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P2X7-like receptor activation in astrocytes increases chemokine MCP-1 expression via MAP kinase

by

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A THESIS

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ABSTRACT

Leukocyte infiltration in the CNS following trauma or inflammation is triggered in part through the action of the chemokine, Monocyte Chemoattractant Protein-1 (MCP-1), in astrocytes. In response to many situations, including trauma, the extracellular concentration of ATP increases. We have discovered that ATP receptor activation in astrocytes plays a central role in MCP-1 upregulation. In cultured astrocytes, activation of purinergic P2X7 receptors leads to the activation of the MAP kinases ERK1, ERK2, and p38. P2X7 receptor engagement also leads to an increase in the expression of the chemokine MCP-1, an effect mediated by MAP kinase cascades. These data show that transmitter receptors in astrocytes may mediate communication with hematopoietic inflammatory cells.

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DEDICATION

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ABBREVIATION	PROPER NAME
α-β-M-ATP	α-β-methylene adenosine 5' triphosphate
2-MS-ATP	2-methylthio adenosine 5' triphosphate
ABC	ATP Binding Cassette
ADP	Adenosine 5'-Diphosphate
AIDS	Acquired Immune Deficiency Syndrome
AMP	Adenosine 5'-Monophosphate
AP-1	Activated protein –1
ASK1	Apoptosis Signal-Regulating Kinase
ASK-1	Apoptosis Stimulating Kinase
ATP	Adenosine 5'-Triphosphate
ATP-γ-S	Adenosine 5'-O-[3-thiotriphosphate]
Bz-ATP	Benzoyl-Benzoyl-ATP
Ca^{++}	Calcium
CaMK	Calcium, Calmodulin Dependent Protein Kinase
cAMP	Adenosine 3',5'-Cyclic Monophosphate
CCR	Chemokine Receptor
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator

CHOP	C/EBP-Homologous Protein
CNS	Central Nervous System
COX	Cyclo-oxygenase
cPLA2	Cytosolic Phospholipase A2
CSF	Cerebro-Spinal Fluid
CXCR	CXC receptor
DIDS	4'4'Diisothiocyanatostilbene-2,2'-disulfonic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EAE	Experimental Allergic Encephalitis
ECL	Enhanced Chemi-Luminescence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-tetraacetic Acid
ERK	Extracellular Signal-Regulated kinase
FCS	Fetal Calf Serum
GABA	γ-Amino-n-butyric Acid

GDP	Guanine di-phosphate
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GRB2	Growth Factor Receptor-bound Protein 2
GSH	Glutathione
GTP	Guanine tri-phosphate
HIV	Human Immonodeficiency Virus
hsp	Heat Shock Protein
ICAM	Intercellular Adhesion Molecule
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JNK/SAPK	c-Jun amino-terminal kinase (JNK) / stress-activated protein kinase
KSR	Kinase Suppressor of Ras
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LTF	Long Term Facilitation
LTP	Long Term Potentiation
MAP	Mitogen Activated Protein

MAP kinase	Mitogen Activated Protein Kinase
MAP-2	Microtubule associated protein – 2
MAPKAP	Mitogen Activated Protein Kinase Activating protein
MAPKAPK	MAP Kinase Activated Protein Kinase
MAPKK	MAP Kinase Kinase
MAPKKK	MAP Kinase Kinase Kinase
MBP	Myelin Basic Protein
MCP-1	Monocyte Chemoattractant Protein-1
MDR	Multi-drug Resistant
MEF2C	Myocyte-Specific Enhancer-Binding Factor 2
MEK	Mitogen Activated Protein Kinase Kinase
MEKK	MAP Kinase Kinase Kinase
MKK	MAP Kinase Kinase
MLK-3	Mixed Lineage Kinase 3
mM	milli-Molar
MNK	Mitogen-Activated Protein Kinase Signal-Integrating Kinase
m-RNA	Messenger Ribonucleic Acid
MT	Metallothionein
NFκB	Nuclear Factor Kappa B

NFAT	Nuclear Factor of Activated T-cells
NK	Natural Killer
NMDA	N-Methyl-D-Aspartate
o-ATP	Oxidised ATP
PACAP	Pituitary Adenylate Cyclase-activating Polypeptides
PAK	p21-activated Kinase
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma 12 Cell Line
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
PI	Pre-incuabtion
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PP2A	Protein phosphatase 2A
pp90rsk	Ribosomal S6 protein kinase

PPADS	Pyridoxal–phosphate-6-azophenyl-2,4-disulphonic acid
PVDF	PolyVenyl diFlouride
PYK	Proline rich tyrosine kinase
Rb2	Reactive Blue 2
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SH2	Src-homology-2
SOS	Son of Sevenless
TAK	TGF-beta homologous protein
Th	T-helper
TNF	Tumor Necrosis Factor
TNP-ATP	2'- (or 3') -O-(2,4,6-trinitrophenyl)-ATP
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAF	TNF Receptor Associated Factors
TTBS	Tween-Tris Buffered Saline
UTP	Uridine Tri-Phosphate
μM	micro-Molar
VCAM	Vascular Cell Adhesion Molecule

Introduction

Astrocytes in the CNS:

Glial cells were originally thought of only as a "glue" responsible for holding neurons in place [1]. Ramón y Cajal and Pio del Río-Hortega later divided glial cells into three families, namely oligodendrocytes, microglia and astrocytes [1]. Of the three types of glia, astrocytes are the most numerous type in the CNS [2]. Classically thought to exist solely in a supportive and nutritive role, they are now known to perform a diverse array of functions in the CNS.

In the developing brain astrocytes serve as axonal guides and as nutrient reservoirs. It is thought that neuronal extension may involve astrocytic extracellular matrix proteins that create a pathway for neuronal growth [3]. While providing the roadmap, the astrocyte also secretes a variety of growth factors that may trophically support the neuron and contribute to synaptogenesis. As during growth, CNS injury causes astrocytes to up-regulate the secretion of variety of growth factors and cytokines, which in turn can lead to an increase/decrease in the survival of surrounding neurons and glia.

In addition to cytokine and growth factor release astrocytes possess a number of potent uptake mechanisms for ions and transmitters that enables the maintenance of extracellular homeostasis. Classically, astrocytes are well known for their ability to buffer extracellular potassium concentrations. Neuronal activity leads to an increase in the extracellular potassium concentration, which can reach levels of up to 12 mM in the normal brain [4].

Astrocytes possess membrane pumps (such as the Na/K-ATPase pump) that, unlike their neuronal counterparts, do not saturate at these high ionic concentrations. The combined activity of membrane pumps, potassium channels, and gap junctions that link multiple astrocytes allows the clearance of potassium and a subsequent re-distribution of charge over large distances [4]. The discovery that astrocytes also possess uptake mechanisms for glutamate, glycine, taurine, GABA, adenosine, histamine, and other neurotransmitters [5] extends the spectrum of potential astrocyte influence.

Astrocytes have been shown to express an almost full complement of neurotransmitter receptors. Although traditionally thought not to respond to the same signals as neurons, the presence of these receptors, especially *in vivo*, suggests a role for astrocytes in neurotransmission. The presence of many of these receptors and receptor uptake mechanisms proximal to synapses or within synaptic clefts lends credence to an *in vivo* function [5]. Indeed, activation of some neurotransmitter receptors on astrocytes has been shown to induce membrane depolarization and subsequent activation of astrocyte calcium channels which may then engage a variety of signal transduction mechanisms [4].

Interestingly, astrocytes may also participate directly in signalling. Many astrocytes are equipped with the ability to generate self-propagating calcium waves that can propagate from cell to cell [6]. In this fashion, neighbouring astrocytes may respond to a distant stimulus. The generation of intercellular calcium signalling has been found to be dependent on ATP receptors, and it is speculated that ATP may function as an

extracellular signalling molecule that after release, induces calcium transients in neighbouring cells [6]. Alternatively, astrocytes may also signal via intercellular gap junctions that directly transport the electrical signal from cell to cell [6]. Astrocytes may also communicate with other cell types, as studies using models of the blood brain barrier demonstrate calcium wave propagation between astrocytes and endothelial cells [7]. Bi-directional communication with neurons has also been established. Stimulation of astrocytes leads to calcium entry and subsequent release of glutamate that can then induce measurable currents in apposed neurons [8]. Recently, a role for astrocytes in synaptic plasticity and neurotransmission was suggested based on evidence indicating that astrocytic calcium transients induced by GABA potentiate inhibitory transmission in CA1 pyramidal neurons [8].

CNS insult induces the production of antioxidants, which may cause further damage. A role for astrocytes in antioxidant metabolism is now emerging based on evidence indicating that antioxidant molecules such as metallothionein (MT), glutathione (GSH), apolipoprotein D, and bilirubin localise preferentially to astrocytes [5]. In response to injury, astrocytes selectively up-regulate these proteins [5] [9]. A role for astrocytes in heavy metal toxicity has also been postulated based on the ability of antioxidants such as metallothionein to bind to these metals [5].

Astrocytes serve a number of other roles in the CNS, including roles in inflammation and the immune response, which will be discussed throughout this thesis.

Functions of ATP in the CNS

ATP has long been known as a mediator of intracellular function. That ATP could function outside the cell was first postulated in the 1960's after the observation of a non-cholinergic non-adrenergic response in the autonomic nervous system [10]. Over the next several years ATP-evoked synaptic potentials were observed in a number of neuronal types, further strengthening the hypothesis. The search for unambiguous evidence of ATP's role as an extracellular signalling molecule culminated ultimately with the cloning of various members of the P2-purinoceptor (ATP receptor) family [10].

ATP has been implicated in number of CNS processes and pathologies including memory, pain transmission, and schizophrenia [11]. ATP's role in memory and learning is highlighted by work which shows that ATP potentiates Long Term Potentiation (LTP) and facilitates glutaminergic transmission in the hippocampus [11]. ATP's role in CNS disorders such as schizophrenia is suggested by the fact that antipsychotic drugs may act in part by inhibiting ATP gated Ca^{++} channels [11]. Further, application of exogenous ATP to dopaminergic neurones has been shown to result in dopamine release [11], which likely potentiates the schizophrenic state. Lastly, ATP may also modulate pain sensations in the nervous system as purinergic receptors have been localised to nociceptive neurones and inhibitors of purinergic receptor activity are effective in blocking pain transmission [11, 12].

On a cellular level, ATP is known to cause the proliferation of a number of cell types, to lead to necrosis/apoptosis in others, and to serve a recently discovered role in the immune system [13]. In astrocytes, application of extracellular ATP stimulates the elongation of processes, induces DNA synthesis, and leads to differentiation [14]. ATP also has a number of effects on the immune system including the induced release of a variety of cytokines, potentiation of macrophage killing of mycobacterium, the priming of neutrophils to release superoxide anion, the modulation of leukocyte adhesion, and many others [13] [15].

Sources of extracellular ATP in the CNS

Physiological ATP release has been documented in a variety of cell types. For example, ATP is stored and co-released by neurones with other neurotransmitters such as noradrenaline or acetylcholine [10]. Recently it has been shown that microglia release ATP in response to LPS stimulation [16]. Queiroz et al. have shown that glutamate application caused ATP release in astroglial cells [17]. In addition, the levels of ATP and other purines have been shown to increase up to 200 fold following ischemia [18]. Trauma would be expected to produce even higher levels of extracellular ATP as the intracellular ATP pool (3-5mM) spills out onto surrounding cells.

Mechanisms of ATP release from astrocytes

Membrane rupture as occurs during trauma is perhaps the simplest conceptual escape route for ATP from the cell. More subtle mechanisms of ATP release involving ATP

transporter proteins or synaptosomal release machinery are currently a topic of intense research. Of these, the ATP binding cassette (ABC) family of transporters, including the multi-drug resistant (MDR1) gene product p-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) are thought to be present in astrocytes [19, 20]. Inhibitors of the CFTR have been shown to limit ATP exit from astrocytes [19] while the p-glycoprotein ATP channel is part of a well characterised mechanism of ATP release in a variety of cell types [21]. GAP junctional proteins of the connexin family have also been implicated in the ATP release pathway. In glioma cells, for example, expression of these gap junction proteins has been shown to up-regulate ATP release 10-15 fold [22].

Purinergic receptors: receptors for extracellular ATP

Extracellular ATP exerts many biological effects by acting on cell surface P2-purinergic receptors (P1 receptors are receptors for Adenosine). These receptors are ubiquitously expressed and exist in almost all tissue and organ systems [23]. Prior to the cloning of purinergic receptors, the various receptors in each group were divided pharmacologically based on agonist and antagonist sensitivities. The varying sensitivities of P2 purinoceptors to a diverse array of agonists and antagonists is currently exploited in functional studies aiming to ascribe a particular effect to a certain class of purinergic receptor.

ATP receptors were originally divided into two major classes, the P2X and the P2Y. The P2Y class are G-protein linked metabotropic receptors, while the P2X class are ligand-gated ion channels [10]. Other classes of P2 purinoceptors have recently been added and include the P2T and the P2U class of receptors. The P2T receptors seem to exist primarily in platelets and respond preferentially to ADP, with ATP acting as an antagonist. The P2U class of receptors shows equipotency to stimulation with either UTP or ATP [10]. A list of purinoceptors and their pharmacological agonists and antagonists are presented below.

Table 1 : G protein – coupled purinergic receptors

Receptor	P2Y	P2U	P2T
Natural agonist	ATP	ATP and UTP	ADP
Pharmacological agonists	2-MS-ATP> ATP >>>α-β-M-ATP	UTP>=ATP	ADP
Pharmacological antagonists	Suramin, Rb2		ATP, Suramin

Abbreviations: 2-MS-ATP (2-methyl-thio-ATP); αβMATP (alpha beta methyl ATP)

Table 2 : Purinergic ion channel receptors (P2X family)

Receptor	P2X1/P2X3	P2X2/P2X4/P2X5/P2X6	P2X7
Natural agonist	ATP	ATP	ATP
Pharmacological agonists	2-MS-ATP>= ATP >= α-β-M-ATP>=Bz-ATP>>>>ADP	ATPγS, ATP>2-MS-ATP, Bz-ATP>α-β-M-ATP	Bz-ATP>ATP> ATPγS
Pharmacological antagonists	Suramin, PPADS	Suramin, PPADS	DIDS, Suramin, PPADS, o-ATP. Brilliant Blue G, Calmidazolium, KN-62 (human receptor only).

Abbreviations: 2-MS-ATP (2-methyl-thio-ATP); α-β-M-ATP (alpha beta methyl ATP) o-ATP (oxidised ATP) ; PPADS (Pyridoxal–phophate-6-azophenyl-2,4-disulphonic acid) DIDS (4'4'Diisothiocyanatostilbene-2,2'-disulfonic Acid) ; Rb2 (Reactive Blue 2).

Tables adapted from Chen et al. [10], Watson et al. [24], North and Surprenant [25], and Jiang et al. [26].

The P2X family of purinergic receptors

The P2X family of purinoceptors comprises 7 types of receptors, numbered P2X1 through P2X7. The pharmacological properties of P2X receptors are summarised in table 2. All P2X receptors are non-selective cation channels permeable to sodium, potassium and calcium [10]. Interestingly, these receptors are found in every tissue examined making them the most ubiquitously distributed ion channel receptor family known [27]. Structurally the P2X family consists of two transmembrane spanning domains with a large extracellular loop [27]. Both the N-terminal and C-terminal ends are located intracellularly [28]. The intracellular C-terminal end shows the least sequence conservation between the individual P2X family members and is thought to be responsible for the differences in permeability characteristics [27].

Pharmacologically the P2X family shows three distinct pharmacological phenotypes. The first group is comprised of the P2X1 and P2X3 receptors which are activated preferentially by 2-MS-ATP, ATP and α-β-M ATP and are sensitive to inhibition by TNP-ATP [28]. The second class is comprised of the P2X2/P2X4/P2X5 and P2X6 receptors, which show little activity in response to α-β-M ATP. Suramin and PPADS are effective antagonists at the P2X2 and P2X5 receptors, and less potent at P2X4 and P2X6 [28]. Receptors that do not adhere to these phenotypic guidelines are thought to result from heteropolymerization of multiple receptor types. Co-expression experiments

demonstrate that P2X2 and P2X3 may heteropolymerize, as might the P2X4 and P2X6 receptor groups [28].

The P2X7 receptor

The newest member of the P2X family is the recently cloned P2X7 (P2Z) receptor. A number of properties set this receptor apart from other members in the P2X class. It shows less tendency to desensitise or rectify than the other P2X receptors, and it is strongly influenced by the extracellular concentrations of calcium and magnesium [28]. The agonist profile is also quite different, as Benzoyl-benzoyl-ATP (Bz-ATP) is a stronger agonist at this receptor than ATP [28]. The immune system seems to be the predominant area of expression of the P2X7 receptor, although recently it has also been shown to be present in a number of organ systems including the CNS [29]. Specifically the P2X7 receptor has been localised to microglia, neurones, and most recently to astrocytes [29].

Structure of the P2X7 receptor

Structurally, the P2X7 receptor shares the least sequence homology with the other P2X receptors [30], showing only 35-50% similarity. It also demonstrates the least inter-species conservation with the rat version being only 80% identical to human P2X7 [31], [30]. It is hypothesised that many P2X7 receptors physically interact to form a channel pore, although there is no firm evidence in this regard at present [30, 31].

P2X7 receptor and cell death

By virtue of its extended C-terminal end (the longest of any P2X receptor) the P2X7 receptor is endowed with the unusual property of being able to form a pore in the membrane. When opened, this allows the passage of large molecules such as Ethidium Bromide or Lucifer Yellow into the cell, and intracellular contents to exit [30]. Continuing activation of the P2X7 receptor can, by this route, eventually lead to cell swelling and cell lysis. Pizzo et al. first ascribed the cell lysis effect of ATP to the P2X7 receptor in 1992 when they demonstrated that ATP cytotoxicity correlated with the presence of P2X7 receptors [32]. Since that time, the P2X7 receptor has been conclusively linked to cell death in a number of different systems. Interestingly, other P2X receptors such as the P2X2 and the P2X4 receptors have recently been shown to have pore-forming ability in response to prolonged ATP administration, however activation of these receptors does not cause lysis [33].

Apoptosis has also been documented extensively in response to activation of the P2X7 receptor [34]. Rat glomerular mesangial cells, myeloic cells, cultured mesangial cells, microglial cells, and many other cell types undergo P2X7-mediated apoptosis [34, 35]. Ferrari and co-workers have demonstrated that caspase inhibitors effectively inhibit chromatin condensation and DNA fragmentation induced by P2X7 receptor activation [35].

The mechanism whereby the same receptor causes death by lysis or death by apoptosis is unknown, although it is postulated that it depends on the permeability state of the P2X7 receptor. Surprenant et al. have shown that the pore-forming properties of the P2X7 receptor can be distinguished from its properties as a non-selective cation channel [36]. Given that the P2X7 receptor shows little desensitisation, it is plausible that continuous stimulation, at levels insufficient to create a pore, may increase the intracellular calcium concentration sufficiently to evoke an apoptotic response [34]. If, on the other hand, the stimulus causes P2X7 pore formation, death by leakage of intracellular material and lysis is more likely.

P2X7 receptor signalling

P2X7 receptor signalling mechanisms have been sparsely studied, perhaps because the P2X7 receptor carries no known signalling motif [37]. el-Moatassim and Dubyak first demonstrated downstream signalling by P2X7 with their discovery that phospholipase-D was activated in response to receptor stimulation [38] . This PLD activity seems to be independent of the pore-forming property of the receptor although it does correlate with calcium influx through the channel. Downstream effects of P2X7 mediated PLD activation are largely unknown although PLD activation has been shown to be an integral element in the potentiation of macrophage killing of mycobacterium in response to ATP, and has been postulated to form part of the cytolytic pathway initiated by the P2X7 receptor [39].

Phospholipase A2 (PLA2) has recently been discovered as a P2X7 receptor target. Alzola et al. have demonstrated that P2X7 receptor activation results in activation of both calcium dependent and calcium independent PLA2 activities in ductal cells of the rat submandibular gland [40]. This up-regulation of PLA2 activity was found to be necessary for ATP induced Kallikrein secretion in these cells [40].

The transcription factors NFκB and NFAT are intimately involved in a number of cellular processes including cytokine/chemokine production and apoptosis. Using ligated P2X7 receptors Ferarri et al. have demonstrated the activation of the transcription factor NFκB, a key regulator of proinflammatory genes, following activation by the P2X7 receptor [37]. Interestingly, the subunit composition of NFκB induced by P2X7 receptor activity was a p65 homodimer, which is distinct from the heterodimers formed by classical inflammatory mediators. The authors infer that the varying subunit composition likely results in the modulation of a different group of NFκB dependent genes than those affected by other inflammatory mediators [37]. This same group has also implicated nuclear factor of activated T-cells (NFAT) in P2X7 signalling. Of interest, the NFAT group of transcription factors modulate the production of several cytokines including IL-2, IL-4, GMCSF, TNF, and others [41].

Several caspases are also downstream from the P2X7 receptor. Caspases 1, 3 and 8 become activated and subsequently cleave lamin B and PARP in response to P2X7

activation in microglial cells. Inhibition of these caspases prevents apoptotic damage but does not inhibit P2X7 mediated necrotic damage [42].

All of the MAP kinase pathways have very recently been shown to become activated in response to P2X7 stimulation. In microglial cells the p38 pathways and the ERK pathway were recently inferred to be downstream of P2X7 based on their activation by the P2X7 receptor agonist Bz-ATP [43]. The JNK/SAPK also becomes activated in response to P2X7 stimulation [44].

Immunity and the P2X7 receptor

The P2X7 receptor has been implicated in many immune functions. Interleukin-1 beta (IL-1b), for example, is released from microglial cells in response to P2X7 stimulation [16]. Interestingly, IL-1b release induced by classic stimuli such as lipopolysaccharide (LPS) is also sensitive to P2X7 inhibition, likely because LPS administration causes ATP release which can then act in a paracrine/autocrine fashion to activate P2X7 [42]. Nitric oxide production by macrophages in response to LPS may also be a P2X7 receptor dependent event [45]. TNF expression in response to P2X7 stimulation has also recently been documented in microglial and dendritic cells [43] [46].

The fusion of macrophages to form multinucleated giant cells may depend on the P2X7 receptor as macrophage cell lines expressing high levels of the P2X7 receptor show higher rates of fusion and the specific P2X7 receptor inhibitor oxidized ATP (o-ATP)

prevents this fusion [47]. Recently a study by Mutini et al. also ascribes an antigen-presenting role to the P2X7 receptor as immature dendritic cells exposed to the P2X7 receptor antagonist o-ATP were hindered in their ability to stimulate antigen specific Th lymphocytes [48].

Macrophage efficiency is also altered by the P2X7 receptor. The killing of mycobacterium, for example, is enhanced when macrophages are stimulated with ATP. This effect of ATP has been ascribed to the P2X7 receptor as inhibitors of P2X7 function such as o-ATP, suramin, DIDS, and Amiloride all extinguish this response [15]. Interestingly, it seems that certain mycobacteria counter the effects of P2X7 activation by releasing ATP scavenging enzymes that effectively sequester extracellular ATP [49].

Introduction to MAP kinase

Mitogen activated protein kinase was first discovered in 1988 as a serine/threonine kinase responsible for phosphorylating and activating ribosomal protein S6 kinase [50]. MAP kinases are pleiotropic signaling molecules in that their activities regulate a number of different cellular responses. The acronym MAP reflects the fact that the MAP kinases are Mitogen Activated Proteins. In that respect, they serve to relay mitogenic signals from the cell membrane to the nucleus through the phosphorylation of a wide variety of targets. Some enzymes which are phosphorylated by the MAP kinases include p90rsk, MAP kinase kinases, MAPKAP-2, Raf-1, the EGF receptor, and cPLA2 [50]. In addition to cellular enzymes, MAP kinase also phosphorylates cytoskeletal proteins such as tau, MAP-2 and h-

Caldesmon [50]. Finally, transcription factors such as Elk-1, c-myc, c-fos, ATF-2 and others [50] comprise the nuclear pool of MAP kinase substrates.

MAP kinase activation is effected by a number of stimuli that include almost all of the known growth factors that bind tyrosine kinase receptors, lymphokines that engage tyrosine kinase receptors, several agonists that stimulate the hydrolysis of polyphosphoinositides and phosphatidylcholine leading to activation of protein kinase C, and inhibitors of protein-serine/threonine phosphatases, protein-tyrosine phosphatases [51]. Inactivation of the MAP kinases occurs through a combination of serine/threonine phosphatases (such as PP2A) and tyrosine phosphatases (such as CL100) [52]. Interestingly, the MAP kinases control the production of some of their own phosphatases, with MAP kinase activation leading quickly to their synthesis [53]. Protein synthesis inhibitors, therefore, are effective agents in prolonging MAP kinase responses [53].

The MAP kinase family currently is divided into more than 12 members [54]. The MAP kinases are divided into three distinct cascades based on sequence homology, agonists and antagonist profiles, and the effects of activation [54]. The first to be discovered were the Extracellular Signal Regulated Kinases (ERK) of which ERK1 and ERK2 have been studied in the most detail. Classically the ERK pathways are responsible for cell growth, proliferation, and survival. Other members of the MAP kinase family include the Jun-N-terminal kinases (JNK) and the p38 MAP kinases. There appears to be extensive redundant

signaling especially between the JNK and p38 MAP kinase groups, and both are canonically associated with apoptotic events and cellular stress signaling.

Activation of the MAP kinases occurs by dual phosphorylation on tyrosine and threonine residues within a conserved TXY motif [54]. Phosphorylation on either the threonine or tyrosine residue alone increases MAP kinase activity approximately 10 fold, while dual phosphorylation results in 3000-fold stimulation. Dual specificity kinases that phosphorylate the MAP kinases are aptly termed the MAP kinase kinases (MKK). To date, there are seven different isoforms of the MAP kinase kinases [55]. MEK 1 and MEK 2 were the earliest to be discovered and serve as MKKs of the ERK cascade [55]. MKK4 and MKK7 are regulators of the JNK cascades while MKK3 and MKK6 preferentially phosphorylate and activate members of the p38 group of MAP kinases [55]. MKK5 activates ERK5, although little has been studied in relation to this obscure ERK isoform [55].

Activation of the MAP kinase kinases is mediated by dual phosphorylation on threonine and serine residues by the action of MAP kinase kinase kinases (MAPKKK). Members of the MAPKKK family include MAPKKK1-4, apoptosis stimulating kinase (ASK-1), mixed lineage kinase 3 (MLK-3), Raf-1, A-Raf, and B-Raf [55].

Although phosphorylation on multiple residues leads to the activation of the MKKKs the exact mechanisms and specific sequence targets are less well understood. Proven upstream

regulators of the MKKKs include p21-activated kinase (PAK), and an extensive family of low molecular weight G-proteins that include the Ras and Rho protein families [55].

The ERKS – ERK1 and ERK2

More is known about the Extracellular signal Regulated Kinases than any other MAP kinase group. Five members comprise the ERK group of MAP kinase proteins, numbered ERK1 through ERK5. Of the five members, only ERK1 and ERK2 have been studied in detail.

Signaling to the ERKs by receptor tyrosine kinases

Classically ERK1/2 are recruited in response to growth factor stimulation of a receptor tyrosine kinase. Binding of a ligand to a tyrosine kinase growth factor receptor causes the receptor to dimerize and trans-autophosphorylate on multiple tyrosine residues. These phosphorylated tyrosines serve to attract signaling proteins harboring src-homology-2 (SH2) domains to the receptor. For example, phosphorylated tyrosines and the surrounding residues serve as sites for protein-protein interactions with the SH2-domain-containing adapter molecules GRB2 and SHC. The association of GRB2 to the receptor stimulates the recruitment of SOS (son of sevenless) to the receptor-adapter complex. SOS binding to GRB2 then causes SOS induced Ras activation through the exchange of GTP for GDP on Ras. Downstream of Ras lies Raf, the first member of the ERK kinase cascade. It is believed that activated Ras facilitates the translocation of Raf to the plasma membrane

leading to its activation via ill-defined mechanisms (all three Raf members have been shown to translocate in response to activated Ras [54, 57]). The identity of the kinase(s) responsible for activating Raf is currently unknown although both serine/threonine kinases and tyrosine kinases may be involved [53]. Activated Raf then serine phosphorylates and activates MKK. As mentioned above MKK is a dual specificity kinase, and phosphorylates ERK1/ERK2 on both tyrosine and threonine residues. This dual phosphorylation then leads to increased ERK activity. Recently, another member of this cascade, termed Kinase Suppressor of Ras (KSR) has been discovered. KSR interacts with multiple proteins in the ERK cascade including Raf, MEK and ERK and it is therefore postulated that it acts as a scaffold to facilitate protein-protein interaction [58].

Signaling to the ERKs by G-proteins

In addition to receptor tyrosine kinases, G-protein coupled receptors can also activate ERK1/ERK2. G-proteins are heterotrimeric complexes composed of alpha, beta, and gamma subunits. G-protein activation occurs through an exchange of GDP for GTP on the alpha subunit, causing the alpha subunit to dissociate from the beta-gamma subunits. Although the exact mechanism by which G-proteins induce ERK1/ERK2 activation is still a matter of debate, both beta-gamma subunits and alpha subunits of various G-proteins have been shown to influence ERK1/ERK2 signaling [59, 60][61, 62].

Beta-gamma subunits released from the Gi family of receptors (e.g. thrombin, LPA, m2 muscarinic) have been shown to induce activation of ERK1/ERK2 by causing activation of Ras [59, 60]. This action has been shown to occur through beta-gamma subunit mediated interaction with the adapter protein Shc, which thus may serve as a link between G-protein and tyrosine kinase mediated pathways [61, 62]. Beta-gamma subunits from the Gq family (e.g. m1muscarinic, platelet activating factor receptor) have also been shown to mediate Ras dependent ERK1/ERK2 activation and may exert their effect though direct translocation of Raf to the plasma membrane [60], or through a tyrosine kinase sensitive pathway similar to Gi-mediated ERK1/ERK2 activation [63]. Alternatively, Gq may activate ERK1/ERK2 by inducing PKC stimulation, as PKC has been shown to directly phosphorylate and activate Raf [60, 63].

In most cell types Gs is considered to have an inhibitory effect on MAPK activation. This inhibitory effect is most likely mediated through the elevated levels of cAMP induced by Gs alpha subunit stimulation of adenylyl cyclase. Increases in cAMP lead to activation of PKA which may in turn disrupt the association between Ras and Raf [64]. Studies indicating that PKA inhibits MAPK activation even in the presence of v-Raf open the possibility that PKA may also exert an effect downstream of Raf [64].

In some cells however, it appears that cAMP can serve to stimulate ERK1/ERK2 [65]. ERK1/ERK2 in PC12 cells, for example, has been shown to become activated in response

to treatment with the adenylyl cyclase activator forskolin, or through the addition of cAMP analogues [66]. Paradoxically, other studies on PC12 cells have shown the opposite results, with cAMP effectively abrogating ERK1/ERK2 signaling [67]. Although the pathway whereby cAMP activates ERK1/ERK2 is unclear, studies using dominant negative Ras mutants in PC12 cells point to a cAMP target upstream of Ras [66].

Signaling to the ERKs by Calcium:

Increases in intracellular calcium either by release from intracellular pools or through the opening of membrane calcium channels causes ERK activation [54]. Calcium, calmodulin dependent protein kinase (CaMK) II is postulated to facilitate ERK signaling by inhibiting enzymes that hydrolyze the GTP bound to Ras (recall that Ras is inactivated by GTP hydrolysis) [54, 68]. CaMK II could also act on the ERKs by engaging a tyrosine kinase receptor such as the EGF receptor [54, 69]. In this model, increases in intracellular calcium induce the dimerization of the EGF receptor which then signals to the ERK cascade by the classical pathway explained above. Calcium-sensitive guanine nucleotide exchange factors that stimulate the GDP to GTP exchange on Ras have also been implicated as ERK effectors [54, 70]. Finally, a group of Ca^{++}-sensitive tyrosine kinases have been shown to activate the ERKs. Members of this group include PYK2 and Src [54, 71].

Expression of the ERKs in the CNS:

Both ERK1 and ERK2 are expressed in the CNS. In-situ hybridization studies reveal that ERK2 is preferentially expressed in the cerebral cortex, olfactory bulb, hippocampus, amygdala, basal ganglia, basal nucleus, thalamus, hypothalamus, brain stem nuclei, cerebellum, and spinal cord neurons [72, 73]. ERK 1 expression was found to be elevated in the olfactory bulb, cortex, hippocampus, amygdala, nucleus basalis of Maynert, substantia nigra, hypothalamic and brainstem nuclei, cerebellum and some spinal cord neurons ([72, 73]. In the hippocampus ERK2 is expressed in all regions, while ERK1 is localized to the dentate gyrus ([72, 73]. Astrocytes contain both ERK1 and ERK2 as evidenced by western blotting with ERK antibodies and activity assays of ERK activity [74].

p38, a mediator of cell stress

Much less is known about the p38 group of MAP kinases than the ERKs. p38 activation is elicited by cell stress signals and by cytokines. Osmotic shock, DNA damage, X-rays, hydrogen peroxide, and growth factor withdrawal all strongly activate p38 MAP kinase [75]. Cytokines such as TNF-α and IL-1β also activate p38 [75].

Cell signaling to the p38 cascade involves at least two members directly upstream, MKK3 and MKK6 [75]. Interestingly, there are at present no MKKKs that selectively activate p38

[75]. TAK1, ASK1 and MTK1 do strongly activate p38 but are non selective in that they also modulate other MAP kinase signaling cascades [75].

Signaling to p38: The TNF receptor and small molecular G-proteins.

Binding of TNF-α results in the trimerization of the TNF-α receptor. This oligomerization leads to the recruitment of a number of proteins, such as the TNF receptor associated factors (TRAFs). Binding of TRAF-2 and TRAF-6, in particular, seems to be a particularly potent signal for p38 activation [76]. Signaling element between TRAF and the MKKKs are unknown although ASK1 has recently been implicated. ASK1 activation then leads to activation of the dual specificity kinases MKK3/MKK6 which in turn activate p38 [75] .

The Rho family of small G-proteins are involved in many cellular processes including cytoskeletal organization, gene transcription, and membrane trafficking [75]. Various cell surface receptors have been shown to activate the Rho family of g-proteins. The Rho family members Rac and cdc42 in particular seem to be strong upstream regulators of the p38 kinase cascade [75].

Downstream of p38: Targets and cellular effects:

Few *in vivo* targets of p38 are currently known. MAP kinase activated protein kinase (MAPKAPK) is a target of p38 phosphorylation and also seems to be involved in regulating p38 localization within the cell. Phosphorylation of MAPKAPK may trigger nuclear export of p38 as activated p38 binds to MAPKAPK and the complex exits the nucleus [77]. p38

regulated/activated kinase is another target of p38. This kinase becomes activated in response to cellular stressors and phosphorylates hsp27 *in vivo* [77, 78]. Transcription factors phosphorylated by p38 include ELK1, ATF2, and SAP1 [77]. Other proteins on which limited data exists but are thought to be possible targets of p38 include MNK1/2, CHOP, MEF2C, and strathmin [77].

A number of cytokines depend on p38 activation for their synthesis. TNF-α, IL-1, IL-6, iNOS, and MCP-1 are examples of pro-inflammatory mediators that are linked to p38 activation. To this effect, p38 is currently being explored by the pharmacological industry as a therapeutic target for the down-regulation of the inflammatory response [77]. In addition, p38 has also been shown to be involved in toxic shock as inhibitors of p38 improve the survival of mice challenged with LPS [77]. Further, p38 is intricately involved in cell death signaling. In many cell types p38 activation is a trigger for entry into the apoptotic pathway, and down-regulation of p38 can rescue a cell from apoptosis [79].

Role of the MAP kinases in the CNS

Synaptic plasticity:

Activation of NMDA channels followed by calcium influx is thought to be a crucial event in synaptic plasticity. Importantly, ERK mediates the expression of a number of early response genes that become activated in response to NMDA activation [80, 81]. For example, ERK becomes activated in response to conditions that induce long term

potentiation (LTP) in the hippocampal slice [80, 82] and inhibition of the ERK pathway prevents the induction of LTP in region CA1 of the hippocampus [80, 83]. In *Aplysia*, long term facilitation (LTF) is also blocked by inhibitors of the ERK pathway [80, 84]. Behavioral experiments confirm ERK's role in synaptic processes. Blockage of Ras, the upstream activator of the ERK pathway, leads to decreases in long term memory formation [80, 85]. Further, ERK activity is up-regulated in many experimental protocols that induce memory formation, an event that is blocked by specific inhibition of the ERK pathway [80, 82].

Cell death:

ERK exerts a protective effect against apoptosis in many cell types in the CNS [79, 86]. PC12 cells undergo apoptosis after withdrawal from NGF, a process than can be halted by constitutively activating the ERK cascade [79]. Activation of the ERK pathway in primary cultures of cerebellar granule neurons following the addition of the pituitary adenylate cyclase-activating polypeptides PACAP-27 and PACAP-38 has been shown to inhibit apoptosis [86]. Patterns of ERK activation also point to a neuroprotective role in the CNS. After seizure or ischemic damage in the hippocampus for example, ERK activity is markedly elevated [80, 87, 88].

Infection in the CNS:

The bacterial cell wall component LPS is a potent activator of the ERK cascade. Following ERK activation a number of cytokines are up-regulated as the tissue recruits inflammatory mediators to the site of infection. Interleukin 1beta, IL-6, TNF and many other cytokines have been shown to be ERK dependent [89-91]. The ERK cascades also influence viral entry. Reovirus, for example, has recently been shown to preferentially infect cells with an activated Ras pathway [92], although the mechanism whereby reovirus is facilitated is unknown. ERK also effects HIV pathogenesis as blocking ERK activity decreases HIV infectivity and upregulating ERK activity has been shown to increase HIV activity [93]. The mechanism behind ERK modulation of HIV infectivity is believed to be by phosphorylation of the HIV reverse transcription complex which is necessary for HIV to dissociate from the cell membrane and translocate to the nucleus [93].

Introduction to the chemokines:

Chemokines are small secreted proteins with molecular weights between 8 and 12 kD which serve a critical role as homing signals for immune cells. Chemokines are released in response to a wide variety of stimuli including growth factors, cytokines, bacterial or viral infection, trauma and many other insults. Although initially chemokines were thought to be expressed only in select areas in a manner restricted to immune cells, it now seems likely that almost every cell has the capacity for chemokine secretion [94]. This widespread nature

of chemokine synthesis correlates with its putative role as a signal of cellular stress or damage.

Different chemokines selectively attract different leukocytes to the site of interest. Monocyte chemoatractant-1 (MCP-1), for example, has chemotactic activity mainly for monocytes, and also perhaps plays a role in the recruitment and/or activation of T-lymphocytes, basophils, and natural killer (NK) cells [95]. Interleukin-8 (IL-8) on the other hand shows little or no ability to attract monocytes but serves as a potent stimulus for neutrophil migration [95].

The chemokine family is divided into four groups based on the location and pattern of four conserved cysteine residues. The CXC family, also termed the alpha chemokine family, is characterized by two conserved cysteine residues (nearest the N-terminal) separated by a single amino acid. The CC chemokines, also called beta chemokines, have the two nearest N-terminal cysteines side by side [96]. The CXC and the CC chemokines were the first to be discovered, and recently two other chemokine groups, the δ group (lymphotactin) and the γ group (neurotactin) have been added [97]. Among members of the CXC chemokines there appears to be a preference for neutrophil attraction while the CC group shows more specificity for monocytes. More recent evidence indicates, however, that much crossover between the various families exists [97].

Chemokines exert their physiological effects by binding to chemokine receptors. These receptors are seven transmembrane domain spanning G-protein coupled receptors [97]. Receptors for the CXC chemokines are termed simply CXC receptors (CXCR) and are

currently numbered CXCR1 though CXCR4. Receptors for the CC family are similarly designated CCR1-8. Although in some instances a particular chemokine is specific to a particular chemokine receptor, many chemokines bind multiple receptors and many receptors can bind multiple chemokines. This promiscuity of action is thought to play a part in the overlapping roles exhibited by many members of the chemokine family [97].

Monocyte chemmoatractant-1 (MCP-1)

One of the first chemokines to be cloned was MCP-1. It belongs to the CC family of chemokines and binds to the CCR2 and CCR4 chemokine receptors. Early studies described expression of this chemokine in monocytes/macrophages, endothelial cells and fibroblast although now it is presumed that most cell types have the capacity to secrete MCP-1 [94].

The signal transduction pathway leading to MCP-1 synthesis is unclear. The promoter contains binding sites for both NFκB and AP-1, and co-operative binding by both of these transcription factors is required for MCP-1 expression in some cell types [98]. As such, any stimuli that activate NFκB and/or AP-1 could potentially activate the transcription of MCP-1. Recently p38 MAP kinase has been implicated as an upstream regulator of MCP-1 as the specific p38 inhibitor SB203580 decreased MCP-1 mRNA levels in response to IL-1 in mesangial cells [99].

Secretion of MCP-1 results in monocyte activation and recruitment. When MCP-1 binds to its receptor on monocytes a cascade of events ensues which includes secretion of various

cytokines such as IL-1 and IL-6 and the activation of adhesion pathways [94]. That monocytes are activated by MCP-1 is also highlighted by studies showing that MCP-1 added to monocytes increases their ability to inhibit DNA synthesis in certain tumor models [94].

Upon binding by MCP-1 the CCR2 receptor likely engages either the Gi or Gq family of G-proteins, leading to increases in PLC and inhibition of cAMP generation. Other downstream effects include activation of PKC, PI3K, influx of extracellular calcium, and activation of MAP kinase family members [94]. Pertussis toxin (Gi inhibitor), P-I-3-kinase inhibitors or MAP kinase inhibitors can block monocyte recruitment in response to MCP-1 [94, 100].

MCP-1 in the CNS:

The increase in MCP-1 expression is an early step in the progressive inflammatory response in neuronal tissue [97, 101, 102] and is critical in promoting the invasion of inflammatory monocytes and leukocytes into the brain [103-105]. In the absence of damage or inflammation, MCP-1 mRNA is normally not found in the CNS.

Inflammation results in the accumulation of a number of toxic metabolic products and reactive oxygen species that can intensify cellular damage. In this regard, various observations support a central role for MCP-1 in CNS inflammation. For example, transgenic mice overexpressing glial-specific MCP-1 show pronounced monocyte and

macrophage infiltrate [106]. Also, intrahippocampal injection of various chemokines including MCP-1 results in monocyte recruitment to the site of injection. In a study by Bell et al. [107] MCP-1 was found to be the most potent stimulus of monocyte recruitment among a wide variety of tested chemokines. The importance of MCP-1 in inducing inflammation is also highlighted by MCP-1 receptor (CCR2) knockout studies, where such animals show striking deficits in monocyte recruitment in various inflammatory models [108, 109]. Finally, functional studies demonstrate that neutralization of MCP-1 using an antibody specific for MCP-1 attenuates monocyte chemotaxis in the CSF of meningitis patients [110] and migration of monocytes following HIV-1 Tat induction of MCP-1 in transmigration assays [111].

MCP-1 in disease:

MCP-1 is also thought to be involved in the progression of several diseases. For example, in multiple sclerosis MCP-1 is localised to astrocytes in scars and in surrounding tissue [112-114]. In an animal model of multiple sclerosis, experimental allergic encephalopathy, interruption of MCP-1 expression reduces disease progression [115]. There may be a role for astrocyte production of MCP-1 in HIV as well because HIV-1 Tat-stimulated astrocytes produce MCP-1 and MCP-1 is elevated in the CSF of patients with AIDS dementia [103, 116]. In addition, astrocytomas and glioblastomas express MCP-1 and soluble MCP-1 antibodies have been demonstrated to block monocyte recruitment induced by these tumours [97]. That chemokines can also modulate

neovascularization and tumour growth [97] in other systems speaks to the relevance of MCP-1 in CNS neoplastic disease.

MCP-1 in Ischemia and Trauma:

Trauma to the CNS triggers a cascade of reactions leading ultimately to the generation of inflammatory infiltrate. Importantly, MCP-1 is the predominant chemokine up-regulated following CNS trauma. In addition, following ischemia there is also a rapid expression of MCP-1 mRNA as detected by RT-PCR or in situ hybridization [117-119]. This increase precedes and is thought to promote the invasion of monocytes and inflammation in ischemic tissue [120]. Notably, in both ischemia and trauma the astrocytes have been shown to be the principal cell expressing MCP-1 following the insult [121-123].

Astrocytes release MCP-1

Astrocytes are thought to be the predominant source of MCP-1 following a diverse array of CNS insults [122, 123]. Stab injury induces local MCP-1 expression within three hours after injury, which precedes the inflammatory infiltrate, thus suggesting a parenchymal source of MCP-1 production [101]. In these same experiments other common chemokines were undetectable at this early time point [101]. In situ hybridization studies implicate astrocytes as the earliest and most predominant source of MCP-1 following implantation injury, nitrocellulose stab injury [122], and other insults [123]. Interestingly, reactive astrogliosis may be intimately tied to MCP-1 expression as the level of MCP-1

production seems to correlate with the degree of astrogliosis following trauma [101] and MCP-1 co-localizes with increases in GFAP staining (indicator of astrogliosis) in some inflammatory models [124]. Although a wealth of evidence exists supporting the central role of the astrocyte in MCP-1 expression and recruitment of hematogenous cells to the site of injury, the signals that initiates MCP-1 expression in astrocytes are largely unknown.

Hypothesis and Objectives:

Hypothesis:

We hypothesize that the P2X7 purinergic receptor is present on astrocytes. Activation of this receptor, as might occur in trauma and inflammation due to high extracellular ATP, may activate a number of intracellular kinase pathways that may include the MAP kinase family of proteins. We also propose that P2X7 activation may act to communicate with the immune system by leading to the production of chemokine synthesis in astrocytes.

Specific questions:

1. Do astrocytes express the P2X7 receptor?

Astrocyte cultures were used as a convenient model system in this study. Immunocytochemistry using P2X7 receptor antibody in conjunction with an antibody to GFAP (astrocyte specific marker) was employed to answer this question.

2. Can activation of the P2X7 receptor lead to ERK1, ERK2, and p38 activation?

To determine if these kinases were activated in response to P2X7 receptor activation we employed Western Blotting with phospho-specific antibodies. These antibodies recognise only the active form of the kinases, and as such levels seen on Western Blots are indicative of total cellular activity.

In order to more accurately determine the activation of the ERKs an *in vitro* ERK activity assay was also performed. Many different purinergic agonists and antagonists were measured for their ability to activate the ERKs in order to establish that the P2X7 receptor was the most likely receptor responsible for any observed increase in ERK activity.

3. Do astrocytes in culture produce MCP-1?

Immunohistochemistry with anti-MCP-1 antibody was employed on cultured astrocytes grown on coverslips to answer this question.

4. Does the activation of the P2X7 receptor lead to increased expression of MCP-1 mRNA?

RT-PCR was employed to investigate a possible link between P2X7 receptor activation and MCP-1 m-RNA expression. Through the use of a number of pharmacological agonists and antagonists, we hoped to delineate which purinergic receptor was responsible for an increase in MCP-1.

5. Are the MAP kinases involved in the link between the P2X7 receptor and MCP-1 expression?

The use of specific inhibitors of the ERK and p38 cascades was used to address this issue. Following the application of the inhibitors, cultures were measured for differences in MCP-1 expression relative to controls.

MATERIALS AND METHODS:

Cell culture

Astrocyte cultures were prepared from 1 day-old Sprague Dawley rats (University of Calgary) using modifications of standard techniques [125, 126]. All procedures conformed to guidelines laid down by the Canadian Council on Animal Care. Briefly, neonates were decapitated and the brain exposed. The cortex was dissected free of the underlying brain tissue under sterile conditions and the meninges and pia mater were removed. The tissue was dissociated by mechanical trituration, the resulting cell suspension plated onto glass coverslips and grown in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (1:1) with 10% fetal calf serum. Media was changed twice per week. As previously described [127], immunocytochemical characterisation of cultures indicated that > 95% of cells stained positive for glial fibrillary acidic protein, confirming that the primary cells present are astrocytes (Data not shown).

Immunocytochemistry:

Please note that immunocytochemistry figures were by/with the help of Dr. J. Armstrong in the laboratory of Dr. MacVicar. Astrocyte cultures were grown on poly-ornithine coated glass coverslips and were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (4° C; 15 minutes), rinsed in Phosphate buffered saline, and pre-incubated for 1 hour in phosphate buffered saline (PBS) containing 5% normal donkey serum. Coverslips were incubated overnight (4° C) in PBS containing 0.5% BSA, rabbit anti-MCP1 (1:3,000; Serotech) or rabbit anti-P2X7 (1:3,000; Alamone Labs) and mouse anti-GFAP

(1:5,000; Pharmigen), rinsed and incubated overnight (4° C) with Cy^2-conjugated donkey anti-mouse IgG and Cy^3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories 1:1,000). Coverslips were mounted with FluorSave (Calbiochem) and imaged on a LSM510 attached to a Axioplan 2 upright microscope (Carl Zeiss). Photographs of cells were enhanced using Photoshop 5.

Culture stimulation:

Astrocyte cultures were plated in equal numbers into 6 well plates and allowed to grow to confluency. Once confluent the medium was switched to DMEM with 0.5% FCS for 48-72 hrs to reduce the background MCP-1 expression and MAP kinase activation. Following the period of starvation the test compounds were added to the cultures. In all cases, vehicle control (either 1/1000 DMSO or H_2O) was added to matched cultures in the same 6 well plate for an equivalent time period. Following stimulation the media was removed and analyzed (Elisa) and/or the cells processed for further study.

Western immunoblotting

Confluent astrocyte cultures were starved in 0.5% FCS DMEM for 48-72 hours. Following stimulation cultures were washed with ice-cold PBS and harvested on ice with cold lysis buffer containing 50mM β-glycerophosphate, 1mM EGTA, 2mM $MgCl_2$, 0.5% Triton-X 100, 100uM Sodium Orthovanadate ($NaVO_4$), 1mM DTT, and 2ug/mL each of aprotinin and leupeptin. The supernatant was removed and protein concentration determined by means of the Bradford DC microtitre plate assay. The samples were then

boiled (5 min) and subjected to SDS/Page [128] on 15% w/v acrylamide gels. The protein was then transferred to PVDF and blocked in TTBS (0.05% Tween 20; 25mM Tris-Base; 62.5mM NaCl - solution, pH 7.5) containing 5% w/v skim milk, then incubated in primary antibody. For MAP kinase blots, anti-rat MAP Kinase R2 (Erk-1-CT; 1/2000 dilution) or anti-phosphospecific MAP kinase (1/1000) was used. For p38 blots we used the anti-phospho p38 (1/1000) and anti-p38 antibodies (1/1000). The secondary antibody employed was anti-rabbit Ig, horseradish peroxidase linked (H+L) whole antibody (1/3000). Finally, membranes were washed and probed using the Supersignal Substrate Western Blotting ECL detection kit.

ERK activity assays

This assay measures phosphotransfer by the ERKs onto a substrate peptide corresponding to residues 663-673 of the EGF receptor. This EGFR peptide assay offers a selectivity advantage over the classic Myelin Basic Protein (MBP) substrate assay. After protein determination [129], 20uL supernatant samples were mixed with a 20uL of reaction buffer consisting of 50mM β-Glycerophosphate, 100uM $NaVO_4$, 20mM $MgCl_2$, 200uM ATP, 10ug/mL PKI (PKA inhibitor), 1mM EGTA, 1ug EGFR peptide and 1 uCi [γ-^{32}P] ATP. This mixture was incubated at 32°C for 15 minutes and stopped with the addition of 10uL of 25%(w/v) TCA. 40 uL of this mixture was then spotted onto p81 filter paper and washed 4 times in 75mM phosphoric acid followed by once in acetone. After drying samples were counted on a beta-counter.

RNA extraction and RT-PCR

Please note that PCR for data in this thesis were actually performed by the laboratory of Dr. R. Ransohoff. Culture stimulation and Trizol preparation was done by W. Panenka in Dr. MacVicar's laboratory. Samples were dissolved in Trizol, total RNA extracted and RT-PCR performed as described by McTigue et al. (1998) [130]. Briefly samples were ethanol precipitated, the pellets dissolved in 100µl RNase-free H_2O and RNA concentration determined spectrophotometrically. RT-PCR dot-blot hybridisation analysis was then performed. PCR products were denatured and transferred to nylon membranes and hybridised with nick-translated cDNA inserts. Hybridisation signals were quantified by Phosphoimager (Molecular Dynamics) using a blinded protocol.

Reverse Trancription

1 µg of RNA was treated with DNAase according to the manufacturer's instructions (GIBCO- BRL, Gaithersburg, MD). First strand cDNA was synthesized using 1 mg of DNAase treated RNA, oligo dT primers, and SuperScript IITM. Amplified PCR products of α-tubulin transcripts were analyzed on ethidium bromide-stained agarose gels to confirm the presence of intact RNA in all samples and verify that, in each sample, the cDNA synthesis reaction generated products capable of being amplified in the PCR.

Optimization of PCR conditions and generation of standard curves

A fragment of the rat MCP-1 transcript (~400 bp) was amplified in RT-PCR reactions using gene specific primers (5*CCTGTTGTTCACAGTTGCTGCC3* and 3*TCTACAGAACTGCTTGACGGTGGTTG5*). The product of this reaction was purified (PCR purification kit, Qiagen, Valencia, CA), and the concentration of the amplified fragment was quantitated by spectrophotometry. Five serial ten-fold dilutions of this fragment (from 2 pg/ml to 0.2 fg/ml) were prepared, amplified by PCR and labeled with SYBR Green (Roche, Indianapolis, IN), which yields a bright fluorescence on binding to double-stranded nucleic acids; this fluorescence abruptly diminishes upon denaturation of DNA strands during melting-curve analysis. PCR and analysis to generate standard curves were performed in 20 µl reactions in glass capillaries, using a LightCycler (Roche), and LightCycler3 software, according to the manufacturer's instructions. For each reaction, melting-curve analysis was used to detect the synthesis of non-specific products. Negative controls (omitting input cDNA) were also used in each PCR run, to confirm the specificity of PCR products. To optimize PCR conditions, standard-curve reactions were performed at varying annealing temperatures, Mg^{2+} concentrations, with or without FastStartTM (Roche). At optimal conditions for PCR, standard curves were linear across serial ten-fold dilutions and the melting curve analysis indicated synthesis of a single homogeneous product of expected melting temperature.

PCR and real-time analysis

Standard curves were generated with each set of samples. The PCR reaction in 20 µl contained 2 mM Mg^{2+}, 0.25 µM each of forward and reverse primer (identical with those used to generate the template for standard curves), 1x FastStart DNA Master SYBR Green I (Roche) containing Taq DNA polymcrasc and 2 µl cDNA synthesis reaction product. Reaction conditions for PCR were: denaturation at 95°C for 7 min; 40 cycles of amplification by denaturing at 95°C for 15 sec, annealing at 60°C for 5 sec, extending at 72°C for 15 sec. The accumulation of products was monitored by SYBR Green fluorescence at completion of each cycle. Analysis was performed on LightCycler3 software and results are expressed as the crossing point at which accumulation of PCR products became exponential. Using the standard curve, this value was converted to pg/ml. Reaction conditions for melting curve analysis were: denaturation to 95°C at 20°C/scc without plateau phase, annealing at 65°C for 15 sec, denaturation to 95°C at 0.1°C/sec, with continuous monitoring of SYBR Green fluorescence

Statistical analysis

Raw data in the form of scintillation counts for the MAP kinase assays or Phosphoimager Densitometry units for PCR were subjected to the Freidman two-way analysis of variance by ranks. When an F value was less than 0.05, a comparison against the control or Bz-ATP stimulated groups was done using a one-tailed Wilcoxon sign rank test. In all cases a p value of 0.05 or less was considered significant and is indicated on the appropriate

figures with a star. Error bars in all figures represent standard error. All experiments represent a minimum N=4 and also represent data from at least two separate animals.

Materials

All culture reagents were purchased from Gibco BRL (Burlington, Ont.). PD 98059 and SB 203580 were purchased from Calbiochem (La Jolla, CA). Antibody sources were as follows: Phospho-specific p38, phosphospecific ERK1/2 and anti-p38 were from New England Biolabs (Mississauga, Ontario). Secondary antibody was obtained from Amersham (Oakville, Ontario, anti-rabbit IgG). BioRad-DC protein assay reagents were also obtained from Bio-Rad labs (Mississauga, Ontario). All other drugs were purchased from SIGMA (Oakville, ONT).

RESULTS

The P2X7 receptor is present in astrocyte cultures

Electrophysiological studies suggest that the P2X7 receptor is present in astrocytes [131]. Immunostaining with anti-P2X7 receptor antibody demonstrated that all of the GFAP positive astrocytes were immunopositive for P2X7 receptors (figure 1 a,b,c,d,e,f). Punctate P2X7 receptor-immunoreactivity was observed over the entire cellular membrane of GFAP-immunoreactive astrocytes. It can also be noted that in various parts of Figure 1 slight punctate P2X7 receptor immunoreactivity is evident in the absence of GFAP staining. This could possibly indicate astrocytic processes that do not stain effectively with our GFAP stain or perhaps non-specific background staining with the P2X7 antibody. Western blots using P2X7 antibody and immunostaining using only the secondary antibody confirmed the specificity of the P2X7 antibody (data not shown).

Purinergic receptors activate ERK1/ERK2 and p38

Little is known about the signalling pathways downstream of P2X7-receptor activation. In particular, the activation of the ERKs and p38 in response to P2X7 receptor stimulation in astrocytes has not been demonstrated. We used immunoblotting with phosphospecific antibodies to ERK1, ERK2 and p38 and found that all three MAP kinases were activated in cultures treated with the purinergic agonist Bz-ATP (fig 2a, b). Bz-ATP can potently activate the P2X1 receptor, the P2X2 receptor and the P2X7 receptor. Application of Bz-ATP caused a rapid increase in the amount of phosphorylated ERK1, ERK2 and p38 within 5 to 15 min. In figure 2a the bottom band indicates phosphorylated ERK2 (p-

ERK2) running at a molecular weight of approximately 42 kD. The upper band in figure 2a indicates phosphorylated ERK1 at a molecular weight of approximately 44 kD. In order to confirm that this was indeed activation of the ERKs, phosphotyrosine immunoblotting and gel shift Western blots were also employed (data not shown). The increase was reversible within 3 hr. These results were confirmed and extended using an ERK *in vitro* kinase assay [132]. A 3-fold increase in ERK activity was noted 15 minutes following Bz-ATP application (fig 2 c). Bz-ATP application also produced a significant, dose dependent increase in ERK activity (fig 2 d). Atlhough many studies use concentrations of Bz-ATP in the mM range and claim specificity [133-135] it was felt that a slightly lower concentration would be prudent. From the dose-response data, a concentration of 100uM Bz-ATP was thus chosen for further experiments.

The classification of the purinergic receptor subtype involves the analysis of the effectiveness of several agonists and antagonists [10, 136, 137]. We therefore compared a number of purinergic receptor agonists with respect to their ability to activate ERK1/ERK2 (fig 3a). ATP strongly activates a variety of purinergic receptors, many of which have been linked to the ERKs [138, 139]. The P2Y class of purinergic receptors, for example, is known to lead to ERK activation and this class of purinergic receptor also has been localized to astroctyes [138]. It is possible, therefore, that the equivalent effectiveness of Bz-ATP and ATP represents activation of other ERK-linked pathways other than the P2X7 receptor. Application of ADP produced a smaller effect on ERK activation than ATP, in concordance with its significantly lower effectiveness at

purinergic receptors known to activate the ERKs [24, 138, 139]. AMP and Adenosine are agonists for various other P2 receptor subtypes but do not stimulate the P2X7 receptor [136, 140] and did not stimulate the ERKs. ATPγS is a non-hydrolyzable ATP analogue that weakly activates the P2X7 receptor in addition to its agonist effects on the P2X4 and P2X2 purinoceptor subtypes [24, 36]. An increase in ERK activity was elicited with ATPγS, although this increase was smaller than the increase produced by application of Bz-ATP. Given the specificity of ATPγS it is unknown whether the observed increased in ERK activity reflects P2X7 activation, or activation of other purinoceptors linked to the ERKs. α-β-methyl ATP is predominantly a P2X1-receptor agonist (EC50 1uM). 2-methyl-thio-ATP (2-MS-ATP) is a potent P2X3-receptor agonist (EC 50 0.3uM), P2X2 agonist (EC 50 3uM), and P2X1 agonist (EC 50 1uM) [24, 25]. Both failed to have any significant effect on ERK activity (Fig 3a). These results demonstrate that the observed increase in ERK activity in response to Bz-ATP is likely a P2X7 receptor mediated event.

As the agonist profile implicated the P2X7 receptor we sought to confirm these findings by testing a variety of purinergic receptor antagonists. We thus tested a number of purinergic receptor antagonists for their ability to block the Bz-ATP mediated increase in ERK activity (figure 3b). Bz-ATP mediated ERK activation was significantly blocked by DIDS. DIDS is an ion channel blocker that has been shown to inhibit P2X7 mediated events [141]. The general P2X-receptor antagonist, PPADS [142] and the non-selective P2X/P2Y-receptor antagonist, suramin [143] also blocked the Bz-ATP activation of ERK. Application of o-ATP, a specific and irreversible P2X7-receptor antagonist [144],

also significantly blocked Bz-ATP activation of the ERKs to an extent equivalent to the more general purinergic antagonists. Because there was no significant difference between the ERK attenuation induced by the general purinergic antagonists as compared to the block seen when only the P2X7 receptor was selectively antagonized (by o-ATP), we can conclude that the Bz-ATP effect is dependent on activation of the P2X7 receptors. Taking into account the results of the purinergic agonists, the data form a l profile consistent to that found in other descriptions of P2X7 mediated events [15, 131].

Fig. 1. The P2X7 receptor is present in rat cortical astrocyte cultures.

Astrocytes were confirmed to be the predominant cell type (>95%) in our cultures as indicated by positive staining for the astrocyte specific marker Glial Fibrillary Acid Protein (GFAP) (green) as seen in panel **a** and **c**. The P2X7 receptor is expressed in the cultured astrocytes as evidenced by positive immunostaining with P2X7 receptor antibody (red) seen in **b** and **e.** Colocalisation of GFAP and P2X7 is indicated by double staining in panel **c** and **f**. Note that immunocytochemistry indicated that all GFAP positive cells also expressed the P2X7 receptor. Scale bar = 20 μm.

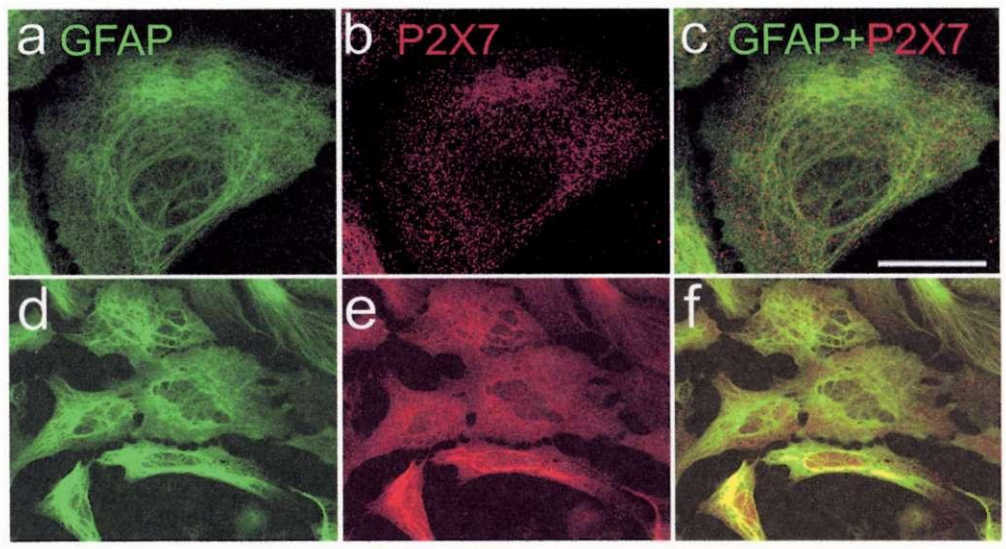

a GFAP
b P2X7
c GFAP+P2X7
d
e
f

Fig. 2. Bz-ATP, a P2X7 receptor agonist, activates the MAP kinases ERK1/ERK2 and p38. In panels **a, b** and **c** rat cortical astrocytes were treated with 100 μM of the purinergic agonist Benzoyl-benzoyl ATP (Bz-ATP) for the time periods indicated. **(a)** Western Blotting with Phospho-specific ERK antibody indicated an increase in the active, phosphorylated form of the ERK proteins **(b).** Phospho-specific p38 antibody indicated an increase in the active, phosphorylated form of p38 following 100 μM Bz-ATP application. Parallel blotting with p38 antibody indicated equal protein loading between lanes **(b).** **(c)** ERK1/ERK2 activity assay confirmed the increased activity of the ERKs in response to Bz-ATP. **(d)** The dose-response curve of Bz-ATP induced ERK1/ERK2 activity demonstrates that activity increased markedly between 10 and 100 μM and continued to increase up to 1,000 μM, the highest concentration tested. Note that time of application for the various concentrations was held constant at 15 minutes. The stars indicate a significant increase from the control group (p value = 0.033 for all conditions indicated with a star, N=4 for all conditions).

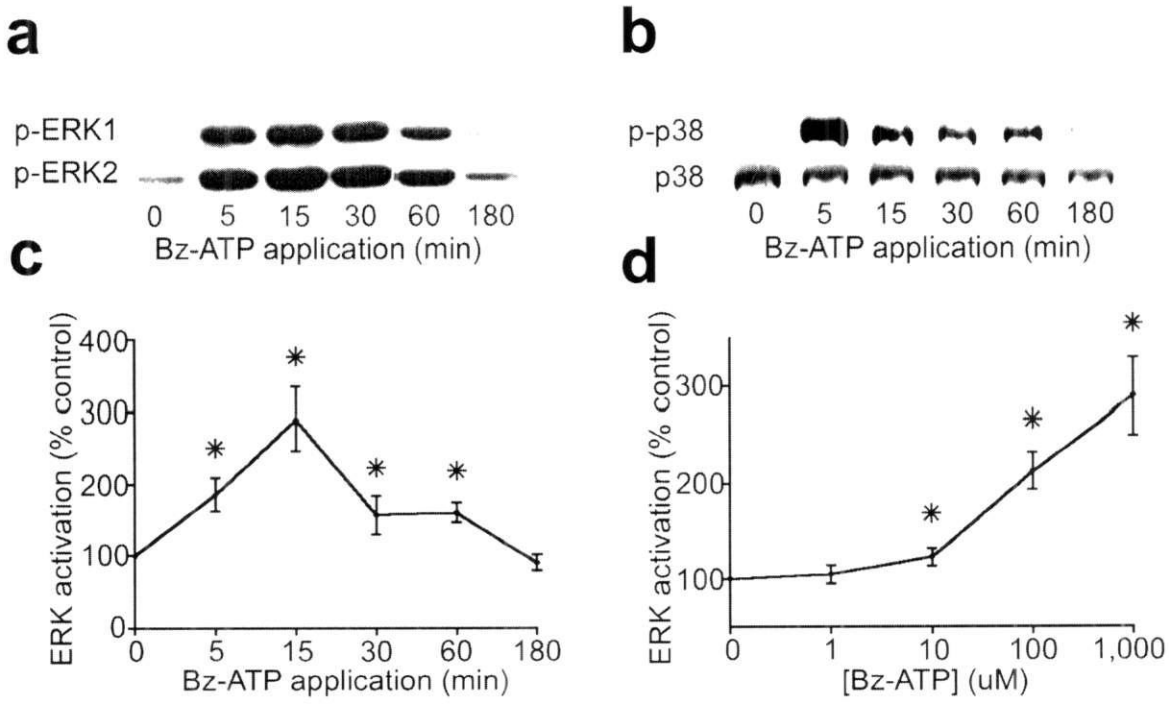
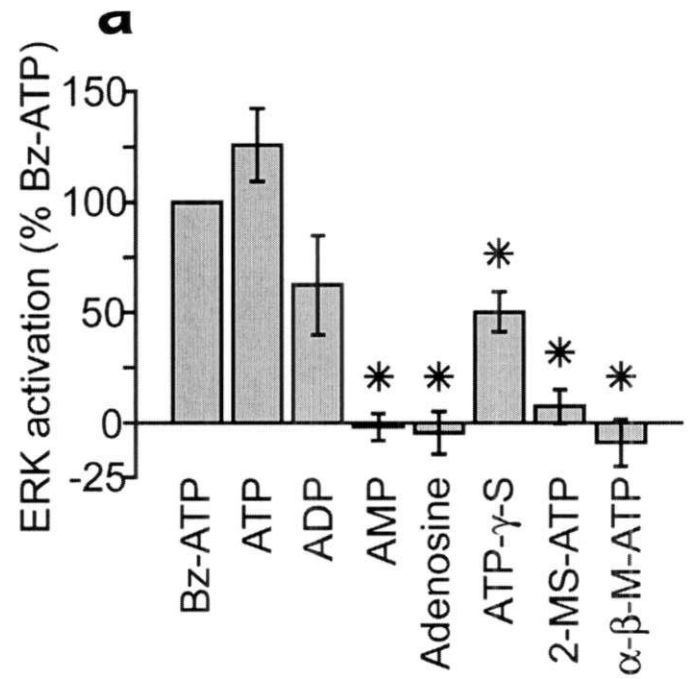
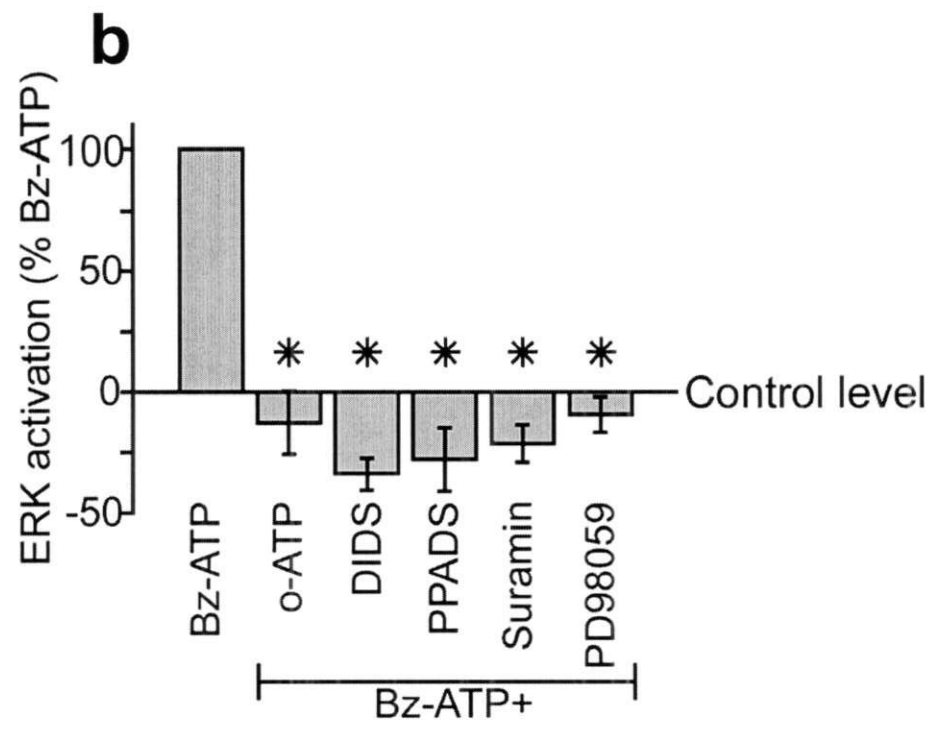

a
p-ERK1
p-ERK2
0 5 15 30 60 180
Bz-ATP application (min)
b
p-p38
p38
0 5 15 30 60 180
Bz-ATP application (min)
c
ERK activation (% control)
400
300
200
100
0
0 5 15 30 60 180
Bz-ATP application (min)
d
ERK activation (% control)
300
200
100
0 1 10 100 1,000
[Bz-ATP] (uM)

Fig 3. ERK activity assays using purinergic receptor agonists and antagonists confirm that the P2X7 receptor activates the ERKs. **(a)** Purinergic agonists demonstrated a profile consistent with P2X7 receptor induced ERK activation. Abbreviations: ATP-γ-S, Adenosine 5'-O-[3-thiotriphosphate] : 2MS-ATP, 2-methylthio adenosine 5' triphosphate : α-β-M-ATP, α-β-methylene adenosine 5' triphosphate. The control level is the level of MAP kinase activation in untreated cultures and was defined as 0%. The level of ERK activation documented with the selective P2X7 receptor agonist Bz-ATP was defined as 100%. All compounds were applied for 15 minutes at a concentration of 100 μM. **(b)** Purinergic receptor antagonists and the ERK antagonist PD98059 diminished ERK activation in response to P2X7 receptor stimulation. All cultures were subjected to 15 minutes of 100 μM Bz-ATP stimulation in the presence or absence of various signaling inhibitors. Concentrations and pre-incubation times of inhibitors were as follows: PD98059, 50 μM, 15 min pre-incubation (PI) : o-ATP, 300 μM, 2 hours PI : DIDS, 200 μM, 2 hour PI : PPADS, 100 μM, 15 min PI : Suramin, 1 mM, 15min PI. Abbreviations: o-ATP, oxidized ATP : PPADS, Pyridoxal–phophate-6-azophenyl-2,4-disulphonic acid : DIDS, 4'4' Diisothio-cyanato-stilbene-2,2'-disulfonic Acid. Stars indicate a significance decrease from the Bz-ATP sample group. P values were found to be 0.033 for all conditions indicated with a star (N=4) except PPADS (p=0.0059, N=7), and o-ATP (p=0.0056, N=7).


a
ERK activation (% Bz-ATP)
150
100
50
0
-25
Bz-ATP
ATP
ADP
AMP
Adenosine
ATP-γ-S
2-MS-ATP
α-β-M-ATP


b
ERK activation (% Bz-ATP)
100
50
0
-50
Control level
Bz-ATP
o-ATP
DIDS
PPADS
Suramin
PD98059
Bz-ATP+

Astrocytes are the source of MCP-1 production

Evidence indicating that MCP-1 production can originate from astrocytes is well documented. However, given that MCP-1 can also be produced by neurons and microglia, it was of central importance in this study to ascertain that the source of MCP-1 production was astrocytic. To this end, we initially measured the purity of our astrocyte cultures using immunohistochemistry with the astrocyte specific marker GFAP and determined that at least 95% of the cells present in culture are astrocytic in origin (results not shown). We then sought to locate MCP-1 within these astrocytes through simultaneous immunohistochemical staining for GFAP and MCP-1. Figure 4 demonstrates co-localization of anti-MCP-1 antibody (**b**) and anti-GFAP antibody (**a**). Further, all cultured cells that expressed GFAP also expressed MCP-1 (results not shown). This furthers previous studies, both *in vitro* and *in vivo*, implicating astrocytes as a predominant source of MCP-1 production in the CNS.

Purinergic receptors induce MCP-1 expression:

The kinetics of MCP-1 induction by Bz-ATP are depicted in figures 5a and 5b. Dose-response curves of Bz-ATP induced MCP-1 production are similar to those seen for Bz-ATP induced ERK activity (Fig 2c) in that MCP-1 mRNA production is evident at 10uM and is maximal at 1000uM, the highest concentration tested (fig 5b). These concentration values are also in accordance with other dose response studies of P2X7 receptor mediated cytokine production [133]. Maximal MCP-1 mRNA expression

occurred between 1 and 2 hours following exposure to Bz-ATP (fig 5a). The temporal kinetics of MCP-1 production in response to Bz-ATP correlate with other investigations of MCP-1 production in cultured astrocytes [116].

In order to establish that the MCP-1 expression was a P2X7 receptor mediated event, we employed the same strategy and compounds used in forming the link between P2X7 and ERK. Bz-ATP is the most effective inducer of MCP-1 production, followed by ATP, ADP, and little or no effect with AMP or adenosine. The other P2X receptor agonists also produced a profile consistent with a P2X7 receptor mediated effect in that ATPγS induced an intermediate response and α-β-M-ATP and 2-MS-ATP showed little efficacy in inducing MCP-1 mRNA production. Figure 6b demonstrates that P2X7 induced MCP-1 production is sensitive to purinergic receptor antagonists. Rank effectiveness of inhibition was suramin>PPADS>DIDS (fig 6b). These agonist and antagonist profiles identify the P2X7 receptor as likely being responsible for the increase in MCP-1.

Unlike the ERK activation profile, ATP produced a significantly lesser increase in MCP-1 production relative to Bz-ATP. It is possible, therefore, that other pathways activated by ATP (such as P2Y mediated ERK activation) do not also activate MCP-1.

Unfortunately, the most specific inhibitor of the P2X7 receptor, o-ATP, was of little use in ascertaining P2X7 receptor identity. As can be seen in figure 7(d) o-ATP only mildly attenuated MCP-1 production. The ineffectiveness of o-ATP inhibition of MCP-1

synthesis likely stems from the ability of o-ATP to independently increase expression of MCP-1 (figure 7c). This effect may be mediated through the p38 pathway as o-ATP lead to phosphorylation of p38 in a time and dose dependent manner (figure 7a,b). Further, application of both the p38 inhibitor SB203580 and o-ATP resulted in a marked decrease of MCP-1 production in response to Bz-ATP (figure 7,d).

Of note in Figure 7 is the large increase in MCP-1 production (10 fold) relative to Bz-ATP induced stimulation from previous figures (maximum approximately four fold – figure 5). This discrepancy should not be taken to indicate that o-ATP is a more effective inducer of MCP-1 production than Bz-ATP. Rather, as the o-ATP group of samples was run separately by PCR and the PCR is semi-quantitative (no internal GAPDH control) it can only be concluded that o-ATP significantly increased MCP-1 production relative to control. A direct comparison of Bz-ATP vs. o-ATP would need to be performed and run in the same PCR run in order to ascertain the differences in effectiveness of these two compounds.

Interestingly, Hide et al. have very recently demonstrated a similar finding in astroglial cells where it was shown that o-ATP increases expression of TNF-α mRNA [43].

The MAP kinases ERK1/ERK2 and p38 are involved in P2X7-MCP-1 signaling

Recently p38 has been implicated in MCP-1 expression following a number of stimuli [145, 146]. These same studies have failed to show a link to the ERK cascade. In our

hands blocking the ERK1/ERK2 pathway with the selective inhibitor PD98059 depressed Bz-ATP-induced MCP-1 production in the astrocyte cultures (fig 6c). SB20358, a selective inhibitor of the p38 MAP kinase cascade, also inhibited MCP-1 production (fig 6c). Greatest inhibition of MCP-1 occurred when both the ERK and p38 inhibitors were applied together (fig 6c). These results indicate that both ERK and p38 may mediate MCP-1 expression.

Fig 4. Rat cortical astrocyte cultures express the chemokine Monocyte Chemoattractant protein–1 (MCP-1). Panel **a** illustrates immunostaining for the astrocyte specific marker Glial Fibrillary Acidic Protein (GFAP). Cultured astrocytes also expressed MCP-1 as seen by immunostaining with MCP-1 antibody **(b)**. Scale bar = 20 μm. Note that all cultured cells staining positive for GFAP also stained positive for MCP-1 (results not shown).

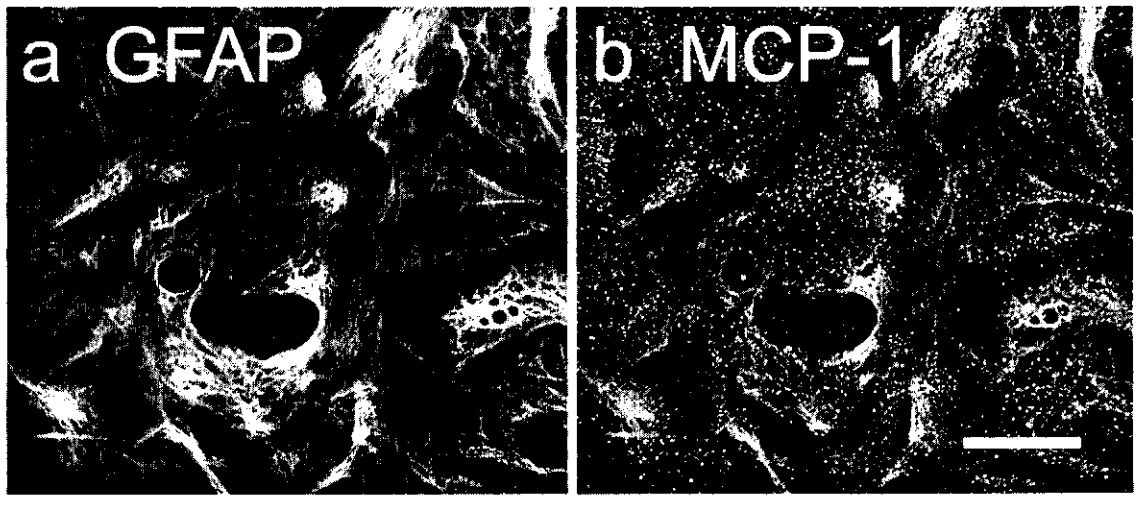
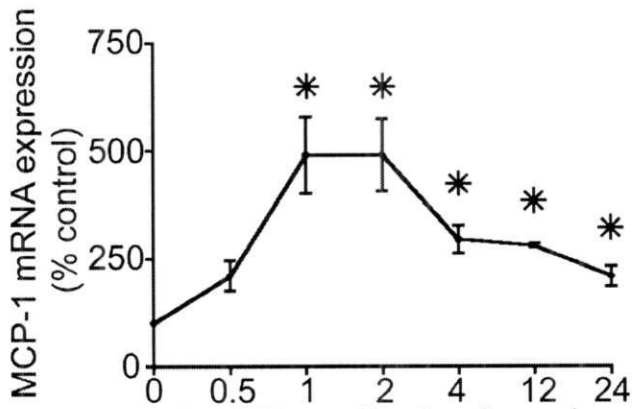

a GFAP
b MCP-1

Fig. 5. Bz-ATP, a P2X7 receptor agonist, induces expression of the chemokine MCP-1. **(a)** MCP-1 expression was up-regulated by P2X7 receptor stimulation. Cultured astrocytes were treated with 100 μM of the P2X7 receptor specific agonist Benzoyl-benzoyl ATP (Bz-ATP) for the time periods indicated followed by RT-PCR analysis for MCP-1 mRNA. **(b)** The dose response curve of Bz-ATP induced MCP-1 upregulation demonstrated that MCP-1 expression increased markedly between 10 and 100 μM Bz-ATP with a lesser increase up to 1,000 μM, the highest concentration tested. Note that time of application for the various concentrations was held constant at 2 hours. Significance increase relative to control is indicated with a star. P value = 0.03 for all conditions where a significant increase was noted, N=4 for all conditions.

a


MCP-1 mRNA expression
(% control)
750
500
250
0
0
0.5
1
2
4
12
24
Bz-ATP application (hours)

b

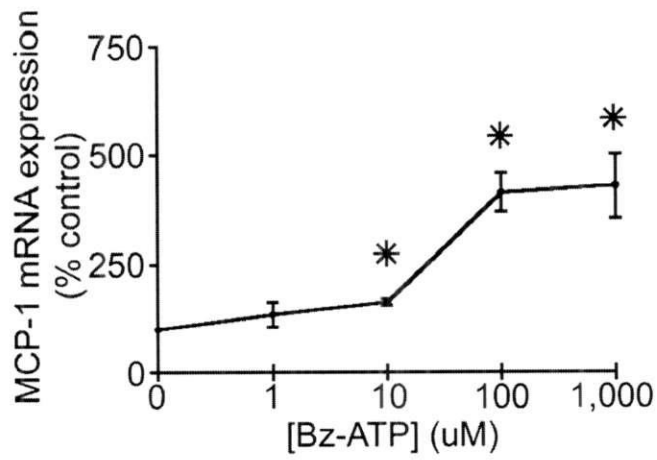

MCP-1 mRNA expression
(% control)
750
500
250
0
0
1
10
100
1,000
[Bz-ATP] (uM)

Fig 6. MCP-1 expression is induced by P2X7 receptor activation and is blocked by ERK and p38 inhibition. **(a)** Purinergic agonists demonstrated a profile consistent with P2X7 receptor induced MCP-1 expression (ATP-γ-S, Adenosine 5'-O-[3-thiotriphosphate] : 2MS-ATP, 2-methylthio adenosine 5' triphosphate : α-β-M-ATP, α-β-methylene adenosine 5' triphosphate). The control level was the level of MCP-1 expression in untreated cultures and was defined as 0%. The level of expression documented with the selective P2X7 agonist Bz-ATP was defined as 100%. All compounds were applied at 100 μM for 2 hours **(b)** Purinergic receptor antagonists revealed a profile consistent with P2X7 receptor involvement in MCP-1 expression. 100 μM Bz-ATP was applied to the cultures in the presence or absence of various signaling inhibitors (o-ATP, oxidized ATP : PPADS, Pyridoxal–phophate-6-azophenyl-2,4-disulphonic acid : DIDS, 4'4'Diisothiocyanatostilbene-2,2'-disulfonic Acid). Concentrations and pre-incubation times of inhibitors are as in figure 2. **(c)** The MAP kinases ERK1/ERK2 and p38 were involved in P2X7 induced MCP-1 expression. The specific ERK inhibitor PD98059 (50 μM) and/or the specific p38 inhibitor SB20358 (25 μM) reduced P2X7 receptor induced MCP-1 signaling **(c).** Significance decrease relative to the Bz-ATP sample group is indicated. P values for all conditions showing a significant decrease were 0.033 (and N=4) except PPADS (p=0.022, N=5), DIDS (p=0.022, N=5), PD98059 (p=0.004, N=9), and SB203580 (p=0.044, N=10).

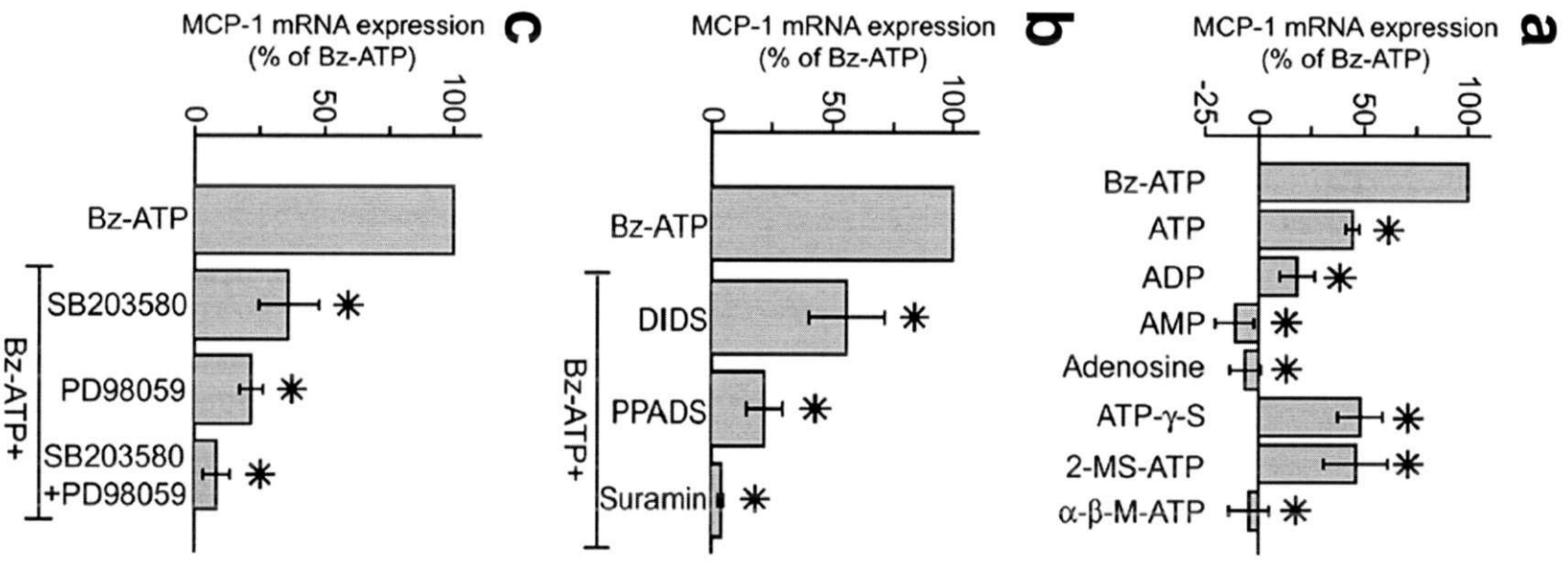
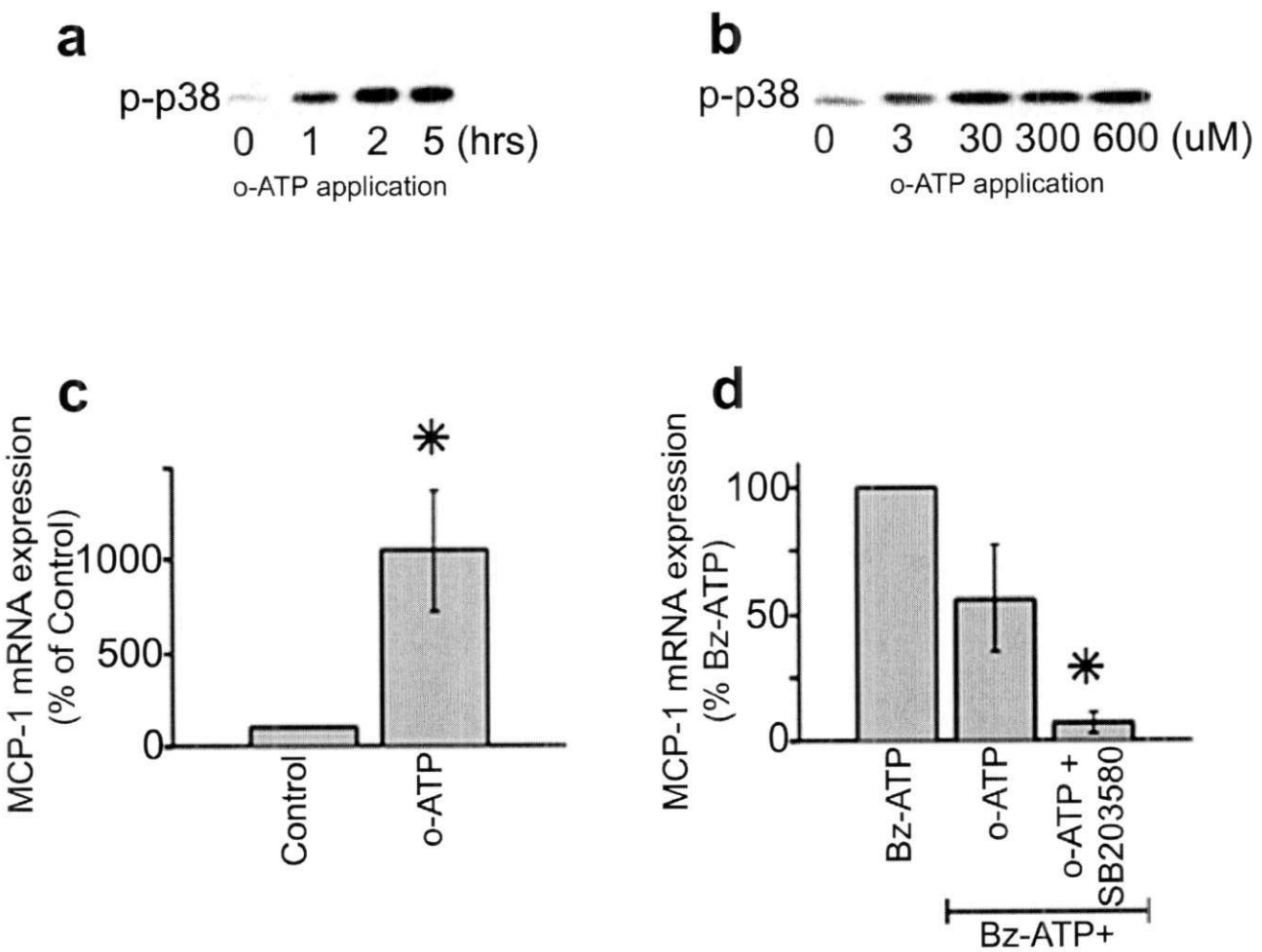

a
MCP-1 mRNA expression
(% of Bz-ATP)
-25
0
50
100
Bz-ATP
ATP
ADP
AMP
Adenosine
ATP-γ-S
2-MS-ATP
α-β-M-ATP
b
MCP-1 mRNA expression
(% of Bz-ATP)
0
50
100
Bz-ATP
Bz-ATP+
DIDS
PPADS
Suramin
c
MCP-1 mRNA expression
(% of Bz-ATP)
0
50
100
Bz-ATP
Bz-ATP+
SB203580
PD98059
SB203580
+PD98059

Fig. 7. o-ATP, a specific P2X7 receptor antagonist, independently activates p38 and increases expression of MCP-1. In panel **a** rat cortical astrocytes were treated with 300 μM of the P2X7 receptor specific antagonist oxidized-ATP (o-ATP) for the time periods indicated. In panel **b** parallel cultures were subjected to varying concentrations of o-ATP for a period of 3 hours. SDS/Page followed by Western Blotting with phospho-specific p38 antibody indicated an increase in phosphorylated (activated) p38 that correlated with time of exposure to o-ATP **(a)** and with increasing concentrations of o-ATP **(b).** In panel **c** astrocyte cultures were treated with 300 μM o-ATP for 5 hours and MCP-1 mRNA measured. As can be seen, o-ATP increased the production of MCP-1. **(d)** Astrocyte cultures were treated with the P2X7 agonist Bz-ATP (100 μM-2 hours) in addition to the P2X7 antagonist o-ATP (300 μM – 5 hours) +/- the p38 inhibitor SB203580 (25 μM – 2 hrs, 15 min). As can be seen, o-ATP mildly attenuates Bz-ATP induced MCP-1 production while further inhibiting the p38 pathway with SB203580 results in marked depression of MCP-1 mRNA synthesis. Significance is indicated with a star and p values are as follows: Increase with o-ATP alone ($p=0.006$, $N=7$), decreased MCP-1 with SB203580 and o-ATP ($p=0.033$, $N=4$).


a
p-p38
0 1 2 5 (hrs)
o-ATP application
b
p-p38
0 3 30 300 600 (uM)
o-ATP application
c
MCP-1 mRNA expression
(% of Control)
1000
500
0
Control
o-ATP
*
d
MCP-1 mRNA expression
(% Bz-ATP)
100
50
0
Bz-ATP
o-ATP
o-ATP + SB203580
Bz-ATP+
*

DISCUSSION

The results in this thesis provide evidence for the involvement of astrocyte P2X7 receptors in the inflammatory response in the brain. We have shown that astrocytes express P2X7 receptors and that activation of these receptors increases expression of the chemokine, MCP-1. The activation of the MAP kinases, Erk1/2 and p38 by P2X7 receptor activation is a critical step in the increase in MCP-1 expression. Purinergic receptors are also apparently activated during cortical trauma because the trauma-induced increase in MCP-expression, which has been shown to occur in astrocytes, was also attenuated by purinergic receptor antagonism.

Possible pathways of ERK activation by the P2X7 receptor:

For a diagrammatic representation of some potential pathways between the P2X7 receptor and MCP-1 production please see figure 8 at the end of the discussion section.

Our data indicate that P2X7 stimulation results in ERK activation. The effectiveness of the selective agonist Bz-ATP and the effectiveness of inhibition seen with o-ATP are strong evidence that the P2X7 receptor is the mediator of the ERK activation. In fact, similar studies often use only these two compounds to implicate the P2X7 receptor. That our evidence is further corroborated with an extensive array of other purinergic receptor mediators further strengthens our conclusion that the ERK activation is a P2X7 mediated event.

There is a paucity of information concerning the possible signaling mechanisms associated with the P2X7 receptor. Our study links P2X7 activation to an increase in the activity of the ERKs. There are a number of possible pathways whereby this activation could occur. As an example, the increase in intracellular calcium following P2X7 receptor activation could lead to an increase in PYK activity and subsequently to ERK stimulation. As demonstrated by Swanson and colleagues, the P2X2 receptor relies on this mechanism for ERK activation. Given the substantial overlap in structure and pharmacology between the P2X2 and P2X7 receptors, this is a likely pathway.

Recently, much interest has been focused on the trans-activation of the EGF receptor as a possible mediary between intracellular calcium concentrations and ERK activity. In PC12 cells activation of the ERKs in response to membrane depolarization was found to be dependent on the transactivation of the EGF receptor, an event that was also linked to an increase in intracellular calcium [147]. Given that P2X7 receptor activation also induces membrane depolarization, it is plausible that EGF receptor trans-activation may be involved in the ERK activation seen in this study.

Alternatively, activation of the ERKs may occur secondarily to activation of PLD or PLA2 in astrocytes. Both of these enzymes have been shown to become activated in response to P2X7 stimulation. PLA2 was recently shown to activate the ERK pathway in renal proximal tubule epithelial cells, an effect that seems to be mediated through

production of arachidonic acid [148]. PLD activation of ERK, through the production of phosphatidic acid, has also been recently documented [149].

Potential implications of ERK activation by the P2X7 receptor

Given the promiscuity of the MAP kinase family, there are several possible implications of P2X7 mediated ERK activation.

Reactive astrogliosis is a process characterised by astrocyte proliferation, hypertrophy, and a shift in expression of inflammatory mediators in response to CNS insult. At present it is unknown how this process contributes to CNS repair, although it has been shown in some models that reactive astrocytes are a prerequisite to axonal growth and guidance following injury. In this study, we have established that the P2X7 receptor is likely involved in the response to CNS insult/trauma. In addition, ATP is well known as an inducer of astrogliosis, although the particular intracellular signals are not clear. Given the role of the ERK family in cell proliferation, differentiation, and gene expression, it is possible that the connection between the P2X7 receptor and ERK signalling plays a role in reactive gliosis.

P2X7 receptor activation has a known apoptotic and necrotic effect although the mechanism by which these occur is unknown. Studies to date have established that P2X7 stimulation may induce the activity of certain caspases and that caspase inhibitors are protective against ATP-induced DNA fragmentation and chromatin condensation [35] in

certain cell types. Many forms of CNS insult including spinal cord injury, traumatic brain injury and ischemia result in widespread apoptotic cell death. The MAP kinases, including ERK, play central roles in the cascade to programmed cell death. In a landmark study, Xia et al. showed that apoptosis of PC12 cells in response to nerve growth factor withdrawal resulted in inhibition of the ERK pathway and apoptosis [79]. Subsequent expression of a constitutively active ERK isoform abrogated apoptosis [79]. ERK's protective effect has also been established in astrocytes, where it has been shown that ERK activation is protective against calcium-reperfusion induced apoptosis [150]. The increase in ERK activity seen in this study may thus present a possible explanation as to why some cells do not undergo apoptosis in response to P2X7 stimulation [13].

ERK activation is involved in the inflammatory response. For example, inhibiting the ERK pathway with the inhibitor PD98059 results in an anti-inflammatory response *in vivo* using a TPA-induced ear edema model [151]. ERK has been shown to be involved in the signal transduction of a number of extracellular signaling elements including COX-2, Leukotriene C4, IL-1, IL-6, IL-8, nitric oxide, TNF-alpha, and others [152, 153]. Interestingly, during disease states such as multiple sclerosis, EAE, and Alzheimer's disease astrocytes express the cell adhesion molecule ICAM-1. In a study by Lee et al. it was shown that ICAM-1 ligation in astrocytes activates the ERK pathway, and inhibition of ERKs in response to ICAM-1 ligation leads to diminished production of IL-1 and IL-6 [154]. PGE-2 expression in response to IL-1 stimulation of astrocytes was also recently shown to be an ERK mediated process [155]. Given that we are suggesting a role for the

P2X7 receptor in trauma and inflammation, it is not surprising that the P2X7 receptor engages the MAP kinase pathways. It is also suggested that the P2X7 receptor, by activating the ERKs, may also trigger the production of many of the classical inflammatory mediators that are associated with MAP kinase expression.

p38 activation by the P2X7 receptor:

The p38 cascade was also activated by the specific P2X7 receptor agonist Bz-ATP. This confirms recent work in microglial cells showing p38 activation in response to Bz-ATP [43]. We sought to link P2X7 signaling to the p38 cascade in astrocytes because: 1.) The P2X7 receptor can induce apoptosis and the p38 cascade is activated in response to many types of cell stressors and is involved in apoptosis, 2.) The p38 cascade is intimately involved in the inflammatory response, and suppressing p38 activation inhibits inflammation in many disease models and, 3.) p38 is involved in the transcription of many cytokines/chemokines that are elevated in response to CNS disease and trauma.

Potential pathways of p38 activation by the P2X7 receptor

Much less is known about the upstream activators of the p38 MAP kinase cascade than of the ERKs. Some potential enzymes upstream of p38 that may be involved include the calcium-calmodulin dependent protein kinases, PYK2, and Ras. In a study by Enslen et al. membrane depolarization of PC12 cells led to an increase in intracellular calcium and subsequent activation of a subgroup of calcium-calmodulin dependent protein kinases, resulting finally in p38 activation [156]. As such, the calcium-calmodulin dependent

kinases are a potential signaling molecule following P2X7 receptor stimulation. Similar to the ERKs, PYK2 can also mediate activation of p38 [157]. Interestingly, the PYK2-p38 pathway has been implicated in ischemic injury and seizure activity in microglial cells and in neurons, although the mechanism of activation is unknown [158]. Since ischemia is followed by an increase in extracellular ATP levels, it is possible that ischemia may promote activation of purinergic receptors which then signal to p38 via PYK2.

Ras activation, classically thought to stimulate the ERK subgroup of map kinases, has also been shown to lead to p38 activation [159]. It is thought that the family of small molecular weight G-proteins (Rac, Rho, etc.) mediates this interaction. Given this crosstalk, many of the potential pathways cited as potential mediators of P2X7 mediated ERK induction may also mediate the signal to p38.

Alternatively, the mechanism of p38 activation may be indirect. The classical stimulator of p38 is TNF-alpha, which has been shown to be released from microglia in response to elevations in extracellular ATP concentrations [43]. The time course of activation of the p38 pathway (5 minutes) however, makes it unlikely that synthesis of other inflammatory mediators is an intervening step.

Implications of p38 activation by the P2X7 receptor:

Cell death

The p38 cascade is best known for its function in apoptosis. In PC12 cells withdrawal of Nerve Growth Factor leads to apoptosis and upregulation of the p38 pathway [79]. In this same study the expression of a dominant negative mutant of p38 led to increased survival [79]. The role of p38 in PC12 cell apoptosis was extended by Dummer et al. where insulin was used to rescue cells from apoptosis, an effect that was mediated by insulin's ability to down-regulate p38 [160]. Apoptosis induced by Fas ligation in T-lymphocytes is also likely a p38 mediated event because p38 activation correlates temporally with apoptosis and expressing a constitutively active form of MKK3 (upstream p38 activator) potentiates apoptosis [161]. Also in T-lymphocytes expression of a constitutively active form of MKK6 (upstream p38 activator) was found to be necessary and sufficient to induce Fas-mediated apoptosis, although this effect was not directly shown to be mediated by p38 [162]. The specific p38 inhibitor SB203580 has been shown to resist apoptosis in a stimulus and cell specific fashion. SB203580 inhibits apoptosis in cerebellar granule cells induced by glutamate, apoptosis in Rat-1 cells and PC12 cells caused by serum deprivation [163].

Other than in PC12 cells, little information exists as to the role of p38 in the CNS. Although it is known that brain damage after cerebral ischemia results in a rapid upregulation of p38 phosphorylation [164], the increase in p38 levels has not been correlated with decreased survival. In astrocytes, there have been no studies suggesting

apoptosis may be mediated by p38. Our data indicate that the P2X7 receptor, a well-known apoptotic-inducing agent, does activate the p38 MAP kinase cascade. In addition, the P2X7 receptor is known to lead to production of TNF-alpha, a pro-inflammatory cytokine that leads to apoptosis in a number of systems. Taken together, it is possible that the mechanism of P2X7 induced apoptosis involves the p38 cascade in astrocytes.

Cell cycle and differentiation.

ATP is a known mitotic and differentiating agent, but the mechanism of this action is unknown. Although the link between p38 and the cell cycle is tenuous, there is some evidence that p38 may also be involved in the cell cycle. Injection of Xenopus embryos with constitutively active p38 induces cell cycle arrest [165] and treatment of yeast cultures with the p38 inhibitor SB203580 hinders proliferation [163]. Differentiation of a number of cell types including PC12 cells to neurons and 3T3-L1 cells into adipocytes has been found to be dependent on p38 [163]. In a number of cell systems, p38 has also been shown to be necessary and sufficient for differentiation [163].

Our data suggest that extracellular ATP activates p38 in astrocytes. Given that ATP is an astrocytic mitogen, the activation of p38 seems counterintuitive, as p38 activation is usually associated with an inhibition of mitogenesis. It should be remembered, however, that at the concentrations used in this study, the ATP analogue Bz-ATP is likely not a mitogenic agent, but rather an apoptotic agent based on previous reports. It would be

interesting to see if lower concentrations of ATP or Bz-ATP similarly induced p38 activation.

Inflammation and p38

Compounds that inhibit p38 have been in development as immuno-suppressive agents for many years. The pharmaceutical industry interest stems from a number of studies showing p38 inhibition has beneficial effects in a number of immune mediated diseases. In models of arthritis SB203580 reduces inflammation and increases bone density, while the higher potency p38 inhibitor SB22025 has been shown to halt arthritic progression [166, 167]. In a mouse inflammatory angiogenesis model p38 inhibition resulted in a 40% decrease in angiogenesis and a concomitant decreased in many inflammatory mediators [167].

p38 also participates in the expression of a number of other enzymes that modify the inflammatory response. IL-1, TNF-alpha, and IL-6 have all been shown to be expressed in response to p38 activation [163]. COX-2, involved in tissue remodeling, is also under transcriptional control of p38 [163]. Oxidation enzymes such as iNOS and the expression of cell surface adhesion molecules also depends on p38 activity [163]. In our study, we focussed solely on the upregulation of the chemokine MCP-1 although undoubtedly other inflammatory mediators such as those listed above may be consequences of P2X7-p38 activation. In fact, in a very recent study by Hide et al. the P2X7 receptor was found to induce TNF synthesis, and p38 inhibition attenuated this effect [43].

Under pathological situations such as multiple sclerosis, or acquired immune deficiency syndrome (AIDS) astrocytes may produce cell adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 that facilitate leukocyte migration across the blood brain barrier [168]. In ischemia, trauma, or inflammation, cytokines such as TNF-alpha and IL-1 are released which likely catalyze the production of these adhesion molecules [169] [170]. In many systems, these pathways are p38 dependent. In a study by Pietersma et al. it was shown that astrocytes challenged with TNF-alpha upregulate VCAM expression, and that this effect is blocked by SB203580 [171]. E-selectin upregulation in endothelial cells in response to TNF has also been shown to be p38 dependent as expression of dominant interfering forms of the p38 pathway suppress E-selectin synthesis [172] Subsequently, p38 was also found to be integral in ICAM-1 expression in B-lymphocytes induced by CD40 engagement [173]. Given that the P2X7 receptor is likely to be activated in response to trauma, it may be possible that the subsequent p38 activity serves to facilitate the immune response by upregulating adhesion molecule synthesis on the astrocyte.

MCP-1 in astrocytes:

Our immunostaining confirmed that MCP-1 expression occurred in our cultured astrocytes. Given the purity of the cultures (>95%) and the intensity of staining, it is highly unlikely that MCP-1 could come from another source. Although this data confirms other well known studies indicating that astrocytes are a major source of MCP-1

expression, to our knowledge it is the first to document immunocytochemically the existence of MCP-1 in cultured astrocytes.

The use of cultured astrocytes provides an easily manipulated experimental procedure to study astrocyte function, however a substantial body of evidence points to some caveats regarding generalizing culture conditions to an *in vivo* model. In particular, it is likely that astrocytes in culture represent a form of astrocyte termed reactive astrocytes that, because of the harsh environment, are essentially perpetually physiologically stressed. This stress causes the astrocyte to express a variety of receptors, and produce a variety of chemokines, cytokines, and growth factors that would not normally be found *in vivo*. To some extent, our study confirms this assumption. Although MCP-1 is not normally expressed without an inciting event in the CNS, essentially all of the cells in culture expressed MCP-1 without stimulus. Although it was originally our intent to compare Bz-ATP stimulated cultures with control cultures using immunohistochemistry to test for a differential pattern of MCP-1 expression, the high basal rate of MCP-1 production made this approach unrealistic.

The P2X7 receptor induces MCP-1 production via the MAP kinases

Many cell types have been shown to release ATP, such as lymphocytes, macrophages, platelets, epithelial cells, endothelial cells, neurons, microglia and astrocytes [46]. By non-lytic mechanisms, these cells have been shown to release up to 15% of their intracellular content, while trauma and lysis can extrude the entire ATP pool (3mM) into

the surroundings. Although extracellular ATP does not exceed approximately 10 μM in the general extracellular milieu, in compartmentalized regions this value likely increases dramatically, and sufficiently to activate many purinergic receptors [46].

We have found that P2X7 receptor stimulation in cultured astrocytes induces MCP-1 expression. This effect is down-regulated by inhibitors of either the ERK or p38 pathways. Using SB203580 it was recently shown that p38 is involved in MCP-1 expression in mesangial cell, and that this effect was independent of NF-kappa-B activity [145]. This result is interesting given that NF-kappa-B has been well studied as a modulator of MCP-1 expression and because the MCP-1 promoter does contain a binding site for NF-kappa-B. Although it is possible that p38 in our system may be acting independent of NF-kappa-B, given the recent demonstration that p38 is required for NF-kappa-B gene expression in other cytokine systems [174] we cannot rule out this pathway.

The MCP-1 promoter also contains an Sp1 binding site. As both the ERKs and p38 have been found to phosphorylate and activate Sp1 mediated transcription factors it is possible that either or both ERK and p38 act to increase MCP-1 production by enhancing Sp1 transcription.

As mentioned above, the MCP-1 promoter sequence contains an AP-1 binding site that acts in co-operation with the NF-kappa-B and Sp1 binding site. Given that ERK is a well-

known activator of the transcription factor AP-1, it stands to reason that ERK could likely be involved in MCP-1 expression. Furthermore, our result of cooperative participation of ERK and p38 in MCP-1 expression fits very nicely with promoter data indicating a co-dependence of MCP-1 expression on both the NF-kappa-B and AP-1 transcription factors.

Interestingly, however, other researchers have failed to show a link between the ERK cascade and MCP-1 expression. ERK was ruled out as an active participant in MCP-1 expression in response to PDGF [175] and IL-1b [145]. These data are, however, consistent with the known variances in MCP-1 signaling between cell types [175].

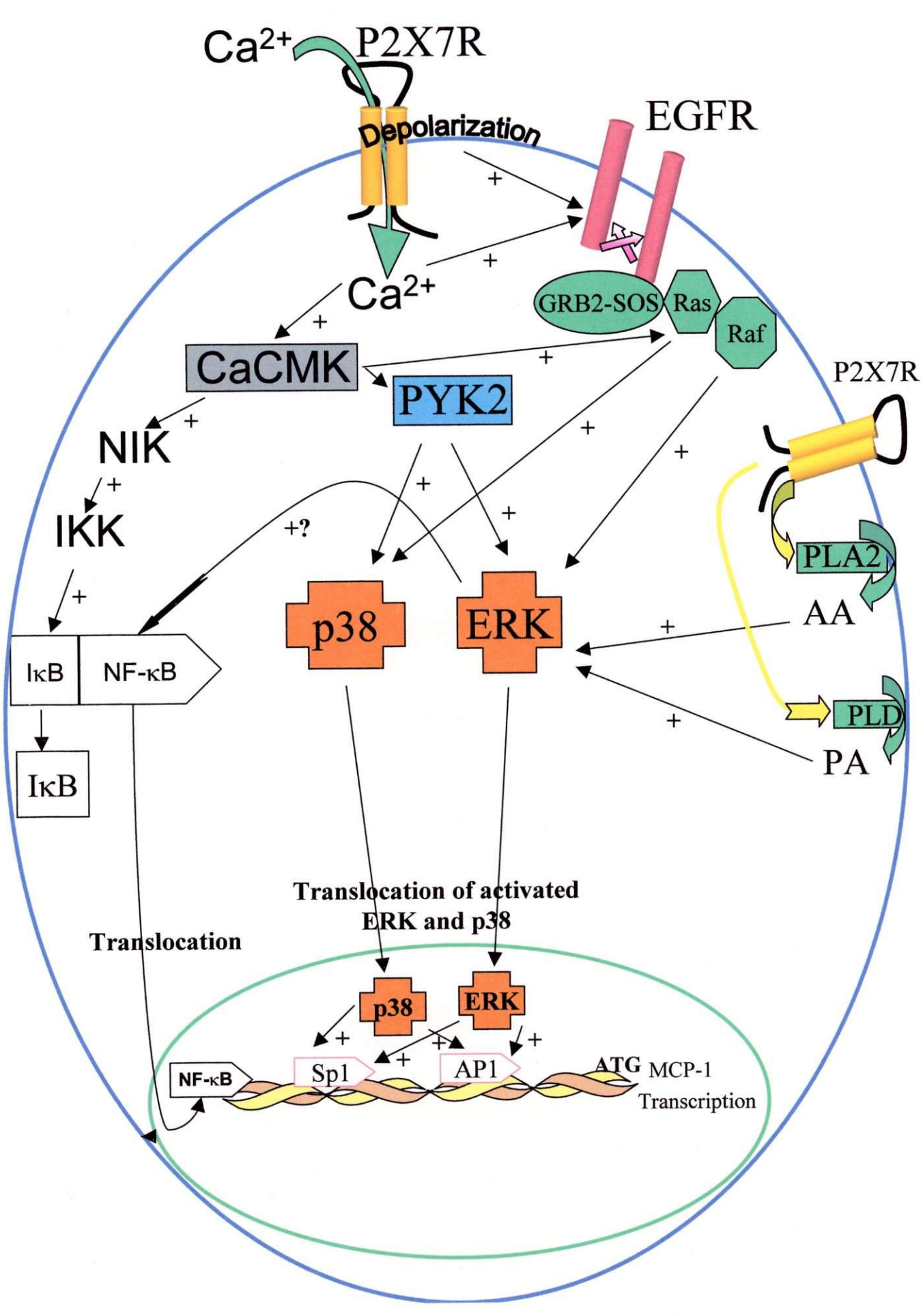
The P2X7 receptor has been linked to the production or release of two other inflammatory mediators, TNF-alpha [43] and IL-1b [16]. Up-regulation of TNF-alpha has been studied in both microglial cells [43] and dendritic cells [46]. In microglial cells this was shown to be mediated by both the ERK and p38 signaling cascades, and to be independent of entry of calcium into the cells [43]. As in our case, the inhibitors PD98059 and SB203580 showed some inhibitory effect individually, and their combined effect was greater than either alone [43] [46].

Significance of MCP-1 production in response to P2X7 stimulation:

Our data presents an intriguing new hypothesis concerning the role of extracellular ATP following CNS injury. Specifically, we propose that ATP may trigger the infiltration of immune cells into the CNS by inducing expression of MCP-1 in astrocytes.

In many disease models, targeted interruptions of MCP-1 expression elicit significant therapeutic benefit [176-178] leading to the possibility that a role for purinoceptors may extend beyond trauma. Atherosclerosis, neoplasia, and HIV are a few examples of disease states where MCP-1 is thought to play a role [95, 179, 180]. Further, MCP-1 seems to be intricately involved in EAE, where it has been demonstrated that MCP-1 is up-regulated at the onset of disease, and abruptly increased during relapse [101]. Interestingly, it has been known since 1985 that suramin is protective in mouse models of EAE [181] although the mechanism was completely unknown. Our results present a likely possibility for suramin's therapeutic effects. Suramin, by inhibiting purinergic receptor activity effectively suppresses MCP-1 production and hence, the progression of EAE. Since purinergic receptor inhibitors are currently in common medical practice, our data raise the attractive possibility that these inhibitors may be of use in the treatment of neuro-AIDS, MS and other CNS disorders, whose pathogenesis includes induction of MCP-1.

Fig. 8. Possible pathways of P2X7 mediated upregulation of MCP-1 expresssion via MAP kinase cascades. + indicates activation. **?** indicates that only speculative data exist. For abbreviations please see Abbreviation section at beginning of thesis.


Ca2+
P2X7R
Depolarization
EGFR
Ca2+
GRB2-SOS
Ras
Raf
CaCMK
PYK2
P2X7R
NIK
IKK
+?
PLA2
AA
p38
ERK
IκB
NF-κB
PLD
PA
IκB
Translocation of activated
ERK and p38
Translocation
p38
ERK
NF-κB
Sp1
AP1
ATG
MCP-1
Transcription

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