mM, 2.19 ± 0.11, $n = 67$). Concentrations above 1 mM (3 mM, 1.99 ± 0.51, $n = 4$; 10 mM, 2.15 ± 0.25, $n = 3$) did not further increase ATP release. The EC_{50} was 234 μM and E_{max} was 2.11 when glutamate was applied alone (Fig. 2a). When 100 μM SP was co-administered with glutamate, potentiation of the level of ATP release was seen even at the lowest concentrations of glutamate examined (30 μM, 2.11 ± 0.43, $n = 3$; 100 μM, 2.39 ± 0.66, $n = 4$). Although ATP release stimulated by co-application of SP with 30 μM and 100 μM glutamate was not significantly greater than that induced by corresponding concentrations of glutamate in the absence of SP, their relative values were comparable with the E_{max} of glutamate alone. Statistically significantly greater levels of ATP release were seen at concentrations of 300 μM glutamate (3.41 ± 0.78, $n = 6$, $p < 0.05$) and above (1 mM, 7.35 ± 0.68, $n = 38$, $p < 0.001$; 3 mM, 7.74 ± 1.40, $n = 4$, $p < 0.01$; 10 mM, 7.96 ± 1.51, $n = 3$, $p < 0.05$). The EC_{50} was 347 μM, which was comparable to that obtained with application of glutamate in the absence of SP. However, the E_{max} was 7.91, which was appreciably higher than that obtained with glutamate alone (Fig. 2a).

To examine whether the interaction between SP and glutamate was dependent on the concentration of SP, ATP release was measured on co-application of various concentrations of SP with a constant 1 mM glutamate, and compared with that elicited by the ATP release stimulated by 1 mM glutamate alone. No significant increases above the glutamate-only level were seen with co-application of 1 μM (1.65 ± 0.16, $n = 3$) or 10 μM (1.63 ± 0.13, $n = 3$) SP. Significant increases were seen with higher concentrations of SP (30 μM, 3.47 ± 0.53, $n = 4$, $p < 0.05$; 100 μM, 7.35 ± 0.68, $n = 38$, $p < 0.001$; 300 μM, 7.52 ± 0.92, $n = 7$, $p < 0.001$). The EC_{50} of SP was 34 μM and the E_{max} was 7.60 (Fig. 2b). Consequently, SP was used at a concentration of 100 μM and glutamate at 1 mM for the remaining studies.

The time dependence of the potentiation of glutamate-stimulated ATP release by SP was investigated by applying glutamate at different intervals after SP application. SP

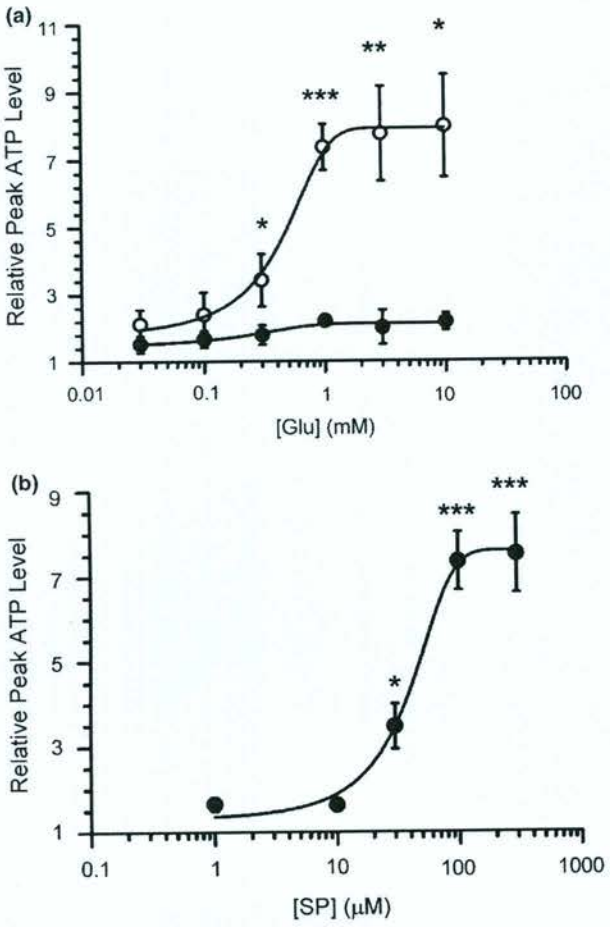

Fig. 2 Potentiation of glutamate-stimulated ATP release by SP is concentration dependent. (a) Glutamate dose dependently stimulated ATP release (black circles) and the degree of modulation by SP (100 μM) increased with increasing concentrations of glutamate (white circles). At each concentration of glutamate examined, values obtained with SP were compared with those without SP. (b) SP dose dependently increased glutamate-stimulated ATP release when co-applied with 1 mM glutamate. At each concentration of SP used, values were compared with the ATP release level obtained with application of 1 mM glutamate alone. Values are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's two-sample two-tailed *t*-test).

potentiated glutamate-stimulated ATP release when the two agonists were co-applied (0 min, 7.35 ± 0.68, $n = 38$). However when glutamate was added 30 s (2.10 ± 0.21, $n = 3$), 2 min (2.23 ± 0.29, $n = 3$), 5 min (2.13 ± 0.39, $n = 3$), 10 min (2.27 ± 0.35, $n = 3$) and 15 min (1.76 ± 0.29, $n = 3$) after SP, no potentiation was observed. Therefore SP and glutamate were co-applied in further experiments. An examination of the degree of potentiation when SP was applied at different intervals after glutamate application could not be performed. This was because levels of ATP release were increasing after application of glutamate, and this rendered comparison of potentiation on addition of SP beyond the already increasing levels impossible between experiments. It was not examined whether the time dependency of the potentiation was inherent in the mechanisms leading to the modulation or whether it was attributable to the breakdown of SP by endopeptidases known to be expressed on spinal cord astrocytes (Lentzen and Palenker 1983; Marcel *et al.* 1990).

Neurokinin receptors mediate the potentiation of glutamate-stimulated ATP release by SP

SP is known to bind three subtypes of neurokinin receptors. These are the neurokinin-1, -2 and -3 receptors (Regoli *et al.* 1994). The selective neurokinin-1 agonist [Sar^9, $Met(O_2)^{11}$]-SP (100 μM; 2.06 ± 0.45; $n = 4$), the selective neurokinin-2 agonist [β-Ala^8]-neurokinin A fragment 4–10 (64 μM; 1.74 ± 0.51; $n = 3$) and the selective neurokinin-3 agonist senktide (100 μM; 1.83 ± 0.21; $n = 3$) did not significantly potentiate glutamate-stimulated ATP release (Fig. 3a). However when [Sar^9, $Met(O_2)^{11}$]-SP and [β-Ala^8]-neurokinin A fragment 4–10 were co-applied with glutamate, a significant potentiation of glutamate-stimulated ATP release was observed (3.51 ± 0.38; $n = 3$; $p < 0.05$) (Fig. 3a). Furthermore, when all three neurokinin agonists were co-applied with glutamate, greater potentiation of glutamate-stimulated ATP release was recorded (4.85 ± 0.94; $n = 6$) (Fig. 3a). The neurokinin-1 and -2 preferring antagonist spantide I (3 μM) significantly reduced SP and glutamate-stimulated relative ATP release to levels seen when glutamate was applied alone (2.03 ± 0.31; $n = 4$; $p < 0.001$) (Fig. 3b). Similarly, the neurokinin-1 preferring antagonist L703606 (10 μM) abolished the potentiating effects of SP (1.90 ± 0.25; $n = 4$; $p < 0.001$) (Figs 3b and c).

Glutamate activates AMPA receptors to bring about ATP release

The contribution of NMDA, AMPA, kainate and mGlu receptors to glutamate-stimulated ATP release in the absence of SP was examined. Application of 50 μM AMPA stimulated ATP release (2.41 ± 0.34; $n = 9$) whereas 5 mM kainate ($n = 4$), 1 mM tACPD ($n = 4$), a mGlu receptor agonist, and 5 mM NMDA ($n = 3$) did not lead to ATP release (Figs 4a and b). In agreement with these results, glutamate-stimulated ATP release was significantly reduced by 50 μM GYKI52466 hydrochloride, an AMPA receptor antagonist (1.16 ± 0.16; $n = 5$; $p < 0.001$) and 20 μM CNQX, an AMPA and kainate antagonist (1.2 ± 0.07; $n = 4$; $p < 0.001$ (Figs 4c and d). However, it was not significantly effected by 30 μM GAMS, a kainate receptor antagonist (1.78 ± 0.22; $n = 4$), 500 μM MCPG, a mGlu 1, 2, 3 and 5 receptor antagonist (1.85 ± 0.21; $n = 4$), and 40 μM MK-801, a NMDA receptor antagonist (2.39 ± 0.44; $n = 5$) (Fig. 4c). AMPA (1 mM) induced slow inward currents which were similar to those induced by glutamate in all

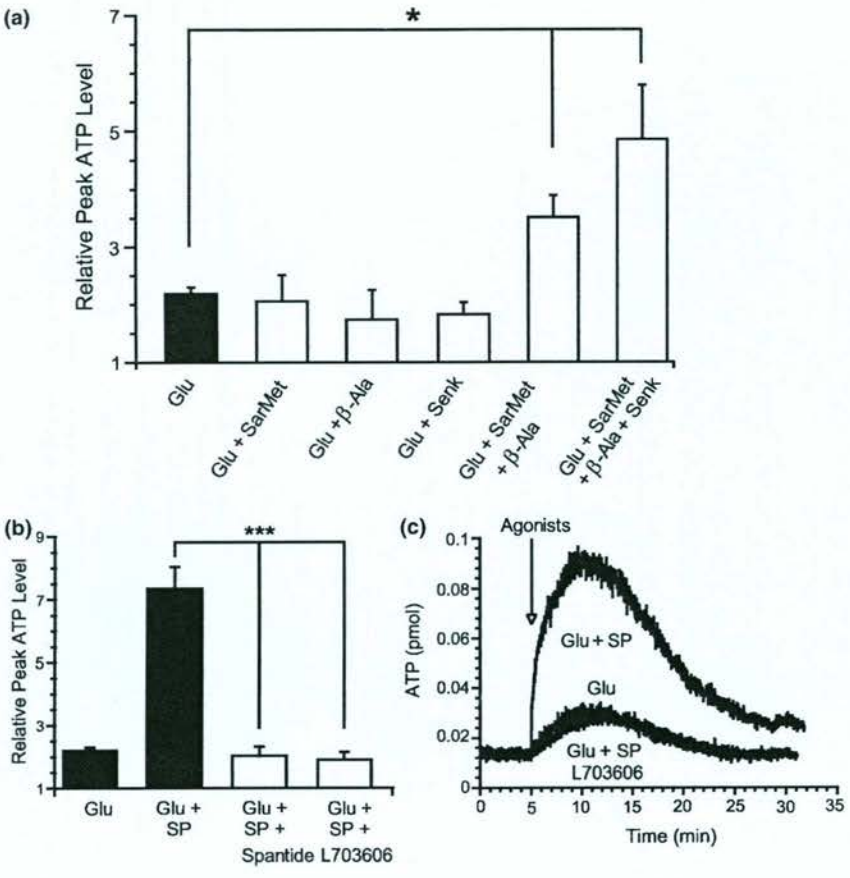

Fig. 3 SP acts on neurokinin receptor subtypes to potentiate glutamate-stimulated ATP release. (a) Summary histogram of the effect of neurokinin receptor agonists [Sar^9, $Met(O_2)^{11}$]-SP (SarMet; neurokinin-1 receptor agonist; 100 μM), [β-Ala^8]-neurokinin A fragment 4–10 (β-Ala; neurokinin-2 receptor agonist; 64 μM) and senktide (Senk; neurokinin-3 receptor agonist; 100 μM) on glutamate-stimulated ATP release. (b) Summary histogram of the effect of spantide I (neurokinin-1 and -2 preferring antagonist; 3 μM) and L703606 (neurokinin-1 preferring antagonist; 10 μM) on ATP release stimulated by co-application of SP and glutamate. (c) Representative time courses of ATP release stimulated by addition of glutamate, glutamate and SP, and glutamate and SP in the presence of L703606. The time courses resulting from glutamate and glutamate plus SP in the presence of L703606 overlap. Values are mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$ (unpaired Student's two-sample two-tailed *t*-test).

spinal cord astrocytes tested (50–75 pA; $n = 3$) (Fig. 4e). NMDA (1 mM; $n = 4$) and tACPD (1 mM; $n = 5$) did not produce inward currents in spinal cord astrocytes (Fig. 4e).

To examine whether glutamate receptor subtypes were available on the membrane of spinal cord astrocytes to be bound by glutamate, immunocytochemistry was conducted. The AMPA glutamate receptor 2/3 subunit was found in fine clusters throughout the cytoplasm and membrane of all GFAP-positive spinal cord astrocytes imaged ($n = 35$ astrocytes; Fig. 5a). In contrast, the AMPA glutamate receptor 4 subunit was localized specifically to an area overlapping and immediately surrounding the H 33342-stained nuclei of spinal cord astrocytes ($n = 36$ astrocytes) (Fig. 5b). The mGlu 1α5 receptor stained positively on 8.8% of imaged GFAP-positive astrocytes ($n = 29$ of 329 astrocytes) and was distributed in thick clusters throughout the membrane and cytoplasm of spinal cord astrocytes (Fig. 5c). The NMDA receptor glycine co-agonist subunit NR1 was found localized to an area overlapping the H 33342-stained nuclei of spinal cord astrocytes in 64% of cells imaged ($n = 74$ of 115 astrocytes) (Fig. 5d), whereas the distribution of NR1 also extended throughout the cytoplasm in the remaining cells imaged ($n = 41$ of 115 astrocytes) (Fig. 5d). The co-localization of glutamate receptor 4 and NR1 with astrocytic nuclei persisted despite the use of a variety of immunocytochemical methods and antibodies.

SP selectively enables glutamate to stimulate ATP release by binding NMDA and mGlu receptors

SP is known to enhance the activity of some neuronal glutamate receptor subtypes, such as the NMDA receptor (Randic *et al.* 1990). It was therefore determined whether SP changes the ability of glutamate receptor agonists to stimulate ATP release. Co-application of SP and 500 μM NMDA led to ATP release (2.29 ± 0.26; $n = 9$) (Figs 6a and b), as did co-application of SP and 1 mM tACPD (2.15 ± 0.23; $n = 7$) (Fig. 6a). Application of 500 μM NMDA, 1 mM tACPD and 100 μM SP led to further increases in ATP release (3.34 ± 1.02; $n = 6$) (Figs 6a and c). No ATP release was seen on co-application of SP and kainate (5 mM; $n = 3$) (Fig. 6a). Co-application of SP and AMPA (50 μM; 2.46 ± 0.49; $n = 5$) led to ATP release, which was not significantly different from that oberseved with application of AMPA alone (Fig. 6a).

The potentiation by SP of glutamate-stimulated ATP release was significantly reduced by 500 μM MCPG (3.48 ± 0.50; $n = 5$; p = 0.001) (Fig. 6d) and 40 μM MK-801 (3.57 ± 0.64; $n = 6$; $p < 0.001$) (Fig. 6d). Furthermore,

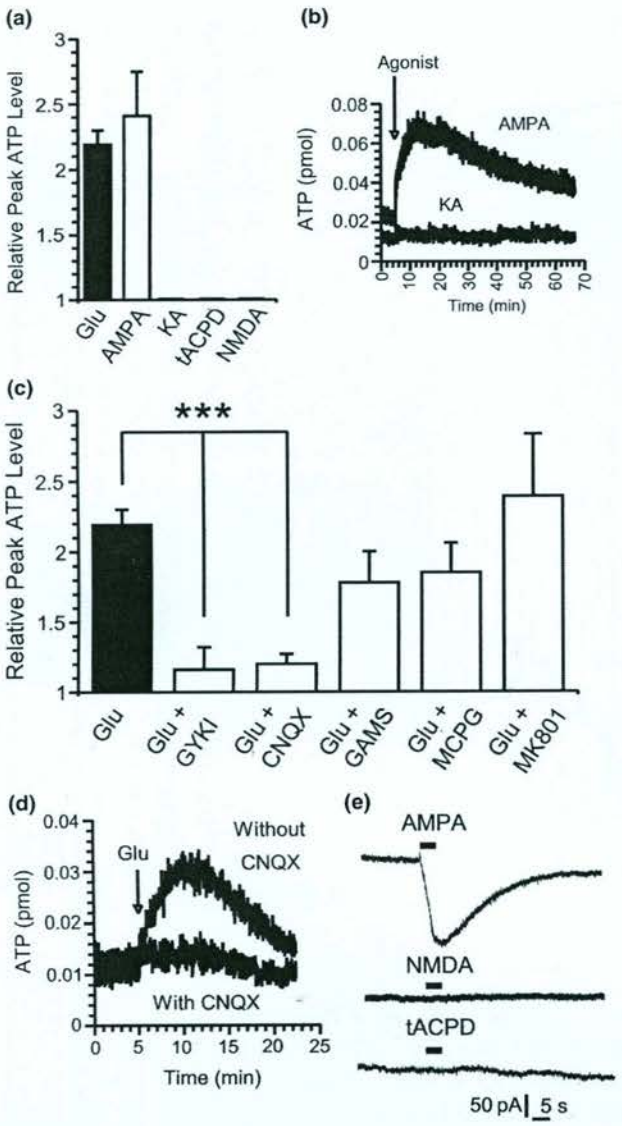

Fig. 4 Glutamate stimulates ATP release by activating AMPA receptors. (a) Summary histogram of the effects of glutamate receptor agonists glutamate (1 mM), AMPA (50 μM), kainate (KA; 5 mM), tACPD (mGlu receptor agonist; 1 mM) and NMDA (5 mM) on ATP release from spinal cord astrocytes. (b) Time course of the effect of AMPA and kainate on ATP release. (c) Summary histogram of the effects of glutamate receptor antagonists GYKI52466 (GYKI; AMPA receptor antagonist; 50 μM), CNQX (AMPA and kainate receptor antagonist; 20 μM), GAMS (kainate receptor antagonist; 30 μM), MCPG (mGlu receptor antagonist; 500 μM) and MK-801 (NMDA receptor antagonist; 40 μM). (d) Time course of the effect of CNQX on glutamate-stimulated ATP release. (e) Representative traces of patch-clamp recordings from spinal cord astrocytes on application of AMPA (1 mM), NMDA (1 mM) and tACPD (1 mM). The black bars above the current traces indicate the duration of addition of the agonists. Values are mean ± SEM. ***$p < 0.001$ (Student's unpaired two-sample two-tailed *t*-test).

SP and glutamate co-stimulated relative ATP release levels were reduced to glutamate-only levels when cells were preincubated with both 500 μM MCPG and 40 μM MK-801 (2.13 ± 0.41; $n = 4$; $p < 0.001$) (Fig. 6d).

The possibility that SP may have assisted in the modulation of NMDA receptors by increasing extracellular levels of glycine or by potentiating the function of the glycine co-binding site of the NMDA receptor was examined. The potentiation of NMDA (500 μM)-induced ATP release by SP was not significantly changed by preincubation with the glycine co-binding site antagonist 7-chlorokynurenic acid (5 μM; 1.76 ± 0.27; $n = 6$). Another possible mechanism by which SP may have modulated the NMDA receptor was by depolarizing the cell and removing the Mg^{2+} block. However, because of the Mg^{2+} dependence of the bioluminescence enzymes, it was not possible to conduct experiments in which the concentration of Mg^{2+} was varied.

Multiple intracellular signalling pathways are involved in the potentiation of glutamate-stimulated ATP release by SP

Neurokinin receptors are G protein-coupled receptors linked to four cell signalling pathways: PLC, PLA_2, PLD and adenylyl cyclase (Nakajima *et al.* 1992; Rayner and Van Helden 1997; Torrens *et al.* 1998). Activation of PLC leads to cleavage of PIP_2 to produce inositol triphosphate (IP_3) and diacyl glycerol. IP_3 can liberate intracellular Ca^{2+} stores. Increased intracellular Ca^{2+} and diacyl glycerol as well as PLA_2 activation can activate PKC. Potential roles of each of these cell signalling components were explored.

The PKC inhibitor chelerythrine chloride (20 μM) abolished the effects of SP on glutamate-stimulated ATP release (2.09 ± 0.53; $n = 6$; $p < 0.001$) (Fig. 7a), as did the PLA_2 inhibitor aristolochic acid (50 μM; 2.40 ± 0.16; $n = 4$; $p < 0.001$) (Figs 7a and b). The potentiation of glutamate-stimulated ATP release by SP was also significantly reduced by 0.5% 1-butanol, a PLD antagonist (3.44 ± 0.87; $n = 5$; $p < 0.01$), and 1 mM LiCl, an inositol monophosphate as inhibitor (3.93 ± 0.73; $n = 8$; $p < 0.01$). However, these antagonists did not fully abolish the effects of SP (Fig. 7a). Thapsigargin (1 μM), an endoplasmic reticulum Ca^{2+}-pump activator inhibitor, also decreased the potentiation (5.43 ± 0.56; $n = 6$; $p < 0.05$). However, thapsigargin reduced the potentiation to the same levels of ATP release as the inactive form of thapsigargin, thapsigargin epoxide (1 μM; 5.55 ± 1.16; $n = 4$). The cell-permeable adenylyl cyclase antagonist DDA (50 μM; 8.63 ± 1.66; $n = 5$) did not significantly affect SP and glutamate-stimulated ATP release (Fig. 7a). Furthermore, the potentiation of NMDA-stimulated ATP release by SP was significantly decreased by 20 μM chelerythrine chloride (1.12 ± 0.33; $n = 4$; $p < 0.05$) (Fig. 7c), but was not significantly affected by 0.5% 1-butanol (2.54 ± 0.68; $n = 4$) (Fig. 7c).

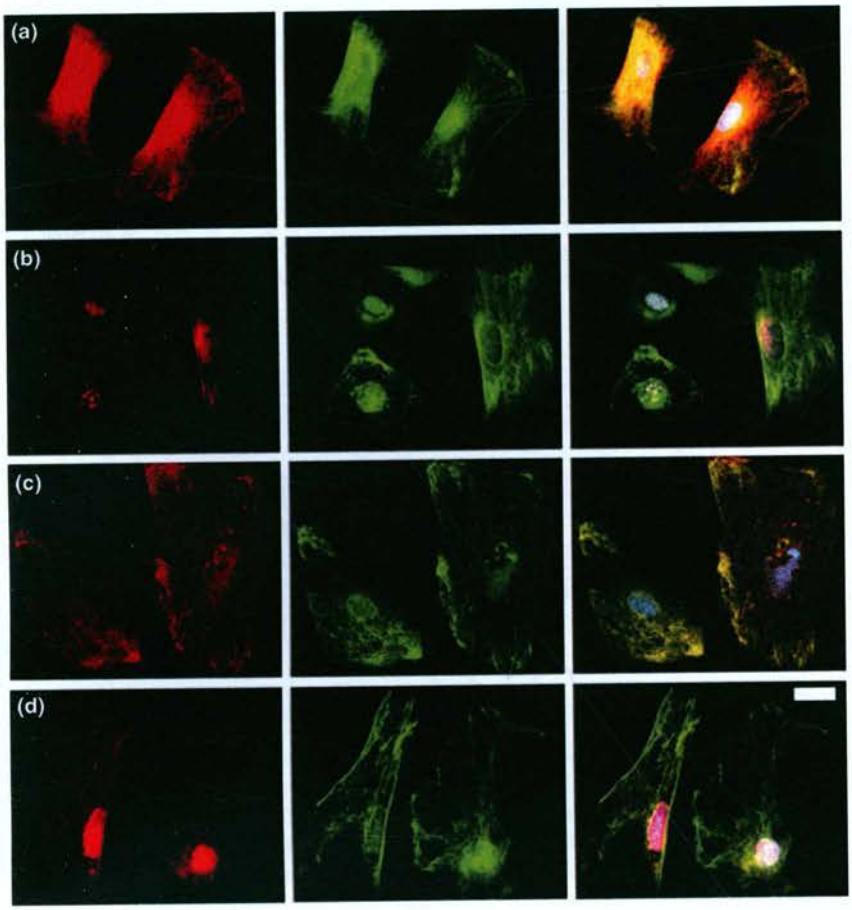

Fig. 5 Representative images of AMPA receptor subunit glutamate receptor 2/3 staining (a), AMPA receptor subunit glutamate receptor 4 staining (b), mGlu 1α/5 receptor staining (c) and NMDA receptor subunit NR1 staining (d) of spinal cord astrocytes. Images on the left show glutamate receptor subtype staining in red, those in the centre show astrocyte intermediate filament marker GFAP staining in green, and those on the right images are merged images with additional H 33342-staining of the nucleus in blue. Co-localization of glutamate receptor and GFAP staining appears yellow, whereas co-localization of glutamate receptor and nuclear staining appears violet. Scale bar is 20 μm and applies to all images in the figure.

As NMDA and some AMPA receptors are Ca^{2+} permeable, the role of extracellular Ca^{2+} influx in ATP release was examined. Astrocytes incubated in a Ca^{2+}-free bath solution containing the Ca^{2+} chelator EGTA (5 mM) had significantly reduced glutamate-stimulated ATP release levels (1.30 ± 0.07; $n = 5$; $p < 0.001$) compared with glutamate-stimulated ATP release from astrocytes in medium containing 500 μM Ca^{2+}, which was used throughout the studies described previously (Fig. 7d). A possible non-receptor source of Ca^{2+} influx on application of glutamate may have been voltage-dependent Ca^{2+} channels. However, a voltage-dependent Ca^{2+} channel blocker, $CdCl_2$, did not significantly affect glutamate-stimulated ATP release (1 mM; 2.40 ± 0.54; $n = 3$).

The potentiation of NMDA-stimulated ATP release by SP was significantly decreased in Ca^{2+}-free medium containing 5 mM EGTA compared with the relative peak ATP release level seen when NMDA and SP were added to cells in medium containing 500 μM Ca^{2+} (1.10 ± 0.14; $n = 4$; $p < 0.001$) (Fig. 7d). Influx of extracellular Ca^{2+} was shown to be sufficient to induce ATP release as the Ca^{2+} ionophore A23187 triggered ATP release (10 μM; 2.38 ± 0.64; $n = 3$) (Fig. 7d).

Discussion

Receptors involved in glutamate-stimulated ATP release and its potentiation by SP

The glutamate receptors involved in generating glutamate-evoked inward currents and ATP release are primarily of the AMPA class as the effects of glutamate on ATP release were blocked by an AMPA receptor antagonist, but were not significantly blocked by antagonists to kainate, NMDA or mGlu receptors. Furthermore, only AMPA evoked inward currents and stimulated ATP release. In agreement with findings in spinal cord astrocytes *in situ* (Brand-Schieber *et al.* 2004), immunocytochemistry showed the existence of AMPA glutamate receptor 2/3 subunits on the surface of these cultured spinal cord astrocytes. The glutamate receptor 4 subunit of the AMPA receptor was not found on spinal cord astrocyte membranes. Glutamate receptor 4 subunits have been selectively identified on the endfeet of astrocytes surrounding blood vessels (Brand-Schieber *et al.* 2004), which were of course absent from our cultures.

Surprisingly ATP release was not elicited by kainate, which is known to activate AMPA receptors. Kainate has been reported to cause small membrane potential changes in

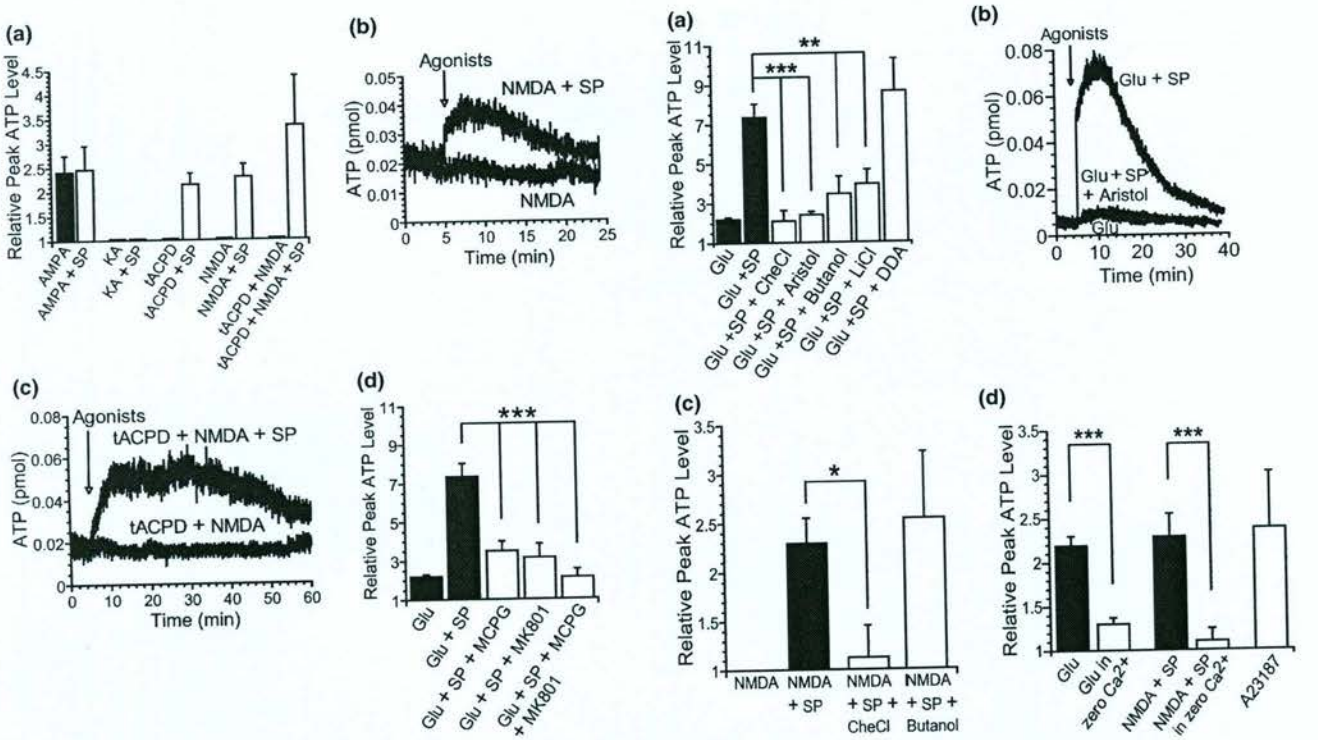

Fig. 6 SP allows glutamate to stimulate ATP release through NMDA and mGlu receptors. (a) Summary histogram of the effect of SP (100 μM) on the amount of ATP release stimulated by glutamate receptor subtype agonists AMPA (50 μM), kainate (5 mM), tACPD (1 mM) and NMDA (500 μM), and co-application of NMDA and tACPD. (b, c) Time course of the effect of SP on (b) NMDA-stimulated and (c) NMDA plus tACPD-stimulated ATP release. (d) Summary histogram demonstrating the effect of application of MCPG (500 μM), MK-801 (40 μM) and co-application of MCPG and MK-801 on SP + glutamate-stimulated ATP release. Values are mean ± SEM. $^{***}p < 0.001$ (Student's unpaired two-sample two-tailed *t*-test).

Fig. 7 Modulation of glutamate-stimulated ATP release by SP requires Ca^{2+} influx and activation of multiple intracellular signalling pathways. (a) Summary histogram of the effect of intracellular signalling molecule antagonists chelerythrine chloride (CheCl; PKC inhibitor; 20 μM), aristolochic acid (Aristol; PLA_2 inhibitor; 50 μM), 1-butanol (PLD inhibitor; 0.5%), LiCl (PIP_2 synthesis inhibitor; 1 mM) and DDA (adenylyl cyclase inhibitor; 50 μM) on the potentiation of glutamate-stimulated ATP release by SP. (b) Typical time course of the effect of aristolochic acid on the potentiation of glutamate-stimulated ATP release by SP. The time courses of ATP release stimulated by glutamate and glutamate plus SP in the presence of aristolochic acid overlap. (c) Summary histogram of the effect of chelerythrine chloride and 1-butanol on the potentiation of NMDA-stimulated ATP release by SP. (d) Effect of Ca^{2+}-free medium with 5 mM EGTA (zero Ca^{2+}) on glutamate-stimulated and NMDA plus SP-stimulated ATP release, and the effect of Ca^{2+} ionophore A23187 (10 μM; $n = 3$) on ATP release. Values are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's unpaired two-sample two-tailed *t*-test).

spinal cord astrocytes, although it has not been determined whether these responses are due to the action on kainate or AMPA receptors (Ziak *et al.* 1998). Consequently, we hypothesize that, in the present case, either kainate does not activate spinal cord astrocyte AMPA receptors to the extent needed to produce ATP release or AMPA receptors on spinal cord astrocytes are insensitive to kainate, possibly owing to a primary structure differing at key amino acid sites that regulate the kainate sensitivity of AMPA receptors (Rodriguez *et al.* 2005).

Although NMDA and mGlu receptor agonists did not elicit ATP release when applied alone, when they were each co-applied with SP a release of ATP was observed. SP had no effect on the ATP release responses of spinal cord astrocytes to AMPA and kainate. These results suggest a model whereby glutamate stimulates ATP release through AMPA receptors, but in the presence of SP it can also stimulate ATP release through NMDA and mGlu receptors, leading to the observed potentiation of glutamate-stimulated ATP release.

In further support of this conclusion, simultaneous antagonism of NMDA and mGlu receptors completely abolished the potentiation of glutamate-stimulated ATP release by SP. Furthermore, with regard to the concentration studies carried out, the E_{max} value of glutamate-stimulated ATP release in the presence of SP was much higher than that in the absence of SP. This suggests that SP enhanced the number of ATP release-linked glutamate receptors in the membrane.

Immunocytochemistry identified NR1 subunits and mGlu 1α/5 receptors expressed on the surface of a portion of spinal cord astrocytes, in accordance with previous reports (Aicher

et al. 1997; Silva et al. 1999), yet the fact that glutamate alone does not stimulate ATP release through these receptors suggests that they are not functionally linked to the ATP release pathway. How SP links these receptors to the ATP release pathway remains unclear. In the case of the NMDA receptor, one contribution SP may make is to change the receptor such that glutamate does not rely on glycine co-binding to activate the NMDA receptor, as the potentiation of NMDA-stimulated ATP release by SP occurred even in the presence of a glycine co-binding-site antagonist. In addition, PKC is known to enhance the activity of NMDA receptors (Liao et al. 2001) and arachidonic acid downstream of PLA_2 activation is known to enhance the activity of mGlu receptors (Collins et al. 1995). As both of these signalling molecules are known to be activated on binding neurokinin-1 (Rayner and Van Helden 1997; Monastyrskaya et al. 2005) and are central to the effects of SP in this study, SP may possibly potentiate the activity of these receptors to a degree where they can activate enough signalling molecules to release ATP.

Surprisingly, the SP and NMDA co-stimulated response occurred despite the presence of Mg^{2+} at a concentration known to inhibit neuronal NMDA receptor-mediated responses (Ziak et al. 1998). There are several examples of NMDA-mediated responses in glial cells that occur at a physiological level of Mg^{2+}, such as NMDA-stimulated ATP release from cortical astrocytes (Queiroz et al. 1997) and NMDA-induced inward currents in spinal cord astrocytes from rats younger than 14 days (Ziak et al. 1998). As suggested by Ziak et al. (1998), perhaps glial NMDA receptors contain Mg^{2+}-resistant subunits such as the ε3 subunit.

To bring about potentiation of glutamate-stimulated ATP release, it is likely that SP acted synergistically on multiple neurokinin receptor subtypes. A neurokinin-1 receptor preferring antagonist abolished the effects of SP, suggesting that an action on the neurokinin-1 subtype was necessary for the effects of SP. However a selective neurokinin-1 receptor agonist was not sufficient to potentiate glutamate-stimulated ATP release. Similarly, selective stimulation of neurokinin-2 or -3 receptors was not sufficient to potentiate glutamate-stimulated ATP release. It was only when agonists to the neurokinin receptor subtypes were applied in combination that potentiation was observed. This suggests that the neurokinin-1 receptor is necessary but not sufficient for the actions of SP on glutamate-stimulated ATP release, and that a synergistic action on neurokinin-2 and/or neurokinin-3 receptors is also necessary for the potentiation. This is consistent with reports of SP acting as an agonist at all three neurokinin receptor subtypes (Regoli et al. 1994; Drew et al. 2005) and with reports of functional neurokinin-1, -2 and - 3 receptors on spinal cord astrocytes (Wienrich and Kettenmann 1989; Zerari et al. 1998).

It was unlikely that the ATP found in solution after addition of agonists was due to release of adenosine and subsequent actions of adenosine kinase to produce ATP because antagonists to an adenosine transporter and adenosine kinase did not effect SP and glutamate-stimulated ATP release. Potential mechanisms of direct ATP release were not explored in this study, but previous studies have reported direct ATP release from astrocytes through P2X7 receptors (Suadicani et al. 2006), connexin hemichannels (Cotrina et al. 1998; Stout et al. 2002), exocytosis (Abdipranoto et al. 2003; Coco et al. 2003) and anion transporters (Abdipranoto et al. 2003; Anderson et al. 2004).

Ca^{2+} influx and various intracellular signalling pathways are involved in glutamate-stimulated ATP release and its potentiation by SP

Significant decreases in the glutamate-evoked release of ATP in the presence of SP were obtained on blocking PKC, PLA_2, PLD and IP_3, but not adenylyl cyclase. It is known that activation of neurokinin-1 receptors activates PKC in human embryonic kidney 293 cells (Monastyrskaya et al. 2005) and PLA_2 is also increased on activation of neurokinin-1 receptors on endothelial cells (Rayner and Van Helden 1997). SP increases PLD, PLC and phosphatidylinositol hydrolysis in Chinese hamster cells and astrocytes (Marriott et al. 1991; Torrens et al. 1998) but it does not increase cyclic adenosine monophosphate in cortical astrocytes (Rougon et al. 1983).

ATP release evoked by activation of NMDA receptors in the presence of SP was greatly reduced in Ca^{2+}-free external solution, indicating that Ca^{2+} influx is necessary for this effect. It is unlikely that this influx of Ca^{2+} ions is due to activation of neurokinin receptors as we were unable to detect any inward currents in spinal cord astrocytes in response to applied SP. It is known that SP can directly enhance PLC and phophatidylinositol hydrolysis in membrane preparations independently of Ca^{2+} (Nakajima et al. 1992). We suggest therefore that the dependence of the effect of SP on NMDA-stimulated ATP release involves an influx of Ca^{2+} through the NMDA receptor. In addition, influx of Ca^{2+} was centrally involved in glutamate-stimulated ATP release as Ca^{2+} influx was sufficient to elicit ATP release and removal of extracellular Ca^{2+} greatly attenuated glutamate-stimulated ATP release. This Ca^{2+} influx could be directly through non-glutamate receptor 2-containing AMPA receptors or as a result of Na^+ influx through AMPA receptors activating the Na^+/Ca^{2+} exchanger (Smith et al. 2000).

From the present findings it would seem that SP-potentiated glutamate-evoked ATP release is dependent on two pathways. The first is a direct action of glutamate on AMPA receptors resulting in influx of Ca^{2+}. An additional pathway is recruited by SP through its actions on neurokinin receptors, producing activation of PKC, PLA_2, IP_3 and PLD. As a result of this activation glutamate is able to bring about ATP release by binding NMDA and mGlu receptors.

Possible applications of the potentiation of glutamate-stimulated ATP release by SP from spinal cord astrocytes

SP and glutamate-containing nerve terminals are found in descending bulbospinal neurons in the ventral horn (Hokfelt *et al.* 2000) and in the intermediolateral column (Helke *et al.* 1982; Kapoor *et al.* 1992). The results of the present study suggest that a consequence of SP and glutamate release in these areas may be increased release of ATP from astrocytes. As these areas are rich in purinergic receptors (Kanjhan *et al.* 1999; Deuchars *et al.* 2001; Deng and Fyffe 2004), this may lead to an increase in purinergic transmission in these areas, with possible functional consequences such as increased phrenic motor innervation of the diaphragm (Miles *et al.* 2002).

SP and glutamate are also found in nociceptive nerve terminals in the dorsal horn, from which they are both released by relatively high frequencies of nerve stimulation, and an increase in their release is seen during inflammatory pain (Sasaki *et al.* 1998; Allen *et al.* 1999; Honore *et al.* 2000). Their concomitant release during heightened activity in nociceptor terminals enhances pain transmission in the dorsal horn of the spinal cord (Mjellem-Joly *et al.* 1992; Zahn and Brennan 1998; Ishizaki *et al.* 1999). This study suggests that one consequence of increased glutamate and SP release during inflammatory pain may be increased ATP release from astrocytes in close apposition to these terminals. This ATP may then act on P2X3, 4 and 7 receptors in the dorsal horn, the activation of which has been reported to lead to release of pro-inflammatory cytokines, brain-derived neurotrophic factor and neurotransmitters, and consequently chronic pain (Kennedy *et al.* 2003; Bianco *et al.* 2005; Coull *et al.* 2005). Hence this increased ATP release from astrocytes in response to SP and glutamate may be one mechanism that contributes to the pain-enhancing effects of these transmitters.

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