Purinergic Modulation of Neurotransmission

by

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Abstract

Signalling through adenosine- and adenine-nucleotide-gated receptors (purinoceptors) regulates a vast array of physiological processes in the central nervous system, including synaptic transmission. The research presented here describes a role for mitogen-activated protein kinases (MAPKs) in mediating molecular signalling cascades initiated by activation of purinoceptors in the hippocampus. In particular, it is proposed that MAPK activation is a requirement for purinoceptor-mediated presynaptic inhibition of neurotransmission. The three primary findings presented here are: 1) P2X7 receptors are localized on mossy fiber terminals where they function to inhibit mossy fiber-CA3 synaptic transmission in a pathway requiring p38 MAPK activation; 2) Adenosine A1 receptors exist in a complex with p38 MAPK in which A1 receptors activate p38 MAPK to decrease Schaffer-collateral-CA1 synaptic transmission; and 3) Sequential activation of C-Jun N-terminal kinase (JNK) following p38 MAPK activation is also required for A1 receptor-mediated synaptic depression. Immunocytochemistry was used to demonstrate that P2X7 receptors are abundant on presynaptic terminals of mossy fiber synapses in the rat hippocampus. Western blotting was used to show increases in the phosphorylation state of p38 MAPK and JNK following A1 receptor stimulation in the CA1 region. Co-immunoprecipitation showed that A1 receptors are physically associated with p38 MAPK and JNK in the hippocampus. Synaptic function was assessed using field excitatory postsynaptic potentials (fEPSPs) evoked in stratum lucidum in the CA3 region or in stratum radiatum in the CA1 region of rat hippocampal slices. Selective stimulation of P2X7 receptors with the agonist Bz-ATP potently decreased mossy fiber-CA3 synaptic depression and this was blocked by the P2X7 antagonist oxidized-ATP, but not by the
P2X<sub>1-3,5,6</sub> antagonist, PPADs or the P2Y antagonist, RB2. Bz-ATP-induced synaptic depression was blocked by the p38 MAPK inhibitor SB203580. Stimulation of A<sub>1</sub> receptors with exogenous adenosine, endogenous adenosine released during hypoxia, or the agonist N<sup>6</sup>-cyclopentyladenosine (CPA) depressed evoked fEPSPs in the CA1 region. These inhibitory responses were blocked with the A<sub>1</sub> receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), the p38 MAPK inhibitors SB203580 and SB202190, and the JNK inhibitors SP600125 and JNK Inhibitor V. These results suggest that the inhibitory actions of purinoceptors requires the activation of p38 MAPK and JNK in the hippocampus.
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<th>Description</th>
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<tbody>
<tr>
<td>A/C</td>
<td>Associative/commissural</td>
</tr>
<tr>
<td>A_1</td>
<td>(G_{i/o}) protein-coupled adenosine receptor</td>
</tr>
<tr>
<td>A_{2A}</td>
<td>(G_{o/off}) protein-coupled adenosine receptor</td>
</tr>
<tr>
<td>A_{2B}</td>
<td>(G_s) protein-coupled adenosine receptor</td>
</tr>
<tr>
<td>A_3</td>
<td>(G_{i/o}) protein-coupled adenosine receptor</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ARL 67156</td>
<td>An ecto-ATPase inhibitor</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1 (JNK MAPKKK)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bz-ATP</td>
<td>2'3'O-(4-benzoylbenzoyl)-ATP</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis; field 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis; field 3</td>
</tr>
<tr>
<td>CADO</td>
<td>2-chloroadenosine; (A_1) receptor agonist</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ca_v</td>
<td>Voltage-dependent calcium channel</td>
</tr>
<tr>
<td>CCPA</td>
<td>2-chloro-(N^6)-cyclopentyl-adenosine; (A_1) receptor agonist</td>
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<td>Cdc42</td>
<td>Small GTPase belonging to the Rho family</td>
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<td>CHA</td>
<td>Cyclohexyladenosine; (A_1) receptor agonist</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein Kinase II</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COS-7</td>
<td>African Green Monkey SV40-transfected kidney fibroblast cell line</td>
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<tr>
<td>CPA</td>
<td>(N^6)-cyclopentyl-adenosine; (A_1) receptor agonist</td>
</tr>
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<td>Cterm-ab</td>
<td>P2X_7 antibody directed towards the intracellular C-terminus</td>
</tr>
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<td>DHPG</td>
<td>3,5-dihydroxyphenylglycine; group I mGluR agonist</td>
</tr>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DPCPX</td>
<td>1,3-dipropyl-8-cyclopentylxanthine; (A_1) receptor agonist</td>
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<td>EctoAb-1</td>
<td>P2X_7 antibody (recognizes an extracellular epitope)</td>
</tr>
<tr>
<td>EctoAb-2</td>
<td>P2X_7 antibody (recognizes an extracellular epitope)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid; divalent cation chelator</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(beta-aminoethyl ether)-(N,N,N',N')-tetracetic acid; (Ca^{2+}) chelator</td>
</tr>
<tr>
<td>ENT1</td>
<td>Gene encoding equilibrative nucleoside transporter 1</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory postsynaptic current</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Field excitatory postsynaptic potential</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>(G_i)</td>
<td>Inhibitory; inhibits adenylate cyclase</td>
</tr>
</tbody>
</table>
GIRK  G protein-activated inwardly rectifying K\(^+\) channel
Glaxo P2X\(_{7^{-}}\)  Transgenic mouse with lac\(Z\) gene inserted in beginning of exon 1
GluR  Glutamate receptor
GR79236  \(A_1\) receptor agonist
G\(_s\)  Stimulatory; activates adenylate cyclase to increase cAMP
GTP  Guanosine triphosphate
GW-493838  \(A_1\) receptor agonist
G\(_\alpha\)  G protein alpha subunit; GTPase domain alpha-helical domain
G\(_{\beta\gamma}\)  G protein beta-gamma complex
h  Dentate hilus
HCN  Hyperpolarization-activated cyclic nucleotide-gated cation channels
HEK  Human embryonic kidney
I-II linker  Cytoplasmic loop connecting domain Ca\(_\alpha\) \(\alpha 1\) domains I and II
IP  Immunoprecipitation
ITP  Inosine triphosphate
JNK  C-Jun N-terminal kinase
JNK Inhibitor V  1,3-Benzothiazol-2-yl-(2-(2-(3-pyridinyl)ethyl)amino)-4-pyrimidinyl)acetonitrile; JNK inhibitor
kDa  Kilodalton
L-CCG-I  2-(carboxycyclopropyl)glycine; group II mGluR agonist
LTD  Long-term depression
LTP  Long-term potentiation
luc  Stratum lucidum
MAPK  Mitogen-activated protein kinase
MAPKK  Mitogen-activated protein kinase kinase (a.k.a. MEK)
MAPKKK  Mitogen-activated protein kinase kinase kinase (a.k.a. MEKK)
MEK  MAP/ERK kinase (a.k.a. MAPKK)
MEKK  MEK kinase (a.k.a. MAPKKK)
MF  Mossy fiber
MKK3/6  Member of MEK family; phosphorylates p38 MAPK
MKK4/7  Member of MEK family; phosphorylates JNK
MLK4  mixed-lineage kinase 4 (JNK MAPKKK)
MOPS  3-(N-Morpholino)propanesulfonic acid; 4-Morpholinepropanesulfonic acid; buffer
mRNA  Messenger ribonucleic acid
Na\(_\alpha\)  Voltage-dependent sodium channel
NBQX  1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; AMPA/Kainate receptor antagonist
NMDA  N-methyl-D-aspartate
o-ATP  Periodate oxidized ATP
P1  Adenosine-gated G protein-coupled receptor
P2  Adenine nucleotide-gated receptor
P2X  Ionotropic Adenine nucleotide-gated receptor
P2Y  Metabotropic Adenine nucleotide-gated receptor
p38 MAPK  p38 mitogen-activated protein kinase
PD98059  2'-Amino-3'-methoxyflavone; MEK inhibitor
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Pfizer P2X&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Transgenic mouse lacking the C-terminus of the P2X&lt;sub&gt;7&lt;/sub&gt; gene</td>
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<tr>
<td>Phospho-JNK</td>
<td>Phosphorylated JNK</td>
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<tr>
<td>Phospho-p38</td>
<td>Phosphorylated p38</td>
</tr>
<tr>
<td>P13K</td>
<td>Phosphatidylinositol 3-kinases</td>
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<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>PP2a</td>
<td>Protein phosphatase 2a</td>
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<tr>
<td>PPADS</td>
<td>Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid</td>
</tr>
<tr>
<td>Purinoceptor</td>
<td>P1 or P2 receptor</td>
</tr>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>Rac1</td>
<td>Small GTPase belonging to the Rho family</td>
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<tr>
<td>Rap</td>
<td>Member of Ras superfamily of small GTPases</td>
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<tr>
<td>Ras</td>
<td>Member of Ras superfamily of small GTPases</td>
</tr>
<tr>
<td>RB2</td>
<td>Reactive blue 2</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
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<tr>
<td>Ras-homologous GTPase</td>
<td>Ras-homologous GTPase (Superfamily of small GTPases)</td>
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<td>SB202190</td>
<td>4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; p38 MAPK inhibitor</td>
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<td>SB202474</td>
<td>4-Ethyl-2(p-methoxyphenyl)-5-(4'-pyridyl)-IH-imidazole; negative control for SB203580 and SB202190</td>
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<td>SB203580</td>
<td>4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; p38 MAPK inhibitor</td>
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<td>SB239063</td>
<td>trans-1-(4-Hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyrimidin-4-yl)imidazole; p38 MAPK inhibitor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Small GTPase</td>
<td>G protein; alternately binds GTP and GDP</td>
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<tr>
<td>SP600125</td>
<td>1,9-pyrazoloanthrone; JNK inhibitor</td>
</tr>
<tr>
<td>T-62</td>
<td>A&lt;sub&gt;1&lt;/sub&gt; receptor allosteric enhancer</td>
</tr>
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<td>TAB1</td>
<td>TAK1 binding protein 1</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming-growth factor β-activated kinase 1</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-Trait</td>
<td>Tris buffered saline with Tween 20</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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<tr>
<td>YO-PRO-1</td>
<td>Quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio) propyl]-diiodide; fluorescent dye</td>
</tr>
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</table>
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In addition, I would like to thank Tom Smith for proofreading an earlier version of this manuscript.

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Dedication

To Walter and Susan
Co-authorship statement

Chapter 2: Activation of P2X$_7$-Like Receptors Depresses Mossy Fiber-CA3 Synaptic Transmission Through p38 Mitogen-Activated Protein Kinase

For this study, I contributed field excitatory postsynaptic potential (fEPSP) recordings for figures 2, 4, 6, and 7.

Chapter 3: p38 Mitogen-Activated Protein Kinase Contributes to Adenosine A$_1$-Receptor-Mediated Synaptic Depression in Area CA1 of the Rat Hippocampus

I was the lead researcher on this study. I did all the fEPSP recordings and approximately half of the Western Blotting. I also assisted with the co-immunoprecipitation experiments.

Chapter 4: C-Jun N-Terminal Kinase Regulates Adenosine A$_1$ Receptor-Mediated Synaptic Depression in the Rat Hippocampus

I took a lead role in this study. I contributed all the fEPSP recordings and approximately half of the Western Blot experiments. I assisted with the co-immunoprecipitation experiments as well.
Chapter One: Introduction
1.1. Hypothesis and Objectives

The purpose of this research was to investigate the mechanisms by which adenosine triphosphate (ATP) and adenosine modulate neurotransmission in the hippocampus. The three main areas of investigation were: 1) modulation of mossy fiber-CA3 synaptic transmission by ATP acting at P2X$_7$ receptors; 2) modulation of CA1-CA3 synaptic transmission by adenosine A$_1$ receptors signalling through p38 mitogen-activated protein kinase (MAPK); and 3) modulation of CA1-CA3 synaptic transmission by adenosine A$_1$ receptors signalling through C-Jun N-terminal kinase (JNK). These areas were chosen because signalling by adenosine and adenine nucleotides (purinergic signalling) is crucial for brain function in both physiological and pathophysiological conditions. The hippocampal slice preparation was chosen for these studies because it exhibits well defined forms of synaptic plasticity at identified synapses, is specifically vulnerable to ischemia, and its anatomical organization makes it particularly suitable for extracellular electrophysiological recordings. The hippocampus is not only an ideal model for studying the mechanisms underlying synaptic transmission, but it is also of great functional relevance because it is required for the formation of new memories.
**Hypothesis 1:**

P2X<sub>7</sub> receptors are expressed on mossy fiber terminals where they function to decrease mossy fiber-CA3 synaptic transmission.

**Objectives:**

1. Image the cellular and subcellular distribution of P2X<sub>7</sub> receptors in the hippocampus using immunohistochemistry.
2. Determine whether P2X<sub>7</sub> receptor activation modulates synaptic transmission by recording whole-cell currents and extracellular field excitatory postsynaptic potentials (fEPSPs) evoked in stratum lucidum of hippocampal slices.

**Hypothesis 2:**

Adenosine A<sub>1</sub> receptors decrease CA3-CA1 synaptic transmission through p38 mitogen-activated protein kinase (MAPK).

**Objectives:**

1. Quantify changes in the phosphorylation state of p38 MAPK caused by adenosine A<sub>1</sub> receptor stimulation using Western blot analysis.
2. Resolve whether A<sub>1</sub> receptors and p38 MAPK are physically associated using co-immunoprecipitation.
3. Determine whether inhibiting p38 MAPK decreases the magnitude of A<sub>1</sub> receptor-dependent depression of fEPSPs evoked in the CA1 region of rat hippocampal slices.
**Hypothesis 3:**

Adenosine A₁ receptors decrease CA3-CA1 synaptic transmission through p38 MAPK mediated activation of C-Jun N-terminal kinase (JNK).

**Objectives:**

1. Determine whether inhibiting JNK modulates the magnitude of A₁ receptor-dependent depression of fEPSP amplitudes in the CA1 region of rat hippocampal slices.
2. Quantify changes in the phosphorylation state of JNK in response to A₁ receptor stimulation using Western blot analysis.
3. Analyze the dependence of JNK activation on p38 MAPK activation using pharmacology and Western blot.
4. Determine whether JNK (or phosphorylated JNK) are physically associated with the A₁ receptor in the hippocampus using co-immunoprecipitation.
1.2. Introduction

Extracellular ATP and adenosine are ubiquitous transmitters of chemical signals between neurons. ATP is present in vesicles containing both peptides and small-molecule neurotransmitters and is released by exocytosis at virtually all synapses. Adenosine is a degradation product of ATP metabolism and is arguably the most widespread and potent inhibitor of excitatory synaptic transmission in the nervous system. ATP and adenosine transmission, or purinergic transmission, is highly conserved across species and cell types. ATP and adenosine play functional roles in an array of neural processes that include: regulating sleep and arousal; analgesia; alcohol addiction; epilepsy; and neuroprotection (Dunwiddie and Masino, 2001; Burnstock, 2004). Although the actions of ATP and adenosine in the nervous system have been known for some time, the signalling by which purinergic transmission occurs has not been studied in detail.

1.3. Purinergic receptors in the central nervous system

ATP produces diverse physiological effects by activating multiple P2 receptors. Adenosine is generated following hydrolysis of ATP by the ecto-nucleotidase cascade (Zimmermann and Braun, 1999), which terminates P2 receptor signalling and stimulates G protein-coupled P1 receptors. P1 and P2 receptors are co-expressed in most cells (Matsuoka and Ohkubo, 2004). Considerable effort has gone into distinguishing P1 and P2 receptor-mediated effects using a number of pharmacological and biochemical tools such as metabolically stable P2-receptor agonists, P1 and P2 receptor antagonists, adenosine uptake inhibitors, and adenosine deaminase. Nonetheless, the actions of specific purinoceptor subtypes can be difficult to isolate because of overlapping
pharmacological profiles. For example, it can be a challenge to distinguish P2X\textsubscript{4} and P2X\textsubscript{7} receptor-mediate effects because of the lack of specific antagonists (North and Surprenant, 2000).

### 1.3.1. Adenine nucleotide-gated receptors

P2 receptors include ionotropic P2X and G protein-coupled P2Y receptors. Both receptors exhibit various effects at neuronal and glial cells, and are distributed widely in the central nervous system (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998; North, 2002). In astrocytes, P2Y receptors predominate over P2X receptors and mediate short-term increases in intracellular Ca\textsuperscript{2+} and eventually effects such as proliferation and apoptosis. Both classes of P2 receptors are also expressed in neurons, where P2X receptors mediate fast synaptic responses to ATP and P2Y mediates slow changes in membrane excitability in response to either non-synaptically released ATP or interactions with other receptors. Neuronal P2 receptors also regulate trophic effects such as neurite outgrowth, neuronal maturation, and the expression of new receptors.

#### 1.3.1.1. P2X receptors

P2X receptors form a family of at least seven subunits (P2X\textsubscript{1-7}) and occur as stable trimers (three subunits), hexamers (six subunits), or multimeric assemblies of P2X\textsubscript{2}/P2X\textsubscript{3}, P2X\textsubscript{4}/P2X\textsubscript{6}, or P2X\textsubscript{4}/P2X\textsubscript{5}. P2X\textsubscript{7} receptors form homomeric channels. Each subunit is comprised of two transmembrane domains, an ATP binding site contained on a large extracellular loop, and intracellular N and C termini (North, 2002).

Traditionally, it was thought that P2X\textsubscript{1-6} receptor subtypes were predominantly localized in neurons, whereas P2X\textsubscript{7} receptors were reported only in activated microglia,
macrophages, or lymphocytes, which is consistent with its known role in repairing brain
damage due to immune insult, inflammation, or infarction (North, 2002). In recent years,
it has been proposed that P2X7 receptors are also present in presynaptic excitatory
terminals in the spinal cord and brain (Deuchars et al., 2001; Armstrong et al., 2002;
Atkinson et al., 2002; Lundy et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003;
Cavaliere et al., 2004), although these findings have since generated controversy (see
discussion page 169). Both P2X2 and P2X4 are unequivocally localized at postsynaptic
specializations of synapses in the cerebellum and hippocampus, where the P2X2 receptor
facilitates inward currents in CA1 interneurons (Khakh et al., 2003). In general, P2X
receptors mediate neuronal inward currents throughout the CNS (Illes and Ribeiro, 2004),
supporting the notion that ATP is a fast synaptic transmitter.

P2X receptors may also play a role in synaptic plasticity. When ATP (that is
coreleased with glutamate) acts at postsynaptic Ca2+-permeable P2X receptors, calcium
enters the cell and inactivates NMDA receptors, a process that tonically inhibits the
effectiveness of NMDA receptors in inducing LTP (Pankratov et al., 2002). Indeed,
inhibition of P2X receptors by PPADS greatly facilitates the induction of LTP (Pankratov
et al., 2002). Ca2+-dependent inactivation of NMDA receptors by P2X receptors provides
a mechanism to prevent weak stimuli from inducing LTP.

Astrocytic release of ATP, glutamate, and GABA may also modulate synaptic
transmission. The stimulation of P2X7 receptors on astrocytes leads to astrocytic release
of both glutamate (Duan et al., 2003) and GABA (Wang et al., 2002a), providing a link
between the secretion of ATP and excitatory or inhibitory neurotransmitters and thus
dynamically regulating synaptic transmission.
1.3.1.2. P2Y receptors

P2Y receptors form a family of ten cloned and functionally defined subtypes, of which P2Y₁, P2Y₂, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄ are expressed in the CNS. P2Y receptors contain the seven hydrophobic transmembrane domains typical of G protein-coupled receptors. The diversity of P2Y receptor-mediated functions is probably attributable to the differential expression of each subtype in different brain regions and cell types. For example, P2Y₁ is localized in neurons, and particularly abundant in nucleus accumbens, striatum, caudate nucleus, putamen, globus pallidus, cerebellum, hippocampus, and throughout the cerebral cortex, whereas P2Y₁₂ is predominantly expressed in glia (Illes and Ribeiro, 2004). P2Y₁ and P2Y₂ receptors are also expressed in astrocytes where they mediate the propagation of Ca²⁺ waves between astrocytes induced by the release of ATP (Haydon, 2001).

1.3.2. Adenosine receptors

P₁ receptors include the A₁, A₂A, A₂B, and A₃ subtypes, each of which has a distinct tissue distribution, pharmacological profile, and effector coupling. For example, the Gᵢ protein-coupled A₁ receptor and Gₛ protein-coupled A₂A receptor have opposing effects on adenylyl cyclase activity. The stimulant effects of adenosine receptor antagonists such as caffeine are due to antagonism of A₁ and A₂A receptor subtypes. The A₂A receptor is particularly abundant in the striatum, where it functionally interacts with dopamine receptor signalling.

The A₁ receptor is the predominant subtype found in the CNS, and is abundant in the spinal cord, cerebellum, hippocampus, and cerebral cortex. A₁ receptor stimulation causes decreased release of every classical neurotransmitter, including glutamate, GABA,
acetylcholine, norepinephrine, 5-hydroxytryptamine, dopamine, and others. The effect of A₁ stimulation is most dramatic on excitatory synaptic transmission, which can be completely blocked by adenosine.

A₁ receptors are expressed both pre- and postsynaptically. Presynaptic A₁ receptors are resistant to desensitization whereas postsynaptic A₁ receptors are not. The mechanism by which A₁ receptor stimulation inhibits neurotransmission appears to depend on a G protein-coupled inhibition of N-type Ca²⁺ channels in presynaptic terminals, although adenosine also inhibits spontaneous Ca²⁺-independent release of neurotransmitter (Scanziani et al., 1992). Other mechanisms may contribute as well, as stimulation of postsynaptic A₁ receptors activates G protein-dependent inwardly rectifying K⁺ channels (GIRKs) causing hyperpolarization of the resting membrane potential.

1.4. Sources of extracellular adenosine

A basal purinergic tone, maintained by tonic activation of A₁ and A₂A receptors, permeates most tissues. The concentration of extracellular adenosine under normal conditions is in the range of 25-250 nM. Basal adenosine tone reflects a balance between mechanisms that increase extracellular adenosine pitted against mechanisms that remove adenosine through uptake or metabolism. Unlike ATP, adenosine is not released in vesicles like a classical neurotransmitter. Rather, adenosine reaches the extracellular space by two primary mechanisms: 1) dephosphorylation of adenine nucleotides by ecto-nucleotidases; and 2) release of adenosine through nucleoside transporters.
1.4.1. Extracellular conversion of adenine nucleotides

Ecto-nucleotidases, ecto-phosphodiesterases, and apyrases are widely expressed and rapidly (in less than a second (Dunwiddie et al., 1997)) dephosphorylate any adenine nucleotide to 5’-AMP, which is then dephosphorylated to adenosine by 5’-nucleotidase (Zimmermann, 2000). Adenine nucleotides accumulate in the extracellular space due to co-release of ATP with classical neurotransmitters (White, 1977; Fredholm et al., 1982) or release of cAMP by a probenecid-sensitive transporter (Rosenberg and Li, 1995), both of which are sufficient to cause large increases in extracellular adenosine following hydrolysis by ecto-enzymes (Dunwiddie et al., 1992; Brundege et al., 1997). ATP is also released from glial cells. The rapid and localized production of adenosine by ecto-nucleotidases in close physical proximity to presynaptic inhibitory A1 receptors on the membrane surface is an important determinant of cellular responses to ATP.

1.4.2. Release of adenosine through facilitated diffusion transporters

Equilibration of adenosine concentrations across cellular membranes occurs through facilitated diffusion nucleoside transporters (Boumah et al., 1994). Nucleoside transporters are bidirectional and passive; that is, they do not depend on energy derived from ATP or ionic gradients to transport adenosine. Adenosine concentrations inside cells are normally low due to the high activity of intracellular adenosine kinase (which converts adenosine to 5’-AMP), resulting in a net inward flux through these transporters. Adenosine is released into the extracellular space by nucleoside transporters when intracellular adenosine concentrations rise, as occurs during pathological conditions such as hypoxia/ischemia. Because nucleoside transporters are bidirectional, the regulation of
extracellular adenosine is intimately linked to intracellular adenosine concentrations. As intracellular adenosine concentrations rise, adenosine can no longer be taken up from the extracellular space by nucleoside transporters, and when the concentration of adenosine becomes greater inside the cell than outside the cell, direct efflux of adenosine occurs. Adenosine formation within cells depends on the breakdown of ATP, and occurs as a result of the action of cytosolic-5’-nucleotidase on 5’-AMP (Zimmermann and Braun, 1996). Another source of 5’-AMP is metabolism of cAMP by cAMP phosphodiesterase. In general, adenosine is released when the brain’s ability to synthesize ATP is outstripped by its energy requirements. The large increase in energy requirements that occurs during seizure, or the loss of metabolic substrates that occurs during ischemia, reduces ATP levels and increases the level of adenine nucleotides and adenosine.

1.5. Removal of extracellular adenosine

The primary mechanism by which adenosine is cleared from the extracellular space is by transport of adenosine into cells followed by either deamination to inosine by adenosine deaminase or phosphorylation to AMP by adenosine kinase (Latini and Pedata, 2001). Uptake inhibitors dramatically increase the concentration of extracellular adenosine, indicating that under normal conditions the majority of adenosine in the extracellular space is cleared by uptake (Dunwiddie and Diao, 1994). In support of this concept, the nucleoside transport inhibitors such as propentofylline protect against brain damage after global ischemia by increasing extracellular adenosine (Fredholm et al., 1994). During hypoxia and ischemia, adenosine transporters are not able to remove adenosine, allowing adenosine deaminase activity to assume the primary role in removing adenosine (Lloyd and Fredholm, 1995; Barankiewicz et al., 1997; Dupere et al., 1999).
1.6. Functional roles of ATP and adenosine in the CNS

Purinergic transmission plays a functional role in a vast array of physiological and pathological processes in the CNS. Adenine nucleotide-gated P2 receptors have been implicated in the pain associated with cancer, reflex sympathetic dystrophy, angina, causalgia, lumbar, and migraine, as well as inflammation, seizure induction, astrocyte proliferation, and astrogliosis associated with ischemia and neurodegenerative disorders (Burnstock, 2004).

Observations that awareness and learning were enhanced by caffeine, which is the most widely used psychoactive drug in the world and a classical adenosine receptor antagonist, has long been an impetus into studying the role of adenosine in the nervous system. Numerous lines of evidence now show that adenosine P1 receptors have functions in sleep and arousal, seizure susceptibility, analgesia, locomotor effects, neuroprotection, Parkinson’s disease, anxiety, and alcohol and drug addiction (Dunwiddie and Masino, 2001).

Some of the most important functions of ATP and/or adenosine in normal and pathological physiology are discussed in further detail below.

1.6.1. Pain

Systematic administration of ATP elicits pain responses. The antinociceptive properties of non-selective P2 receptor antagonists (e.g. suramin) may be due to antagonism of P2X3 receptors located on sensory neurons in trigeminal, nodose, and dorsal root ganglia (Burnstock, 1996). In support of a role for P2X3 receptors in pain, nociception and inflammation are reduced in P2X3 knockout mice (Cockayne et al., 2000).
Adenosine also influences pain transmission through multiple mechanisms at peripheral and spinal sites. The A1 receptor is abundant in the dorsal horn of the spinal cord, where the density of receptors is almost as high as it is in the hippocampus (Johansson et al., 2001). A1 receptors are responsible for the analgesic effects of intrathecally administered A1 agonists, which cause antinoception by reducing cAMP levels in sensory nerve terminals (Sawynok, 1998). A1 receptor knockout mice show increased hyperalgesia, and loss of the analgesic effect of adenosine characteristic of wild-type animals (Johansson et al., 2001), although A1 receptors might be more important in chronic pain than acute pain (Wu et al., 2005). In humans, adenosine infusion into the spinal cord decreases post-operative pain (Gordh et al., 1995).

Adenosine A1 receptor activation might be an effective treatment for migraine and cluster headache because it inhibits neurotransmission without concomitant vasoconstriction (Giffin et al., 2003). Two A1 receptor agonists (GR79236 and GW-493838) and an A1 receptor-selective allosteric enhancer (T-62) are of clinical interest for the treatment of migraine and neuropathic pain (Giffin et al., 2003; Li et al., 2003; Zambrowicz et al., 2003).

It should be noted that caffeine has analgesic effects against some (but not all) types of pain (Fredholm et al., 1999), which is contrary to what might be expected based on the above studies showing that A1 receptor agonists are antinociceptive. This discrepancy probably arises because caffeine acts at A2A as well as A1 receptors (Ledent et al., 1997; Huang et al., 2005), and A2A receptors have different effects on pain transmission than A1 receptors. For example, A2A knockout mice exhibit slower responses to hot plate, tail-flick, and tail immersion tests than wild-type mice (Ledent et
al., 1997; Berrendero et al., 2003). In the spinal cord, adenosine released following opioid stimulation acts at both $A_1$ and $A_2A$ receptors to elicit antinociception (Sweeney et al., 1987; De Lander and Keil, 1994), whereas in the periphery, activation of $A_2A$ receptors stimulates nociceptive nerve terminals (McQueen and Ribeiro, 1986). Thus, the increased nociceptive threshold (i.e. increased tolerance for pain) in $A_2A$ knockout mice suggests that peripheral nociceptive $A_2A$ receptor-mediated nociception predominates over spinal $A_2A$ receptor-mediated antinociception. The above observations indicate that adenosine exerts a wide variety of effects on pain depending on the site of action and the subtype ($A_1$ or $A_2A$) of receptor that is activated.

1.6.2. Sleep and arousal

Because caffeine promotes wakefulness, it is logical to assume that adenosine plays a role in sleep and the regulation of arousal. The sleep-inducing effects of adenosine were first described in cats by Feldberg and Sherwood (1954), and have since been repeatedly demonstrated in a variety of species (Haulilca et al., 1973; Dunwiddie and Worth, 1982; Virus et al., 1983; Radulovacki et al., 1984; Radulovacki et al., 1985). Adenosine levels progressively increase during prolonged wakefulness and decrease during recovery sleep (Huston et al., 1996; Porkka-Heiskanen et al., 1997; Porkka-Heiskanen, 1999). The oscillation of adenosine concentrations through the circadian rhythm is consistent with evidence that adenosine release is directly proportional to metabolism and neural activity (Pull and McIlwain, 1972; Van Wylen et al., 1986; Tobler and Scherschlicht, 1990). Consistent with this idea is the fact that metabolic rate during wakefulness is approximately 30% higher than during non-REM sleep (Madsen et al., 1991; Maquet et al., 1992; Madsen, 1993). Because one of the functions of sleep is probably energy
restoration, it has been proposed that adenosine, as a by product of energy metabolism, may play a role in sleep-wake behaviour by serving as a homeostatic regulator of energy in the brain during sleep (Chagoya de Sanchez et al., 1993; Benington and Heller, 1995).

The \( A_1 \) receptor is the primary receptor subtype involved in regulating sleep, as sleep is induced by \( A_1 \) receptor agonists and reduced by antagonists (Virus et al., 1990; Lin et al., 1997; Portas et al., 1997). In addition, an \( A_1 \) receptor antisense construct decreases REM sleep and increases wakefulness when infused into the basal forebrain (Thakkar et al., 2003). Possible mechanisms by which \( A_1 \) receptors induce sleep include tonically inhibiting the firing of long cholinergic neurons by increasing \( K^+ \) conductances (Rainnie et al., 1994) and disinhibiting the GABAergic input to ventrolateral preoptic neurons (Chamberlin et al., 2003). In support of these mechanisms, adenosine dialysis into cholinergic nuclei or the preoptic area \textit{in vivo} promotes sleep and reduces the level of arousal as measured by EEG activity (Rainnie et al., 1994; Portas et al., 1997).

However, it should be noted that the amount of sleep or rebound after sleep deprivation is identical in \( A_1 \) receptor knockout mice as in wild-type controls, even though an \( A_1 \) receptor antagonist reduced sleep in control animals (Stenberg et al., 2003). Thus, it appears that \( A_1 \) receptors are not absolutely required for sleep because other unknown adaptive regulatory mechanisms are capable of taking over in their absence.

In summary, adenosine is an important endogenous homeostatic sleep factor that inhibits wake promoting neurons in the cholinergic basal forebrain via \( A_1 \) receptor activation.
1.6.3. Epilepsy

A role for adenosine as an endogenous modifier of seizures was long suspected because adenosine is a well known inhibitory neuromodulator that suppresses repetitive firing. In support for this notion, seizure susceptibility was reported to increase in the brain when adenosine levels were decreased (Gouder et al., 2004). It is well established that adenosine receptor agonists have anticonvulsant effects (Dunwiddie and Worth, 1982; Barraco et al., 1984; Zhang et al., 1990), whereas antagonists have proconvulsant effects (Dunwiddie and Hoffer, 1980; Ault et al., 1987; Alzheimer et al., 1989). The anticonvulsant effects of adenosine are mainly mediated by A₁ receptors (Murray et al., 1992; Zhang et al., 1994), although the A₂A receptor may play a role in some brain regions as well (De Sarro et al., 1999).

The above evidence suggests that raising the extracellular concentration of adenosine in the brain may be a viable therapeutic intervention against seizures. This is indeed the case, as transplanting adenosine-producing cells into rat brain decreases seizure susceptibility (Huber et al., 2001; Boison et al., 2002). A chronic reduction of A₁ receptors, and hence a loss of tonic inhibition, also occurs in epileptic tissue in both rats and humans, which might explain the hyperexcitability and recurrent seizures that are characteristic of epilepsy.

In further support of a clinical relevance for local adenosine release in epilepsy, mouse myoblasts were recently engineered to release adenosine by genetic inactivation of adenosine kinase and grafted into the lateral brain ventricles of rats kindled in the hippocampus (Guttinger et al., 2005). These grafts conferred complete long-term protection (up to 8 weeks) from the convulsive seizures observed in rats with wild-type
grafts, suggesting that adenosine release from cellular implants is a feasible option for the long-term treatment of focal epilepsies.

### 1.6.4. Alcoholism

The popular belief that caffeine can counteract the intoxicating effects of alcohol has led to the hypothesis that adenosine receptors, and $A_{2A}$ receptors in particular, play a role in mediating the intoxication resulting from acute alcohol administration. In support of this hypothesis, there is a growing body of evidence that adenosine mediates the neuronal responses to ethanol, and thus may be involved in a number of physiological effects such as alcohol-induced ataxia and alcohol addiction (Mailliard and Diamond, 2004).

Extracellular adenosine increases in response to ethanol exposure because ethanol inhibits a specific nucleoside transporter (ENT1) preventing adenosine uptake (Diamond et al., 1991; Krauss et al., 1993). This increase in extracellular adenosine activates $G_{s}$-coupled $A_{2A}$ receptors, leading to increased cAMP, activation of PKA I & II, PKA II translocation to the nucleus, and cAMP-induced gene expression (Mailliard and Diamond, 2004).

Addictive drugs such as ethanol increase dopamine release in the nucleus accumbens, a brain region intimately involved in reward and reinforcement (Hyman and Malenka, 2001). Activation of the dopamine $D_{2}$ receptor in particular has been implicated in the behaviour of alcohol consumption (Phillips et al., 1998). The microinjection of dopamine $D_{2}$ receptor agonists or antagonists into the nucleus accumbens can increase or decrease ethanol self-administration (Hodge et al., 1997). Interestingly, the most abundant expression of $A_{2A}$ receptors in the brain occurs in the medium spiny neurons of the nucleus accumbens (Jarvis et al., 1989; Fredholm et al.,
1998). D₂ receptors and A₂A receptors are coexpressed in these neurons (Fink et al., 1992), where they synergistically activate PKA and cAMP-dependent gene expression (Yao et al., 2002). Ordinarily, Gₛ coupled A₂A and G𝐢 coupled D₂ receptors are antagonistic. The synergistic action of A₂A and D₂ receptors depends on the release of G_i/o βγ dimers (Yao et al., 2002) that potentiate cAMP production via adenylate cyclase isoforms II and IV (Tang and Gilman, 1991; Federman et al., 1992; Inglese et al., 1994; Baker et al., 1999). Blocking βγ dimers by expression of a βγ inhibitor in the nucleus accumbens decreases voluntary consumption of alcohol (Yao et al., 2002), suggesting that the signalling pathways responsible for A₂A/D₂ synergy could be targeted for treating alcoholism.

1.6.5. Neuroprotection

The brain is extraordinarily vulnerable to ischemic events due to its high metabolic rate. A 5 minute interruption of cerebral blood flow is sufficient to kill neurons, whereas it takes 20-40 minutes of ischemia to kill cardiac or kidney cells (Lee et al., 2000a). The very intracellular and intercellular signalling mechanisms that normally make the brain so specialized for information processing become its bane under ischemic conditions, accelerating cell death by rapid energy depletion and excessive excitation (Lee et al., 2000a).

Adenosine is thought to be an endogenous neuroprotective agent in the CNS because it attenuates damage caused by electrical activity (Arvin et al., 1989; Lloyd et al., 1993), excitotoxicity (Arvin et al., 1988; Arvin et al., 1989; Finn et al., 1991; MacGregor and Stone, 1993), hypoxia (Gribkoff and Bauman, 1992; Fowler, 1993b, 1993a), ischemia (Lloyd et al., 1993; Latini et al., 1999a), and even mechanical stimulation
(Mitchell et al., 1995) and methamphetamine-induced neurotoxicity (Delle Donne and Sonsalla, 1994). Increasing extracellular adenosine by preventing its uptake or inhibiting its degradation (by administering adenosine kinase and deaminase inhibitors) protects against excitotoxic or ischemic brain injury (DeLeo et al., 1988; Phillis and O'Regan, 1989; Dux et al., 1990; Lin and Phillis, 1992; Gidday et al., 1995; Johnson et al., 1998). Similar results are obtained by stimulating A<sub>1</sub> (Rudolphi et al., 1992) receptors or blocking A<sub>2A</sub> receptors (Jones et al., 1998), whereas A<sub>1</sub> receptor antagonists exacerbate the resulting neuronal damage (Rudolphi et al., 1992; Zhou et al., 1994).

In animal models of global or focal ischemia, local administration of CADO, an adenosine analogue, attenuates cell death in the CA1 region of the hippocampus (Evans et al., 1987). Specific A<sub>1</sub> receptor activation during global forebrain ischemia (with the agonists CHA, CPA, or CCPA) reduces mortality and neuronal loss in the hippocampus and improves neurological deficits (von Lubitz et al., 1988; Januszewicz von Lubitz et al., 1989; von Lubitz and Marangos, 1990; Von Lubitz et al., 1994; Zhou et al., 1994; Von Lubitz et al., 1996). In contrast, acute administration of A<sub>1</sub> receptor antagonists such as theophylline or DPCPX during ischemia potentiates mortality and degeneration in hippocampal cells and leads to memory impairment (Rudolphi et al., 1987; Boissard et al., 1992; Zhou et al., 1994; Phillis, 1995).

1.6.5.1. **Mechanism of A<sub>1</sub> receptor-mediated neuroprotection**

There are three cellular mechanisms by which A<sub>1</sub> receptor activation leads to neuroprotection: 1) inhibition of voltage-dependent Ca<sup>2+</sup> channels and decreased Ca<sup>2+</sup> influx; 2) inhibition of neurotransmitter release (particularly glutamate); and 3) activation of postsynaptic GIRKs leading to membrane hyperpolarization (Dunwiddie and Masino,
2001). These actions are neuroprotective both because they limit Ca\(^{2+}\) entry, which is a key trigger of excitotoxic damage (Sattler and Tymianski, 2000), and because they reduce metabolic demand, thus preserving ATP stores necessary for pumping Ca\(^{2+}\) out of cells.

Interruption of cerebral blood flow results in a rapid rise in adenosine levels in cortical areas in a number of species (Rudolphi et al., 1992; de Mendonca et al., 2000), which can then activate presynaptic A\(_1\) receptors leading to decreased Ca\(^{2+}\) influx through N-type voltage-dependent Ca\(^{2+}\) channels (Mogul et al., 1993; Yawo and Chuhma, 1993; Mynlieff and Beam, 1994; Wu and Saggau, 1994; Ambrosio et al., 1997; Zhang and Schmidt, 1999; Brown and Dale, 2000; Park et al., 2001; Sun et al., 2002; Wang et al., 2002b; Manita et al., 2004) and thus decreased glutamate release. Excitatory amino acids such as glutamate, which are released immediately after ischemic injury, induce cell death by causing excessive membrane depolarization and a rise in intracellular Ca\(^{2+}\) (Simpson et al., 1992; Martin et al., 1994; Arundine and Tymianski, 2003). The net result of presynaptic A\(_1\) receptor activation is a decrease in NMDA receptor activation and less NMDA-mediated Ca\(^{2+}\) influx into neurons, which is vitally important in achieving neuroprotection.

In addition to activating presynaptic A\(_1\) receptors, adenosine also mediates neuroprotection by activating postsynaptic A\(_1\) receptors. The hypothesis that postsynaptic A\(_1\) receptors are involved in neuroprotection has been tested in a number of models in which A\(_1\) receptor agonists were found to attenuate the neuronal damage induced by NMDA, kainate, quisqualate, or ibotinate (Arvin et al., 1988; Arvin et al., 1989; Finn et al., 1991; MacGregor and Stone, 1992; MacGregor et al., 1993; MacGregor et al., 1996b; MacGregor et al., 1996a; MacGregor et al., 1997; MacGregor et al., 1998).
Moreover, $A_1$ receptor antagonists potentiate kainic acid-induced neurotoxic effects in the hippocampus (MacGregor and Stone, 1992; MacGregor et al., 1993; MacGregor et al., 1996b; MacGregor et al., 1996a; MacGregor et al., 1997; MacGregor et al., 1998).

Postsynaptic $A_1$ receptor stimulation reduces the excitotoxic effects of excitatory amino acids by activating GIRKs and thus counteracting excessive membrane depolarization by increasing $K^+$ efflux (Dunwiddie and Masino, 2001). The result is decreased neuronal $Ca^{2+}$ influx through voltage dependent $Ca^{2+}$ channels. Adenosine may act as a neuroprotector by elevating the threshold for NMDA receptor channel opening and thus maintaining $Ca^{2+}$ homeostasis in postsynaptic neurons.

Given the anti-neuroprotective effects of adenosine receptor antagonists, it would be reasonable to predict that chronic consumption of caffeine would potentiate ischemic injury. Surprisingly, this is not the case. In contrast to the damaging effects of acute administration of caffeine or DPCPX, chronic administration (2-4 weeks prior to ischemic insult) of these compounds lowers neuronal injury as assessed by magnetic resonance and histology (Rudolphi et al., 1989; Sutherland et al., 1991; Von Lubitz et al., 1994; Rudolphi and Schubert, 1997). The beneficial effects of chronic administration of $A_1$ receptor antagonists may be due to upregulation of $A_1$ receptors (Rudolphi et al., 1989), although such a mechanism is still the subject of debate (Jacobson et al., 1996).

Finally, it should be noted that there is no difference in the severity of ischemic damage between $A_1$ receptor knockout and wild type mice, even though acute administration of an $A_1$ receptor antagonist potentiated ischemic damage in vivo in response to the same ischemic insult (Olsson et al., 2004). This is surprising considering the amount of evidence supporting a role for the $A_1$ receptor in neuroprotection, and
highlights one of the pitfalls of using knockout mice to study receptor function: the emergence of compensatory mechanisms.

1.6.5.2. Ischemic preconditioning

Cerebral ischemic preconditioning is a phenomenon in which a brief episode of sublethal ischemia increases the brain’s tolerance for subsequent longer and more severe ischemic insults, presented hours or even days later (Lee et al., 2000a). Ischemic preconditioning was initially described in the heart (Swain et al., 1984), but it occurs in a variety of organs (Hawaleshka and Jacobsohn, 1998). The primary mechanisms underlying ischemic preconditioning in the brain are thought to be adenosine release, activation of A₁ receptors, activation of MAPKs, and the opening of ATP-dependent K⁺ channels (Heurteaux et al., 1995). Adenosine’s involvement in ischemic preconditioning highlights its central role in endogenous neuroprotection.

1.7. Mitogen-activated protein kinases

Integrated function of the cell is achieved through highly interactive networks of protein kinases and other second messenger systems. This is partly achieved by activation or deactivation of regulatory proteins through modulation of protein phosphorylation states. Mitogen-activated protein kinases (MAPKs) are a ubiquitous family of conserved signal transducers within the cellular signalling network. There are three important members of this family: 1) the extracellular signal-regulated kinase (ERK); 2) p38 MAPK; and 3) C-Jun N-terminal kinase (JNK), which are classified according to their phospho-acceptor motif (T/SEY, TGY, and TPY respectively). Each is activated by dual threonine and tyrosine phosphorylation. The ERK group consists of ERK1 (p44 MAPK), ERK2 (p42 MAPK), and ERK3 (p54 MAPK).
MAPK), ERK3α, ERK3β, ERK5, and ERK7. There are three JNK gene products (JNK1, JNK2, and JNK3) and four p38 MAPK gene products (p38α, p38β, p38γ, and p38σ).

Classic MAPK functions include regulation of cell differentiation, cell proliferation, embryogenesis, and cell death. However, a substantial amount of evidence has emerged over the course of the past decade that also implicates MAPKs in ion channel regulation, cardioprotection, neuroprotection, synaptic plasticity, and learning and memory.

### 1.7.1. Classic MAPK cascade

MAPK activation is rapid, allowing cells to respond to environmental changes in a tightly controlled series of three sequential intracellular protein kinase activation steps. The MAPK cascade is initiated by activation of MAPK kinase kinase (MAPKKK), which in turn phosphorylates (and thus activates) MAPK kinase (MAPKK), which then finally activates the specific MAPK. MAPKs are proline-directed kinases, meaning that they regulate a wide array of protein kinases, nuclear proteins, and transcription factors through phosphorylation of serine or threonine residues that are adjacent to proline.

### 1.7.2. Signaling by G protein-coupled receptors to MAPKs

Receptors that transduce signals through activation of heterotrimeric GTP-binding proteins (G proteins), or G Protein-coupled receptors, form the largest superfamily of cell surface receptors encoded by the human genome, with over 1000 members (Flower, 1999). G protein-coupled receptors are activated by a wide array of stimuli, such as polypeptides, chemoattractants, growth factors, hormones, neurotransmitters, phospholipids, odorants, photons, and taste ligands. G protein-coupled receptors contain
a conserved structural motif consisting of seven α-helical membrane spanning regions, which undergo significant changes upon receptor activation (Dixon et al., 1986; Dohlman et al., 1987). The Go subunit is GDP bound and closely associated with the Gβγ heterodimer in the absence of ligand. The Go-GDP/Gβγ heterotrimer is associated with the cytosolic loops of a G protein-coupled receptor. Binding of a ligand to the G protein-coupled receptor induces a conformational change in the Go subunit which results in the release of GDP and binding of GTP in its place. Go-GTP dissociates from the G complex exposing effector interaction sites in the βγ heterodimers. Both Go-GTP and Gβγ are capable of signalling to their respective effectors. Go hydrolysis of the gamma-phosphate moiety of GTP returns the cycle to the basal state (Lambright et al., 1994; Sondek et al., 1994; Lambright et al., 1996; Sondek et al., 1996; Hamm, 1998; McCudden et al., 2005).

There are four major families of G protein α subunits based on their primary sequence similarity:Gs, Gi, Gq, and G12 (Wilkie et al., 1992). Each regulates the activity of several second messenger systems. For example, Gs α subunits activate adenylyl cyclases whereas Gi α subunits inhibit adenylyl cyclases and thus oppositely influence intracellular concentrations of adenosine 3',5'-monophosphate (cAMP) (Simonds, 1999). Gβγ dimers themselves regulate numerous signalling molecules, such as receptor kinases, adenylyl cyclases, phospholipases, phosphatidylinositol 3-kinases (PI3Ks), and ion channels (Clapham and Neer, 1997).

There is extensive evidence that G protein-coupled receptors are capable of activating MAPKs via an intricate signalling network (Gutkind, 2000). The complexity of the signal transduction network leading to MAPK activation is a reflection of the role
of MAPKs as multitasking mediators able to generate a specific response to a diversity of upstream stimuli, depending on the receptor, agonist, and cellular background. The details of the signalling pathways linking G protein-coupled receptors to ERK1/2 activation are known in more detail than signalling to p38 MAPK or JNK. Generally, ERK is phosphorylated by MEK (its MAPKK), which acts downstream of the protein kinase Raf. Signals transduced by G protein-coupled receptors converge on this three kinase module through Ras-GTPase, which activates Raf to stimulate MEK (Kolch, 2000) (Fig. 1.1).

G protein-coupled receptor-mediated stimulation of p38 MAPK and JNK is well documented, but the exact mechanism of activation is not yet clear. Activation of JNK by βγ subunits appears to occur through MKK4 or MKK7 (JNK-specific MAPKKs) (Yamauchi et al., 1999) following activation of two small GTPases of the Rho family, Rac1 and Cdc42 (Collins et al., 1996; Mitsui et al., 1997), whereas Gα12 stimulates JNK through Src-like kinases (Nagao et al., 1999). There is even less knowledge on the mechanism of activation of p38 MAPK by G protein-coupled receptors. Both Gα subunits and βγ dimers can activate p38 MAPK by initiating signalling cascades that converge on a tri-kinase phosphorelay module that is specific to p38 MAPK (Yamauchi et al., 1997). This module phosphorylates p38 MAPK after MKK3 or MKK6 (p38-specific MAPKKs) are phosphorylated by MLK3, MEKK4, ASK1, or TAK1 (p38-specific MAPKKKs) (Gallo and Johnson, 2002).

1.7.3. Involvement of MAPKs in synaptic plasticity

After MAPKs were first identified and found to play a role in response to growth factors and other mitogens to regulate proliferation and differentiation, it was observed that
Figure 1.1. G protein-coupled receptor-dependent activation of MAPKs.

JNK is phosphorylated by MKK4 and MKK7, which can be activated by βγ subunits in a pathway involving the small GTPases Rac and Cdc42. Although less is known about the molecular mechanisms connecting G protein-coupled receptors and p38 MAPK, it has been shown that p38 MAPK can be activated by both α and βγ subunits through MKK3 and MKK6 (Gutkind, 2000).
MAPKs, their upstream regulators, and many of their downstream targets are highly expressed in mature neurons (Boulton et al., 1991), when proliferation and differentiation are no longer required. The discovery that glutamatergic signalling activates ERKs (Fiore et al., 1993; Kurino et al., 1995; Xia et al., 1996) suggested that MAPKs play a role in the normal functioning of the mature nervous system, and led many researchers to explore the role of MAPKs in synaptic plasticity. MAPK involvement in numerous forms of synaptic plasticity in the mammalian brain has since been reported (Thomas and Huganir, 2004).

Long lasting changes in synaptic transmission can occur at CA3-CA1 synapses when brief periods of synaptic activity lead to NMDA-sensitive glutamate receptor opening, increased postsynaptic Ca\(^{2+}\) concentration, activation of intracellular signalling pathways, enlargement of dendritic spines, the appearance of new filopodia, and synaptic delivery and removal of AMPA-sensitive glutamate receptors (Malinow and Malenka, 2002; Sheng and Kim, 2002; Bredt and Nicoll, 2003; Thomas and Huganir, 2004). All three MAPK families have now been implicated in one or more of the above processes underlying synaptic plasticity.

ERK inhibition with MEK inhibitors or dominant-negative Ras expression prevents activity-dependent dendritic enlargement and the appearance of new filopodia (Wu et al., 2001). ERK1/2 is also activated by Ras to signal delivery of AMPA receptors containing GluR1 and GluR2L (long cytoplasmic termini) subunits during LTP (Zhu et al., 2002a). p38 MAPK is activated by Rap1 to control the removal of GluR2 and GluR3 during LTD (Zhu et al., 2002a). JNK activation is required for Rap2-dependent removal of GluR1 and GluR2L during depotentiation (Zhu et al., 2005b).
MAPKs are also involved in other postsynaptically expressed forms of synaptic depression. For example, p38 MAPK and ERK1/2 are required for the induction of LTD by chemical stimulation of group I metabotropic glutamate receptors (Bolshakov et al., 2000; Gallagher et al., 2004). In addition, JNK regulates electrically induced LTD in the dentate gyrus (Curran et al., 2003).

MAPKs may also be involved in presynaptic changes in synaptic transmission. Blocking JNK activity increases baseline synaptic transmission and depresses paired-pulse facilitation (Costello and Herron, 2004), which suggests that endogenous JNK may be involved in the regulation of neurotransmitter release. Moreover, inhibition of JNK and p38 MAPK activity attenuates Aβ- and interleukin-1β-mediated impairment of LTP (Vereker et al., 2000a; Curran et al., 2003; Costello and Herron, 2004). Interestingly, the increases in p38 MAPK and JNK phosphorylation associated with interleukin-1β treatment corresponds to decreased glutamate release from dentate gyrus synaptosomes (Vereker et al., 2000a), further implicating p38 MAPK and JNK in presynaptic mechanisms of synaptic transmission and plasticity in the brain.

1.7.4. Role of MAPKs in cardioprotection and neuroprotection

In heart tissue, numerous studies show that MAPK cascades are involved in cardioprotection. All three MAPKs are activated by ischemia in the heart, but p38 MAPK is the most readily activated by ischemia. p38 MAPK is markedly and transiently activated after only a few minutes of ischemia, and generally returns to control levels after 30 minutes (Yin et al., 1997; Shimizu et al., 1998). A number of studies have reported that p38 MAPK activity is necessary for acute and delayed ischemic and pharmacological preconditioning in the heart (Fryer et al., 2001a; Fryer et al., 2001b;
Schulte et al., 2004; Lasley et al., 2005). However, it has also been reported that p38 MAPK inhibition is neuroprotective (Mackay and Mochly-Rosen, 1999, 2000; Marais et al., 2001; Martin et al., 2001).

1.7.4.1. The role of \( \text{A}_1 \) receptors in p38 MAPK-mediated ischemic preconditioning

There is evidence that p38 MAPK is activated by adenosine \( \text{A}_1 \) receptor stimulation in several tissues, including heart (Haq et al., 1998; Robinson and Dickenson, 2001; Liu and Hofmann, 2003). Adenosine \( \text{A}_1 \) receptor-dependent p38 MAPK activation has been implicated in delayed ischemic preconditioning. The p38 inhibitor SB203580 blocks \( \text{A}_1 \) receptor agonist-induced late phase preconditioning in mouse heart (Zhao et al., 2001b) and human atrial tissue (Carroll and Yellon, 2000; Loubani and Galinanes, 2002). In addition, \( \text{A}_1 \) receptor stimulation induces p38 MAPK activity in rabbit myocardium (Dana et al., 2000). \( \text{A}_1 \) receptor-dependent activation of p38 MAPK is also necessary for delayed preconditioning of rat myocardium \textit{in vivo} (Lasley et al., 2005).

The role of p38 MAPK in ischemic preconditioning in the brain is less clear. p38 MAPK is activated within minutes of transient forebrain ischemia \textit{in vivo} (Sugino et al., 2000), where it induces tolerance to a serious ischemic insult following a brief sublethal ischemic insult in the gerbil hippocampus (Nishimura et al., 2003). However, whether p38 MAPK is specifically activated by the \( \text{A}_1 \) receptor during cerebral ischemia has not yet been tested.

Another type of preconditioning that occurs in the brain is called anaesthetic preconditioning. Volatile anaesthetics, if applied minutes or days before an ischemic insult, can precondition the brain against subsequent ischemic injuries (Kapinya et al.,
2002; Zhao and Zuo, 2004; Bickler et al., 2005; Gray et al., 2005). Interestingly, cerebral anaesthetic preconditioning requires the activation of both $A_1$ receptors (Liu et al., 2006) and p38 MAPK (Zheng and Zuo, 2004). Whether $A_1$ receptors specifically activate p38 MAPK to elicit anaesthetic preconditioning is not known.

1.8. Conclusion

Adenosine and ATP, acting at P1 and P2 purinoceptors, respectively, are implicated in a diverse array of brain functions in both physiological and pathophysiological conditions. Despite the obvious importance of purinergic signalling, little is known of the molecular mechanism by which purinoceptor activation leads to a number of important processes, such as decreased transmitter release and neuroprotection. The goal of the research presented here is to characterize components of the molecular signalling pathways linking purinoceptor activation and decreased neurotransmission in the hippocampus. I propose that MAPKs are activated by purinoceptors and that purinoceptor-MAPK signalling may represent a novel mechanism underlying the regulation of neurotransmitter release in the mammalian brain.

1.9. References


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Chapter Two: Activation of P2X$_7$-Like Receptors Depresses Mossy Fiber-CA3 Synaptic Transmission Through p38 Mitogen-Activated Protein Kinase

A version of this chapter was previously published:

2.1. Introduction

Adenosine 5'-triphosphate (ATP) is released from synapses throughout the peripheral and central nervous system (White, 1977; White, 1978; Jahr and Jessell, 1983; Edwards et al., 1992; Edwards and Gibb, 1993), where it can act on P2X receptors to modulate neurotransmission. P2X receptors are ligand-gated, calcium permeable cation channels (Khakh, 2001) that are activated by extracellular ATP. There are seven known P2X receptor subunits, P2X1-7. Of the seven subunits only P2X7 subunits are thought to function exclusively as homomeric receptors (North and Surprenant, 2000). Activation of P2X7 receptors can lead to the initiation of signaling cascades through second messengers such as phospholipase D (Kusner and Adams, 2000), p38 MAPK (Hu et al., 1998; Hide et al., 2000; Panenka et al., 2001) or the transcription factor NF-kappaB (Ferrari et al., 1997). Recent data suggests that the initiation of these signaling cascades could be mediated through putative protein interactions with the long cytoplasmic C-terminus of the P2X7 subunit (Denlinger et al., 2001; Kim et al., 2001). In some circumstances the pore formed by the P2X7 receptor may allow permeation of large cations (North and Barnard, 1997; North and Surprenant, 2000) that may eventually lead to cytolysis (Di Virgilio, 1995; Baricordi et al., 1999; Mutini et al., 1999).

P2X7 receptors are only activated by high extracellular concentrations of ATP. Low concentrations (nM) of ATP that are ineffective at activating P2X7 receptors are known to increase neuronal excitation and synaptic activity in the nervous system. For example, ATP-induced activation of P2X receptors can evoke single channel cation currents from chick ciliary ganglion nerve terminals (Sun and Stanley, 1996) and enhance the frequency of miniature endplate currents at the frog neuromuscular junction (Fu and
Poo, 1991). Activation of P2X receptors has also been shown to increase the frequency of miniature postsynaptic currents in DRG dorsal horn neuronal cocultures (Gu and MacDermott, 1997; MacDermott et al., 1999) and increase excitation in the hippocampus (Wieraszko and Seyfried, 1989; Inoue et al., 1992; Inoue et al., 1995). On the other hand, high concentrations of ATP (μM) are known to induce a long lasting form of synaptic depression that cannot be explained by the degradation of ATP into adenosine (Wieraszko and Seyfried, 1989). The synaptic depression mediated by high concentrations of ATP could be explained by the activation of presynaptic P2X<sub>7</sub> receptors. Recently Deuchars et al. (2001) reported the presynaptic localization of P2X<sub>7</sub> receptors in the spinal cord and brainstem of the rat. We subsequently investigated the anatomical distribution of P2X<sub>7</sub> receptors in the rat hippocampus. When we discovered that P2X<sub>7</sub> receptors were abundant on hippocampal mossy fiber terminals, we used whole-cell and extracellular field recordings to determine the actions of P2X<sub>7</sub> receptor activation on neurotransmission at this synapse. In the following study we provide physiological and pharmacological evidence that activation of these presynaptic P2X<sub>7</sub> receptors results in rapid and long lasting synaptic depression that is mediated through a p38 mitogen-activated protein (MAPK) signaling cascade.

2.2. Materials and methods

2.2.1. SDS-PAGE & western blotting

Hippocampal proteins were isolated from Sprague-Dawley rats (3-4 weeks old) and homogenized in 0.32 M sucrose. Small (P2) and large (P3) mossy fiber synaptosomal fractions were then isolated according to previously published methods (Terrian et al.,
Proteins were subjected to SDS-PAGE on 10% gels and probed with the following antibodies: rabbit anti-P2X7 polyclonal (1:18,000, Alomone Laboratories, Jerusalem, Israel), rabbit anti-NMDAR1 (1:3,000, Chemicon Temecula, CA) mouse anti-β-tubulin (1:6,000, Sigma, St. Louis, MO) and rabbit anti-synaptoporin (1:30,000, Synaptic Systems, Gottingen, Germany). Immunoreactive signals were visualized using peroxidase-labeled goat secondary antibodies (1:10,000, Jackson Immunoresearch, WestGrove, PA) and enhanced chemiluminescence (Lumi-Light\textsuperscript{plus}, Roche Diagnostics, Mannheim, Germany).

### 2.2.2. Immunocytochemistry

For immunocytochemistry, rats were anaesthetized and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were sectioned in the coronal plane (50 μm) on a vibrating microtome (VT100, Leica, Willowdale, Ont.) and processed for immunocytochemistry using standard procedures (Sloviter et al., 1996). The following primary antibodies were used: rabbit anti-P2X7 (1:3,000, Alomone Laboratories, Jerusalem, Israel) mouse anti-microtubule associated protein (MAP)-2 (1:20,000; Sigma, St. Louis, MO) or anti-syntaxin 1A/B (1:5,000; Stressgen, Victoria, BC). The following secondary antibodies were used: biotinylated SP-donkey anti-mouse or rabbit IgG, Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG or Cy5-conjugated donkey anti-rabbit IgG (1:1000 all from Jackson Immunoresearch, WestGrove, PA). Sections were imaged on an Axioskop LSM510 Laser Scanning Microscope (Carl Zeiss Microscopy, Jena, Germany).
2.2.3. Electrophysiology

Hippocampal slices (300 μm thick) were obtained from 10-30 day old rats, immersed in ice cold artificial cerebrospinal fluid (aCSF; see below) and incubated in a submersion chamber for at least 1 hr at room temperature. For recordings individual slices were transferred to either an interface chamber (Fine Science Tools, Foster City, CA) for extracellular recordings or a submersion chamber for whole cell voltage-clamp recordings. All recordings were done at room temperature. In either chamber, slices were superfused (2 ml/min) with aCSF consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 CaCl₂, and 10 glucose, aerated with 95% O₂/5% CO₂. Extracellular recordings were obtained with glass micropipettes filled with HEPES-buffered aCSF (resistance 1-3 MΩ). Extracellular recordings were filtered at 5 kHz, digitized at 10 kHz using a Digidata1200 interface (Axon Instruments, Foster City, CA) and stored on a Pentium III computer for later analysis using Clampfit (Axon Instruments, Foster City, CA). A bipolar tungsten-stimulating electrode was used to stimulate dentate granule cells, thereby activating mossy fibers. Mossy fiber-CA3 synaptic responses were measured in the stratum lucidum of the CA3 region and distinguished by their characteristic short latency, rapid rise time, large paired-pulse facilitation and >70% inhibition by mGluR agonist L-CCG-I.

Whole cell recordings were obtained using patch pipettes filled with 100 mM cesium methanesulfonate, 10 mM cesium-Bapta, 40 mM HEPES, 5 mM QX-314, adjusted to pH 7.3 with cesium hydroxide (resistance 1-3 MΩ CaCl₂ and MgSO₄ were increased to 4 mM in the aCSF for all whole cell recordings. During paired-pulse facilitation experiments picrotoxin (10 μM) was included in the patch pipette to block
GABA<sub>A</sub> receptors (Nelson et al., 1994; Xiang and Brown, 1998). Series resistance in all recordings was < 20 MQ and data was excluded if series resistance varied more than 15%. All recordings were digitized at 5-10kHz and filtered at 2kHz.

**Statistics.** All statistics were carried out using a paired (correlated groups) t-test except for the comparison between the effect of Bz-ATP and adenosine on slices incubated with SB203580. In this case, a simple one-way analysis of variance (ANOVA) was used.
2.3. Results

2.3.1. P2X$_7$ receptors were found on mossy fiber terminals

Western blotting and immunocytochemistry revealed that P2X$_7$ receptors were abundant on presynaptic terminals of the rat hippocampus. We used a P2X$_7$ antibody that was raised against amino acid residues 576-595 of the rat P2X$_7$ receptor subunit. This antibody recognized a single 70 kDa band in western blots of proteins isolated from the hippocampus (H; Figure 1A-B), small (P2) hippocampal synaptosomes and large, mossy fiber (P3) synaptosomes (Figure 2.1 B). Inclusion of the P2X$_7$ antigenic peptide (1:1) with the antibody blocked detection of the 70kDa P2X$_7$-immunoreactive band. We did not detect any signal in the P3 fraction with an antibody against iba-1, a protein selectively expressed in microglia (Ito et al., 1998). This indicates that microglia did not contaminate our P3 synaptosome preparation (data not shown). Immunocytochemistry with this P2X$_7$ selective antibody revealed dense immunoreactive terminals throughout mossy fiber termination zones in the dentate hilus (h) and stratum lucidum (luc) of CA3 (arrows in Figure 2.1 C). Fainter staining was also observed throughout the hippocampus and may represent immunoreactivity of other presynaptic terminals, or glial cells, such as microglia (Ferrari et al., 1996; Chessell et al., 1997; Di Virgilio et al., 1999) or astrocytes (Kukley et al., 2001; Panenka et al., 2001). Confocal microscopy confirmed that the P2X$_7$-immunoreactive boutons were presynaptic mossy fiber terminals because P2X$_7$-immunoreactivity was co-localized with presynaptic syntaxin 1A/B-immunoreactivity (Bennett et al., 1992; Ruiz-Montasell et al., 1996) (Figure 2.3 G-I) but not dendritic MAP-2-immunoreactivity (Figure 2.2 D-F).
Figure 2.1. P2X$_7$ receptors are located on presynaptic terminals of mossy fiber synapses. A, Western blot of proteins isolated from the rat hippocampus showed that the P2X$_7$ antibody recognized a single band of protein at an approximate molecular weight of 70 kDa. Lane 1 contains Amido Black (AB) stained proteins that were first immunoblotted in lane 2. B, P2X$_7$-immunoreactive bands of protein were present in proteins isolated from whole hippocampus (H) as well as small (P$_2$) synaptosomal and large (P$_3$) mossy fiber synaptosomal preparations. The presence of synaptoporin and relatively low abundance of NMDAR1 and $\beta$-tubulin indicates that P2X$_7$ receptors were highly enriched in the fraction containing mossy fiber terminals (P$_3$). C, Immunocytochemistry with this P2X$_7$ selective antibody revealed dense immunoreactivity throughout mossy fiber termination zones in the dentate hilus (h) and stratum lucidum (luc) of CA3 (arrows). Fainter staining was also observed throughout the hippocampus and may represent immunoreactivity of other presynaptic terminals. Abbreviations: rad, stratum radiatum; l-m, stratum lacunosum-moleculare; m, molecular layer; dgc, dentate granule cell layer. Scale bar=2 mm in C.
Figure 2.2. P2X7 immunoreactivity was not co-localized with dendritic MAP-2 immunoreactivity. D-F, P2X7-immunoreactivity (blue) was found throughout stratum lucidum, however, dendritic MAP2-immunoreactivity (green) did not colocalize with the punctate P2X7-immunoreactivity. Cell bodies were counterstained with ethidium bromide (red). Scale bar=70 µm in D, 50 µm in E, and 30 µm in F.
Figure 2.3. P2X7 immunoreactivity was co-localized with presynaptic syntaxin 1A/B immunoreactivity. G-I, Presynaptic syntaxin 1A/B immunoreactivity (green) was colocalized with the punctate P2X7-immunoreactivity (red) demonstrating that the mossy fiber terminals contained P2X7 receptors. Scale bar=100 μm.
2.3.2. P2X\textsubscript{7} receptor activation depressed mossy fiber-CA3 synaptic transmission

Next, we investigated the effect of P2X\textsubscript{7} receptor activation on synaptic transmission at mossy fiber synapses. First, we recorded evoked postsynaptic field potentials (fEPSPs) in stratum lucidum of CA3 following stimulation of the dentate granule cells (Figure 2.4). To ensure that we were recording from mossy fiber-CA3 synapses we first applied L-CCG-I (20 \textmu M), an mGluR agonist that selectively depresses mossy fiber inputs onto CA3 pyramidal cells (Manzoni et al., 1995; Schmitz et al., 2000). Bath application of L-CCG-I reversibly depressed the amplitude of the fEPSP (Figure 2.4 A). Subsequent bath application of the P2X\textsubscript{7} receptor agonist, 2'3'-O-(4-benzoylbenzoyl) -ATP (Bz-ATP; 30 \textmu M) also depressed the fEPSP (Figure 2.4 A). However, application of L-CCG-I during the peak of the Bz-ATP response did not result in any further depression of the synaptic response (fEPSP amplitude after Bz-ATP was 0.22 \pm 0.12 of control versus 0.17 \pm 0.09 of control in Bz-ATP + L-CCG-I, mean \pm s.e.m, n=3) indicating that P2X\textsubscript{7} receptor activation depressed the same population of synaptic inputs as L-CCG-I. Bz-ATP was also applied alone to monitor the time course of the P2X\textsubscript{7}-mediated synaptic depression without prior L-CCG-I application (Figure 2.4 B). This prevented any potential interactions between progressive drug applications. As shown in Figure 2.4 B, Bz-ATP caused a rapid and long lasting (> 2 hours) statistically significant [t(5)=10.37, p < 0.01] decrease in the fEPSP (fEPSP amplitude following Bz-ATP was 0.30 \pm 0.05 of control amplitude, mean \pm s.e.m., n=6).

In separate experiments, the postsynaptic response was blocked by NBQX and the presynaptic fiber volley was monitored following Bz-ATP application (Figure 2.4 C).
Figure 2.4. The P2X$_7$ agonist, Bz-ATP, depressed mossy fiber fEPSPs but had no detectable effect on the presynaptic fiber volley.

*A*, Averaged sample traces and plots of mossy fiber-CA3 synaptic responses recorded extracellularly from the stratum lucidum during one experiment at the indicated time points. L-CCG-I (20 μM) reversibly depressed the postsynaptic component of the field potential (indicated by asterisk in the first trace). Bz-ATP (30 μM) depressed the L-CCG-I-sensitive component of the fEPSP and coapplication of L-CCG-I did not cause further depression. *B*, Summary of separate experiments in which Bz-ATP was applied without preapplication of L-CCG-I. Averaged sample traces are shown before and after Bz-ATP application. Plot shows the mean values obtained from 6 slices. Bz-ATP depressed the mossy fiber fEPSP amplitude for greater than 2 hours. *C*, Single plot and mean sample traces from a single experiment where the field response was recorded in the presence of NBQX (20 μM) to monitor the presynaptic fiber volley. Bz-ATP had no effect on the presynaptic fiber volley. *D*, Summary of the effects of Bz-ATP on the fEPSP (n=6) and the presynaptic fiber volley (n=5). Asterix indicates statistical significance using a pair t-test, $p<0.01$. Scale bar A: 0.2 mV, 20 msec B: 0.5 mV, 10 msec C: 0.3 mV, 20 msec.
There were no significant \([t(4)=-1.13, p > 0.05]\) alterations in the presynaptic fiber volley as a result of Bz-ATP application (\(n=5\); summarized in Figure 2.4 \(D\)). Furthermore, we visualized mossy fiber terminals using a laser scanning microscope to see if Bz-ATP induced the uptake of YO-PRO-1 via pore dilation and cell lysis (e.g. Virginio et al., 1999). We did not observe any uptake of YO-PRO-1 after Bz-ATP application (data not shown; \(n=2\)). These data suggest that activation of P2X\(_7\)-receptors with Bz-ATP does not induce cytolysis of mossy fiber terminals.

Next, we obtained whole-cell voltage clamp recordings from CA3 pyramidal neurons to determine whether Bz-ATP selectively depressed mossy fiber-CA3 synaptic transmission or had a postsynaptic effect on AMPA receptors. As shown in Figure 2.5, Bz-ATP significantly \([t(5)=22.36, p < 0.01]\) depressed the amplitude of voltage-clamped mossy fiber EPSCs (mossy fiber EPSC amplitude after Bz-ATP was \(0.33 \pm 0.04\) of control, mean \(\pm\) s.e.m., \(n=6\)) but had no statistically significant \([t(4)=2.02, p > 0.05]\) effect on associative/commissural EPSCs (assoc/comm EPSC amplitude after Bz-ATP was \(0.81 \pm 0.10\) of control, mean \(\pm\) s.e.m., \(n=5\)). Associational/commissural responses were evoked by stimulation of stratum radiatum in the presence of L-CCG-I to block mossy fiber synapses. Bz-ATP also had no significant effect on the CA3 whole cell conductance (\(308 \pm 34\) pS before versus \(288 \pm 60\) pS after Bz-ATP) or holding current (\(65.8 \pm 8.2\) pA prior to Bz-ATP application versus \(71.6 \pm 7.9\) pA after Bz-ATP). Therefore, P2X\(_7\) receptor activation selectively depressed mossy fiber synapses and had no direct postsynaptic effect on CA3 neurons.
Figure 2.5. Activation of presynaptic P2X7 receptors with Bz-ATP selectively depressed synaptically evoked mossy fiber currents in CA3.

A, Plots of mean whole cell voltage-clamp recordings in CA3 pyramidal neurons following stimulation of the mossy fiber (MF) pathway (n=6 slices) or the associative/commissural (A/C) pathway (n=5 slices). Bz-ATP significantly depressed the amplitude of voltage-clamped MF EPSCs but had no significant effect on A/C EPSCs. B, Summary of the data presented in A. Asterix indicates statistical significance using a paired t-test, \( p < 0.01 \). C, Average sample traces from evoked responses following stimulation of the MF pathway or the A/C pathway. Scale bar C: 200 pA, 50 msec; 250 pA, 50 msec.
2.3.3. **Bz-ATP-induced synaptic depression was blocked by o-ATP**

To further delineate P2X<sub>7</sub> receptor involvement in this Bz-ATP-induced effect we assessed the ability of Bz-ATP to induce synaptic depression of mossy fiber-CA3 fEPSPs in the presence of the nonselective P2Y antagonist reactive blue 2 (RB2, 30 μM, Figure 2.6 A) or the P2X<sub>1,3,5,6</sub> receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 10 μM, Figure 2.6 B). As shown in Figure 2.6, both RB2 (n=5) and PPADS (n=4) failed to block the effect of Bz-ATP on mossy fiber fEPSPs [t(4)=13.81, p < 0.01 and t(3)=26.5, p < 0.01 respectively]. This data suggests that Bz-ATP-induced synaptic depression was not mediated by the nonselective activation of P2Y receptors or postsynaptically located P2X<sub>1,3,5,6</sub> receptors.

To determine if Bz-ATP-induced synaptic depression was mediated by activation of P2X<sub>7</sub> receptors we applied Bz-ATP in the presence of the P2X<sub>7</sub> receptor antagonist oxidized periodate-ATP (o-ATP, 100 μM, Murgia et al., 1993; Visentin et al., 1999). Consistent with Bz-ATP acting at presynaptic P2X<sub>7</sub> receptors, Bz-ATP-induced synaptic depression was potently inhibited by 2 hour preincubation with the P2X<sub>7</sub> antagonist o-ATP (in matched slices fEPSP amplitude after Bz-ATP was 0.30 ± 0.05 of control versus 0.81 ± 0.12 of control in slices pre-incubated with o-ATP, mean ± s.e.m, see Figure 2.6 C; n=5 for both). This P2X<sub>7</sub>-like pharmacological profile combined with our inability to detect a postsynaptic current in CA3 pyramidal cells suggests that Bz-ATP acted presynaptically at P2X<sub>7</sub> receptors to mediate mossy fiber synaptic depression.
Figure 2.6. The selective P2X$_7$ antagonist o-ATP blocked Bz-ATP induced depression of the mossy fiber-CA3 synaptic responses.

A, Plots and average sample traces of mossy fiber-CA3 synaptic responses recorded extracellularly from the stratum lucidum of CA3 in the presence of the nonselective P2Y antagonist reactive blue 2 (RB2, 30 μM, n=5 slices). RB2 failed to block the effect of Bz-ATP on mossy fiber fEPSPs. B, Similarly, the P2X$_{1,3,5,6}$ receptor antagonist pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADs) failed to block the effect of Bz-ATP on mossy fiber fEPSPs (n=4 slices). C, However, preincubation of the slices with the P2X$_7$ receptor antagonist o-ATP (100 μM, n=5 slices) significantly reduced the magnitude of the Bz-ATP induced depression compared to control (n=5 slices). D, Summary of data present presented in A-C. Asterix indicates significance using a paired t-test, $p < 0.01$. Scale bar A: 0.5 mV, 10 msec.
2.3.4. P2X$_7$ receptor activation increased paired-pulse facilitation

If Bz-ATP depresses mossy fiber synaptic transmission by activating presynaptic P2X$_7$ receptors, then Bz-ATP-induced depression should be associated with an increase in paired-pulse facilitation (PPF, e.g. Regehr et al., 1994; Salin et al., 1996). We monitored PPF while recording whole-cell synaptic evoked currents in CA3 neurons. Consistent with Bz-ATP activating presynaptic P2X$_7$ receptors, we observed a significant [t(4)= -4.65, p<0.01] increase in the ratio of the 2$^{nd}$ EPSC amplitude to the 1$^{st}$ EPSC amplitude immediately after application of Bz-ATP (ratio before Bz-ATP was 1.74 ± 0.05, ratio after was 2.14 ± 0.09, mean ± s.e.m., n=5 slices, Figure 2.7). This data indicates that Bz-ATP decreased the probability of release at mossy fiber synapses.

2.3.5. P2X$_7$ receptor-mediated synaptic depression required activation of p38 MAPK

Recent evidence suggests that MAPK activity is potently activated by synaptic activity and is essential for some forms of synaptic plasticity (Impey et al., 1999). For example, erk1/erk2 MAPK activation is essential for the induction of long-term potentiation and p38 MAPK activity is essential for the induction of long-term depression in CA1 of the hippocampus (Bolshakov et al., 2000). We have recently shown that activation of P2X$_7$ receptors in cultured astrocytes leads to activation of p38 and ERK1/2 MAPK (Panenka et al., 2001). To determine whether MAPK activity was necessary for the synaptic depression induced by Bz-ATP we preincubated the slices (2 hours) with the p38 MAPK inhibitor SB203580 (25 μM) or the ERK1/2 MAPK inhibitor PD98059 (50 μM). Bz-ATP-induced synaptic depression of the L-CCG-I sensitive mossy fiber-CA3
Figure 2.7. Activation of presynaptic P2X\textsubscript{7} receptors increased mossy fiber paired-pulse facilitation (PPF, 50msec).

PPF was monitored by recording whole-cell synaptic currents evoked in CA3 neurons (n=5 slices). \textit{A,} Mean PPF ratio before and after application of Bz-ATP. Asterix indicates statistical significance using a paired t-test, $p < 0.01$. We observed a significant increase in the PPF ratio following Bz-ATP application, which is consistent with Bz-ATP acting on presynaptic P2X\textsubscript{7} receptors. \textit{B,} Average sample traces before and after Bz-ATP application. The lower trace on the right was rescaled so that the first current was the same size after Bz-ATP as it was in control. Scale bar \textit{B;} 200 pA, 50 msec, rescaled traces 70 pA.
postsynaptic response was significantly \[t(3)=0.19, \ p>0.05\] blocked by inhibition of p38 MAPK activity with SB203580 (fEPSP amplitude after Bz-ATP was 0.93 ± 0.12 of control amplitude in slices preincubated with SB203580, \(n=4\), Figure 2.8A). In contrast, preincubation of the slices with the ERK1/2 MAPK activity inhibitor PD98059 failed to block \[t(3)=8.27, \ p < 0.01\] Bz-ATP-induced mossy fiber synaptic depression (fEPSP amplitude after Bz-ATP was 0.24 ± 0.11 of control in slices preincubated with PD98059, \(n=4\), Figure 6B). These data demonstrate that activation of p38 MAPK was necessary for P2X\(_7\) receptor-mediated depression of mossy fiber-CA3 synaptic transmission.

### 2.3.6. Inhibitory effects of adenosine were not mediated through p38 MAPK

ATP and some of its analogs can be rapidly degraded into adenosine by the actions of ectonucleotidase (Dunwiddie et al., 1997; Cunha et al., 1998). Thus, ATP application can inhibit synaptic transmission indirectly through adenosine formation and the activation of presynaptic A\(_1\)-receptors (Dunwiddie et al., 1997; Cunha et al., 1998; Cunha and Ribeiro, 2000; Dunwiddie and Masino, 2001). We could not use an A\(_1\) antagonist such as DPCPX because blocking A\(_1\) receptors results in persistent seizure activity in the CA3 region making it impossible to record stable mossy fiber responses as previously reported (Thummler and Dunwiddie, 2000). Therefore, to determine indirectly whether Bz-ATP-induced mossy fiber-CA3 synaptic depression was mediated through degradation of Bz-ATP into adenosine, we tested whether adenosine inhibits synaptic transmission through p38 MAPK activity. As shown in Figure 2.9, preincubation of the slices with SB203580 (25 \(\mu\)M) blocked Bz-ATP induced mossy fiber synaptic depression (\(n=4\)) but failed to have any effect on adenosine (30 \(\mu\)M) mediated mossy fiber
Figure 2.8. P2X\textsubscript{7} receptor mediated mossy fiber synaptic depression required p38 MAPK activity.

\textit{A-B}, Plots of mean mossy fiber-CA3 synaptic responses recorded extracellularly from stratum lucidum of CA3. \textit{A}, Preincubation of the slices in the p38 MAPK inhibitor SB203580 (25\textmu M) completely blocked Bz-ATP-induced synaptic depression but had no effect on the L-CCG-I-induced depression of the mossy fiber fEPSP (n=4 slices). \textit{B}, Preincubation of the slices with the erk1/erk2 MAPK inhibitor PD98059 (50\textmu M) failed to have any effect on Bz-ATP-induced synaptic depression (n=4 slices). \textit{C}, Summary of data presented in \textit{A-B}. Asterix indicates significance using a paired t-test, p<0.01. Scale bar \textit{A}: 0.5 mV, 10 msec.
Figure 2.9. The p38 antagonist, SB203580, blocked the actions of Bz-ATP but not the inhibition by adenosine (30µM).

A, Plot of an extracellular recording in which the p38 MAPK inhibitor SB203580 blocked the Bz-ATP-induced mossy fiber synaptic depression but failed to block the adenosine-induced inhibition of the mossy fiber fEPSP. B, SB203580 differentially affected the depression induced by Bz-ATP and adenosine. Therefore, adenosine does not exert its inhibitory actions through p38 MAPK and the presynaptic actions of Bz-ATP cannot be explained by the degradation of Bz-ATP into adenosine by ectonucleotidase activity. Asterix indicates statistically significance using a one-way ANOVA, $p < 0.01$. 

[Graph of extracellular recording showing the effects of LCCG, Bz-ATP, and Adenosine on normalized fEPSP amplitude over time.]

[Bar graph showing the normalized fEPSP amplitude for Bz-ATP and Adenosine with SB203580 (25µM) with a star indicating statistical significance.]
synaptic depression (n=4) [in slices preincubated with SB203580 the fEPSP amplitude following Bz-ATP was 0.95 ± 0.15 of control versus 0.15 ± 0.09 of control in adenosine; F(1,6)=21.58, p < 0.01]. Therefore, adenosine does not exert its inhibitory actions through p38 MAPK and the presynaptic actions of Bz-ATP cannot be explained by the degradation of Bz-ATP into adenosine.

2.4. Discussion

The results of the present study demonstrate that activation of presynaptic P2X$_7$ receptors results in the inhibition of neurotransmission at mossy fiber-CA3 synapses through a p38 MAPK -signaling pathway. First, we have used immunocytochemistry to demonstrate that P2X$_7$ receptors are abundant on presynaptic terminals of mossy fiber synapses in the rat hippocampus. Immunocytochemistry with a specific P2X$_7$ antibody resulted in the labeling of small terminal-like puncta throughout the hippocampus. P2X$_7$-immunoreactivity was particularly dense throughout the termination zones of hippocampal mossy fibers where it was completely colocalized with the presynaptic marker syntaxin 1A/B but not the dendritic marker MAP-2 (see Figures 2.2 & 2.3). Syntaxin 1A is known to be present in the presynaptic mossy fiber terminals and syntaxin 1B is present in the mossy fiber axons (Ruiz-Montasell et al., 1996). As demonstrated in Figure 2.3, all of the observed P2X$_7$-immunoreactivity in stratum lucidum was colocalized with the syntaxin 1A labeling of the presynaptic terminal. These data demonstrate that P2X$_7$ receptors are located presynaptically in stratum lucidum of the rat hippocampus. The specific presynaptic P2X$_7$ receptor localization shown here contrasts with the postsynaptic location of other known P2X receptors in the hippocampus of the
rat (e.g. P2X₂, P2X₄ and P2X₆, Le et al., 1998; Rubio and Soto, 2001). These postsynaptically located receptors are likely to contribute to the increase in excitation that is observed in hippocampus following application of low doses of ATP (Wieraszko and Seyfried, 1989).

Consistent with their presynaptic localization, activation of P2X₇ receptors with Bz-ATP completely depressed the L-CCG-I sensitive mossy fiber-CA3 synaptic response in extracellular field recordings. However, no direct effects of Bz-ATP on postsynaptic CA3 pyramidal neurons were observed when the conductance and holding current were monitored during whole-cell voltage clamp recordings. Furthermore, we found no significant effect of Bz-ATP on AMPA receptor mediated associative/commissural synaptic transmission in CA3. This observation is consistent with our conclusion that Bz-ATP selectively activates presynaptic P2X₇ receptors and suggests that at this concentration (30 µM), Bz-ATP did not activate other known postsynaptic P2X receptors (e.g. P2X₂, P2X₄, P2X₆). Although our conclusions support the involvement of P2X₇ receptors in presynaptic depression, the possible contribution of P2X₄ cannot be totally eliminated. The enhancement of PPF during the Bz-ATP-induced synaptic depression is also consistent with a presynaptic site of action (Regehr et al., 1994; Salin et al., 1996) similar to what has been observed during mGluR-mediated depression in CA1 (Fitzjohn et al., 2001). Bz-ATP induced synaptic depression was not blocked by the P2Y receptor antagonist RB2 (30 µM) or the P2X₁,₃,₅,₆ antagonist PPADS (10 µM). Other P2X receptors are antagonized by PPADS at this concentration whereas P2X₇ receptors are not (Surprenant et al., 1996). However, Bz-ATP mediated synaptic depression required P2X₇ receptor activation because little or no synaptic depression was observed when the slices
were preincubated with the irreversible P2X<sub>7</sub> receptor antagonist o-ATP (see Figure 2.6; Murgia et al., 1993; Visentin et al., 1999). Bz-ATP-induced synaptic depression also cannot be explained by the degradation of Bz-ATP into adenosine by local ectonucleotidases because adenosine mediated synaptic inhibition was not blocked by p38 MAPK inhibition whereas Bz-ATP’s actions were (see below).

Mossy fiber synapses contain vesicular ATP, and synaptosomes prepared from mossy fiber synapses release ATP in a Ca<sup>2+</sup> dependent manner in response to K<sup>+</sup>-induced depolarization (Terrian et al., 1989). However, it is not known whether ATP is normally released from mossy fiber synapses or whether P2X<sub>7</sub> receptors are normally activated during the evoked release of neurotransmitters from mossy fiber terminals. It is possible that presynaptic mossy fiber P2X<sub>7</sub> receptors might only be activated during intense periods of mossy fiber activity such as that observed during tetanus or seizure when ATP release might reach levels high enough to activate P2X<sub>7</sub> receptors. Therefore P2X<sub>7</sub> receptors might play an important role in limiting synaptic transmission when mossy fiber synaptic transmission is unusually high.

The results of the present study demonstrate that activation of p38 MAPK is necessary for P2X<sub>7</sub> receptor mediated depression of mossy fiber-CA3 synaptic transmission. Maruyama et al. (2000) have recently demonstrated that p38 MAPK is abundant in the terminals of mossy fiber synapses. As shown in Figure 2.8, Bz-ATP-induced synaptic depression was completely blocked by preincubation of the slices with the p38 MAPK activity inhibitor SB203580 but not the ERK1/2 MAPK activity inhibitor PD98059. The presynaptic mechanism by which P2X<sub>7</sub> receptor-dependent p38 MAPK activity depresses mossy fiber synaptic transmission remains to be determined. However,
recent evidence suggests that p38 MAPK activity is necessary for the inhibition of N-type calcium currents in neuroblastoma cells following bradykinin application (Wilk-Blaszczak et al., 1998). Therefore, it is possible that P2X7 receptor-dependent p38 MAPK activity depresses mossy fiber synaptic transmission through inhibition of calcium channels. However, mossy fiber terminals exhibit predominantly P-type calcium channel-dependent evoked neurotransmitter release and contain few N-type channels (Castillo et al., 1994).

Recent evidence suggests that MAPK activity is potently activated by synaptic activity and is essential for some forms of neuronal plasticity (Impey et al., 1999; Bolshakov et al., 2000). For example, translocation of erkl/erk2 MAPK to the nucleus of the presynaptic neuron is essential for long-term facilitation in Aplysia neurons (Martin et al., 1997) and p38 MAPK is essential for the induction of mGluR-receptor dependent long-term depression in CA1 of the hippocampus (Bolshakov et al., 2000). Interleukin-1β has also been shown to increase p38 activation and modify long-term potentiation in perforant path synapses (Vereker et al., 2000). In the present study, we have shown that the rapid and reversible depression of mossy fiber synaptic transmission by the mGluR agonist L-CCG-I is unaffected by preincubation of the slices with the p38 MAPK inhibitor SB203580. Therefore, the mGluR-induced inhibition of mossy fiber synapses is apparently not mediated by p38 as it is in CA1.

In conclusion, we have provided evidence that P2X7 receptor subunits are abundant on presynaptic terminals of mossy fiber synapses in the rat hippocampus. Activation of these presynaptic P2X7 receptors with the P2X7 agonist Bz-ATP produced a rapid and long lasting synaptic inhibition at mossy fiber-CA3 synapses. This presynaptic
inhibition was mediated by the activation of p38 MAPK, because it was not observed when the slices were preincubated with a p38 MAPK inhibitor. Therefore, the results of the present study demonstrate that unlike any other member of the P2X-receptor family, P2X7 receptors can decrease neurotransmitter release at mossy fiber-CA3 synapses by activating p38 MAPK in the presynaptic terminal.

2.5. References


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Chapter Three: p38 Mitogen-Activated Protein Kinase Contributes to Adenosine A₁-Receptor-Mediated Synaptic Depression in Area CA1 of the Rat Hippocampus

A version of this chapter has been submitted for publication:

3.1. Introduction

Extracellular adenosine concentrations in the brain can increase 30-100 fold following pathological trauma such as head injury, epileptic seizures, and hypoxia/ischemia (During and Spencer, 1992; Bell et al., 1998; Von Lubitz, 1999). Adenosine inhibits glutamate release via A\textsubscript{1} receptors (Fowler, 1989; Katchman and Hershkowitz, 1993; Zhu and Krnjevic, 1993), and may act as an endogenous neuroprotectant by preventing glutamate excitotoxicity (Dunwiddie and Masino, 2001).

There is evidence that p38 mitogen-activated protein kinase (MAPK) can be activated by the adenosine A\textsubscript{1}-receptor via a G-protein-dependent pathway (Robinson and Dickenson, 2001). p38 MAPK is a member of a large family of MAPKs that mediate inflammation, cell proliferation, and apoptosis (Seger and Krebs, 1995; Cobb, 1999). MAPKs are also activated by synaptic activity and are essential for some forms of synaptic plasticity (Impey et al., 1999). In rat hippocampus, p38 MAPK activation mediates metabotropic glutamate-receptor-dependent long-term depression (LTD) (Bolshakov et al., 2000; Rush et al., 2002), as well as Rap-dependent removal of AMPA receptors during LTD (Zhu et al., 2002a). p38 MAPK also mediates inhibition of long-term potentiation by β-amyloid (Saleshando and O'Connor, 2000; Wang et al., 2004) and interleukin-1β (Coogan et al., 1999; Vereker et al., 2000a).

In heart tissue, p38 MAPK is activated by adenosine (Haq et al., 1998), and plays an important role in cardioprotection during ischemia (Weinbrenner et al., 1997; Baines et al., 1998; Baines et al., 1999). A\textsubscript{1}-receptor activation triggers early and delayed ischemic preconditioning (Thornton et al., 1992; Tsuchida et al., 1993) and mediates
myocardial protection by activating p38 MAPK (Zhao et al., 2001b; Schulte et al., 2004). Recent studies have confirmed that A₁ receptor-mediated delayed preconditioning against myocardial infarction is dependent on p38 MAPK in vivo (Lasley et al., 2005).

Two independent lines of evidence also suggest that A₁ receptor-mediated neuroprotection is also dependent on p38 MAPK activity in the brain. A brief exposure to volatile anesthetics such as isoflurane protects the brain against subsequent ischemic insults (Kapinya et al., 2002; Zhao and Zuo, 2004). Isoflurane tolerance against focal cerebral ischemia is dependent on an A₁ receptor-mediated pathway (Liu et al., 2006). Interestingly, isoflurane tolerance against cerebral ischemia requires the activation of p38 MAPK in vivo (Zheng and Zuo, 2004).

Adenosine A₁ receptor depresses synaptic transmission in the hippocampus by inhibiting voltage dependent calcium channels in presynaptic nerve terminals (Wu and Saggau, 1994; Manita et al., 2004). p38 MAPK has also been implicated in presynaptic inhibition, as p38 MAPK activation is necessary for inhibition of N-type calcium current in a G protein-dependent pathway (Wilk-Blaszczak et al., 1998). Although both A₁-receptors (Lee et al., 1983; Fastbom et al., 1987; Tetzlaff et al., 1987; Ochiishi et al., 1999) and p38 MAPK (Lee et al., 2000b; Maruyama et al., 2000) are widely expressed in brain tissue, it is not known whether A₁-receptors can activate p38 MAPK in the brain, or whether p38 MAPK activation plays a role in A₁-receptor-mediated synaptic depression.

In the present study, we have used electrophysiology, Western blot analysis, and co-immunoprecipitation to test whether p38 MAPK activation contributes to the synaptic depression induced by A₁ receptor stimulation in area CA1 of the rat hippocampus. We showed that selective A₁ receptor stimulation in hippocampal slices rapidly elevated
phospho-p38 MAPK and that A1 receptors and phospho-p38 MAPK both exist in the same signaling complex. We also found that protein phosphatase 2a (PP2a) rapidly translocates from the cytosol to the membrane after A1 receptor stimulation in a p38 MAPK-dependent pathway. Finally, we demonstrated that p38 MAPK contributes to adenosine A1-receptor-mediated depression of CA3-CA1 synaptic transmission.

3.2. Materials and methods

3.2.1. Hippocampal Slice Preparation

Sprague Dawley rats (p21-p28) were anaesthetized with halothane and decapitated according to protocols approved by the University of British Columbia committee on animal care. Brains were rapidly extracted and placed into ice-cold oxygenated dissection medium containing the following (in mM): 87 NaCl, 2.5 KCl, 2 NaH2PO4, 7 MgCl2, 25 NaHCO3, 0.5 CaCl2, 25 D-glucose, and 75 sucrose. Hippocampal slices (400μm thick) were cut using a vibrating tissue slicer (VT1000S, Leica, Nussloch, Germany) and maintained for 1-5 hours at 24°C in aCSF containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO4, 26 NaHCO3, 2.5 CaCl2, and 10 D-glucose, and aerated with 95% O2/5% CO2. For electrophysiological recordings, slices were transferred to a submerged recording chamber and allowed to equilibrate for at least 1 hour. The bath solution was perfused with aerated aCSF at a rate of 1.5 – 2 mL/min.

3.2.2. Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten-stimulating
electrode. Glass micropipettes filled with aCSF (resistance 1-3MΩ) were used to
measure CA1 fEPSPs in stratum radiatum. fEPSP signals were amplified 1000 times
with an AC amplifier, band-pass filtered at 0.1–100 Hz, digitized at 10 kHz using a
Digidata 1320A interface board (Axon Instruments, Foster City, CA), and transferred to a
computer for analysis. Data were analyzed using Clampfit 9.0 (Axon Instruments).
Baseline synaptic responses were established by evoking fEPSPs every 30s (0.03Hz) for
at least 20 min. The fEPSP slope was normalized to the mean of the 20 sweeps (10 min)
immediately preceding drug perfusion. The mean normalized fEPSP slope was plotted as
a function of time with error bars representing the standard error of the mean (SEM).
Sample traces are the average of 5 sweeps from a recording that was included in the plot
of the mean normalized fEPSP slope. All bar graphs show the mean normalized percent
inhibition from baseline ± SEM. Statistical significance was assessed using a Student t-
test (p < 0.05).

3.2.3. Immunoprecipitation, Co-immunoprecipitation and
Western Blot Analysis

For biochemical studies, rat hippocampal slices were first incubated with various
treatments (see below) and then lysed in a solubilization buffer (30 min, 4 °C) that
contained 1% NP-40, 20 mM MOPS (pH 7.0), 5 mM EDTA, 2 mM EGTA, 1 mM
phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml
pepstatin A, 1 mM Na3VO4, 30 mM NaF, 40 mM β-glycerophosphate (pH 7.2), 20 mM
sodium pyrophosphate, and 3 mM benzamidine. The tissue homogenates were then
centrifuged at 13,000 x g (20 min, 4 °C) to remove cellular debris, then protein
concentrations of the crude lysates were determined by performing a Bradford assay with
the DC Protein Assay dye (Bio-Rad, Mississauga, ON, Canada). In some experiments, the membrane and cytosolic fractions from hippocampal slices were separated by centrifugation at 13,000 x g for 1 hr at 4°C by omitting the detergent from the solubilization buffer. The proteins from the particulate (membrane) fraction were resolved in normal solubilization buffer (as above) after removal of the cytosolic extract. Hippocampal homogenates were diluted with 1X Laemmli sample buffer and boiled for 5 min. The proteins were resolved in 10% polyacrylamide gel and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Cambridge, ON, Canada). The blots were blocked with 5% non-fat milk in TBST for 1 hr, and the membranes were incubated with primary antibody (see below) overnight at 4 °C. Following four washes with TBST, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (1 hr, room temperature). The membranes were then washed 3-4 X (15 min) with TBST, and proteins were visualized using enhanced chemiluminescence (ECL, Amersham Bioscience, Arlington Heights, IL).

To examine interactions between adenosine A1 receptors and phospho-p38 MAPK, co-immunoprecipitation was performed by first incubating 1 mg extract from hippocampal homogenates with a goat or rabbit IgG (1 hr, 4 °C), then goat or rabbit IgG agarose beads (Sigma) were added to the homogenates for a further 1 hr. In some experiments, ~250 μg of lysates from the membrane or cytosolic fractions were used for the co-immunoprecipitation. The agarose beads were removed by pulse spinning at 6000 rpm for 5 s, and the supernatant was subsequently reacted with an immunoprecipitating antibody overnight at 4 °C. A1 receptor was immunoprecipitated using either a polyclonal goat anti-A1 receptor (5 μg, Santa Cruz, CA) or a polyclonal rabbit anti-A1
receptor (5 μg, Sigma). After overnight pre-incubation of lysates with a polyclonal rabbit anti-phospho p38 MAPK antibody (5 μg, Cell Signalling, Beverly, MA), the p38 MAPK antigen was immunoprecipitated by incubating the immune complexes for >6 hrs at 4 °C with agarose beads conjugated to secondary antibody (rabbit or goat anti-IgG). Agarose beads were then collected by pulse spins, and washed 4 times with wash buffer (solubilization buffer containing 0.1 % NP-40). Proteins from the agarose beads were eluted with 60 μL of 1X Laemmlı sample buffer (Bio-Rad), boiled for 5 min, and resolved in 10% polyacrylamide gels. Proteins were then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Cambridge, ON, Canada).

PVDF membranes were blocked with 5% non-fat milk in TBS (50 mM Tris (pH 7.4), 150 mM NaCl) containing 0.1 % Tween-20 (TBST). The membrane was incubated overnight (4 °C) with the appropriate primary antibody diluted in 5% non-fat milk in TBST containing 0.025% sodium azide. The antibody dilutions are as follows: polyclonal rabbit anti-A1 receptor (1:1000, Sigma), polyclonal rabbit anti-phospho p38 MAPK (1:500, Cell Signalling), polyclonal rabbit pan-specific p38 MAPK antibody (1:500, Cell Signalling), and mouse anti-PP2a (1:500, Cell Signalling). To normalize the protein bands from the membrane and cytosolic fractions, we used a polyclonal rabbit anti-β-actin (1:8000, RBI) or a polyclonal goat anti-GAPDH (1:300, Santa Cruz). The PVDF membranes were washed 3 X 15 min with TBST and then incubated with a mouse, goat, or rabbit horse radish peroxidase-conjugated secondary antibody against IgG (1:3000, Santa Cruz) in 5% non-fat milk. After 3-4 washes with TBST, labelled protein bands were visualized using ECL) and Fluor-S-Max Imaging Software. Densitometry analysis was carried out using ImageJ Software.
3.2.4. Drugs

Adenosine, N6-cyclopentyladenosine (CPA), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel). SB203580, SB239063, SB202190, SB202474, and PD98059 were obtained from Calbiochem (San Diego, CA, U.S.A.), and made up as stock in DMSO before being added to aCSF. The final concentration of DMSO was always ≤ 0.1%.

3.3. Results

3.3.1. p38 MAPK activation increases following A1-receptor stimulation

The selective A1 receptor agonist N6-cyclopentyladenosine (CPA) activates p38 MAPK in rat ventricular myocytes within minutes (Liu and Hofmann, 2003). In the present study, we tested whether CPA treatment increased the level of phosphorylated p38 MAPK in rat hippocampus. Hippocampal slices were treated with either normal aCSF or CPA (500 nM) for time periods ranging from 2 min to 30 min. The activation of p38 MAPK was determined using an antibody that only recognizes p38 MAPK when it is dually phosphorylated at threonine 180 and tyrosine 182. When samples of homogenized hippocampal slices were centrifuged to separate the membrane and cytosolic fractions, we observed that p38 MAPK activation increased in the membrane fraction and decreased in the cytosolic fraction (Fig. 1A,B). In the membrane fraction, the response was maximal after 10 min of CPA exposure (500 nM) and returned partly back to
Figure 3.1. CPA increased p38 MAPK phosphorylation in membrane fractions and decreased p38 MAPK phosphorylation in cytosolic fractions.

Hippocampal slices were exposed to either normal aCSF or CPA (500 nM) for 2, 5, 10, or 30 min. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation for Western blot analysis. Whole-cell hippocampal lysates were used for immunoprecipitation. A, Representative Western blots showing phosphorylated p38 MAPK (p-p38) and β-actin in the membrane and cytosolic fractions. B, Quantitative representation of multiple Western blots showing phospho-p38 MAPK immunoreactivity (mean ± SEM) in the membrane fraction [control (n=5), 2 min (n=5), 5 min (n=11), 10 min (n=11) and 30 min (n=5)] and cytosolic fraction [control (n=5), 2 min (n=5), 5 min (n=15), 10 min (n=9), and 30 min (n=5)]. Data were normalized to the level of protein phosphorylation at time 0 after CPA treatment, and β-actin immunoreactivity was used as a loading control. C, upper panel, Representative Western blot showing that the total amount of p38 did not change in the membrane fraction in response to CPA treatment (500nM; 10 min). C, lower panel, Quantitative representation of total p38 immunoblots (mean ± SEM) showing no significant change in the total amount of p38 (n=5). D, Co-immunoprecipitation showing an association between p-p38 and the adenosine A1 Receptor (A1R) (lane 3). E, Co-immunoprecipitation showing an association between the A1R and p-p38 (lane 2, reverse of E). No association between these proteins was detected when the immunoprecipitating antibodies were omitted (lane 1 of D and E). F, phospho-p38 MAPK immunoprecipitates from membrane (lane 1) and cytosolic (lane 2) hippocampal lysates also showed immunoreactivity to A1 receptor.
antibody. Statistical significance compared with control was assessed using a Student t-test, where * denotes $p < 0.05$ and ** denotes $p < 0.01$. 

**Figure A**

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<th>Time of CPA treatment (min)</th>
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**Figure B**

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**Figure C**

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**Figure D**

- IP: Rabbit IgG
- IP: Rabbit A1R
- IP: Rabbit p-p38

**Figure E**

- IP: Rabbit IgG
- IP: Rabbit A1R
- IP: Rabbit p-p38

**Figure F**

- WB: A1 Receptor
- WB: Phospho-p38
- IP: Phospho-p38

94
baseline by 30 min (the longest time point tested). In the cytosolic fraction, phospho-p38 MAPK decreased maximally at 30 min. Specifically, in membrane fraction CPA increased phosphorylated-p38 (phospho-p38) MAPK immunoreactivity to 148 ± 12% (n=5; p < 0.01) of baseline after 2 min, 135 ± 6% (n=11; p < 0.01) after 5 min, 158 ± 15% (n=11; p < 0.05) after 10 min, and 124 ± 35% (n=5; p > 0.05) after 30 min. In the cytosolic fraction, CPA decreased phospho-p38 MAPK to 98 ± 6% (n=5; p > 0.05) of baseline after 2 min, 85 ± 5% (n=15; p > 0.05) after 5 min, 78 ± 6% (n=9; p < 0.05) after 10 min, and 64 ± 5% (n=5; p < 0.01) after 30 min.

To test whether the total amount of p38 MAPK was increasing in the membrane fraction, we performed a parallel experiment using a pan-specific p38 MAPK (pan-p38 MAPK) antibody. We normalized the pan-p38 MAPK signal to β-actin immunoreactivity and found that CPA (500 nM; 10 min) did not change the total amount of p38 present in the membrane fraction (100 ± 11% of control; n=5; p > 0.30) (Fig 1C).

3.3.2. The adenosine A₁ receptor and phospho-p38 MAPK are physically associated

We performed co-immunoprecipitation experiments to determine whether the adenosine A₁ receptor exists in the same signalling complex as phospho-p38 MAPK. The adenosine A₁ receptor corresponds to a band at approximately 37 kDa in the rat hippocampus (Rebola et al., 2003). Immunoprecipitation with the adenosine A₁ receptor antibody pulled down the adenosine A₁ receptor in whole-cell hippocampal lysates (Fig. 1D, lane 2). Immunoprecipitation with the phospho-p38 MAPK antibody (probed with a polyclonal rabbit anti-A₁ receptor antibody) revealed the A₁ receptor (Fig. 1D, lane 3).
No such bands were present when the immunoprecipitating antibody was omitted (Fig. 1D, lane 1). The reverse immunoprecipitation also confirmed the presence of phospho-p38 MAPK in the adenosine A\(_1\) receptor immunoprecipitates (Fig. 1E, lane 2). A phospho-p38 MAPK band is also present in the positive control lane (Fig. 1E, lane 3). Immunoprecipitation with anti-phospho-p38 MAPK pulled down the A\(_1\) receptor in lysates prepared from both membrane and cytosolic fractions (Fig. 1F). These data suggest that the adenosine A\(_1\) receptor and p38 MAPK physically interact in the hippocampus.

### 3.3.3. A1-Receptor-dependent increases in p38 MAPK activation are blocked by p38 MAPK inhibition and A1 receptor antagonism

The pyridinyl imidazole compound SB203580 is a relatively selective inhibitor of p38 MAPK activity (Cuenda et al., 1995). In heart tissue, increases in p38 MAPK phosphorylation due to adenosine A\(_1\) receptor activation are blocked by SB203580 (Liu and Hofmann, 2003). We tested whether A\(_1\) receptor-induced phosphorylation of p38 MAPK was also blocked by SB203580 in the brain. Hippocampal slices were either incubated in normal aCSF or SB203580 (25 \(\mu\)M) for 1 hour. Slices from each condition were either left untreated or treated with 40 nM CPA or 500 nM for 10 minutes, and then homogenized and centrifuged to separate the membrane and cytosolic fractions. 40 nM CPA (10 min) and 500 nM CPA (10 min) increased phospho-p38 MAPK immunoreactivity to 118 ± 7.2% (n=4; \(p < 0.05\)) and 151 ± 11.7% (n=11; \(p < 0.05\)) of control respectively. SB203580 by itself decreased phospho-p38 MAPK immunoreactivity to 65.8 ± 8.4% (n=4) of control (\(p < 0.01\)) (Fig. 2A,B). Neither 40
Figure 3.2. The increase in p38 MAPK phosphorylation induced by CPA was blocked by p38 MAPK inhibition and A1 receptor antagonism.

Hippocampal slices were either preincubated with normal aCSF or SB203580 (25 μM) for 1 hour and then exposed to CPA (40 nM or 500 nM) for 10 min. The membrane fraction was separated by centrifugation for Western blot analysis. A, Representative Western blots showing phosphorylated p38 MAPK (p-p38) and β-actin in response to CPA in the presence and absence of SB203580 (25 μM). B, Quantified phosphorylated p38 MAPK immunoreactivity (mean ± SEM) for control (n=4), control + SB203580 (n=4), 40 nM CPA (n=4), 40 nM CPA + SB203580 (n=4), 500 nM CPA (n=15), and 500 nM CPA + SB203580 (n=4). C, Representative Western blots showing that the CPA-induced increase in phospho-p38 MAPK was blocked in the presence of DPCPX. D, Quantified phospho-p38 MAPK immunoreactivity for control (n=10), 500 nM CPA (n=10), and 500 nM DPCPX (n=10). Data were normalized to β-actin immunoreactivity. Statistical significance compared with control was assessed using a Student t-test, where * denotes p < 0.05 and ** denotes p < 0.01.
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nM CPA nor 500 nM CPA had an effect in slices pre-treated with SB203580, as phospho-p38 MAPK immunoreactivity decreased to 63.1 ± 6.4% (n=4) and 62.3 ± 7.8% (n=4) of control respectively. Neither condition was statistically different from slices treated with SB203580 alone (p > 0.30).

We also tested whether the A1 receptor antagonist DPCPX prevented the CPA-induced increase in the amount of phospho-p38 MAPK in the membrane fraction. Because DPCPX increases glutamate release in brain slices (Sehmisch et al., 2001; Marcoli et al., 2003), which could potentially activate p38 MAPK through metabotropic glutamate receptors (Rush et al., 2002), we treated slices with tetrodotoxin (TTX) (1.2 µM) for 20 min to block action potentials and thus prevent DPCPX-induced increases in glutamate release. We found that in the presence of TTX, CPA (500 nM; 10 min) increased phospho-p38 MAPK to 143 ± 15% (n=10; p < 0.01) of control levels in the membrane fraction (Fig. 2C,D). This CPA-induced increase in phospho-p38 MAPK was completely blocked (98 ± 15% of control; n=10; p > 0.80) by pre-treatment with DPCPX (500 nM; 20 min) for 20 min prior to CPA application (Fig. 2C,D).

3.3.4. A1-Receptor activation induced translocation of protein phosphatase 2a to the plasma membrane

Adenosine A1 receptor activation causes protein phosphatase 2a (PP2a) translocation and activation in ventricular myocytes (Liu and Hofmann, 2002) in a pathway requiring p38 MAPK (Liu and Hofmann, 2003). We tested whether CPA treatment activated PP2a in rat hippocampal slices. Slices were treated with either normal aCSF or CPA (500 nM) for time periods ranging from 2 min to 30 min, and homogenized and centrifuged to isolate the membrane fraction. Using an antibody recognizing the C-subunit of PP2a, we
Figure 3.3. CPA increases PP2a levels in the membrane fraction.

Hippocampal slices were exposed to either normal aCSF or CPA (500 nM) for 2, 5, 10, or 30 min. The membrane fraction was extracted by centrifugation for Western blot analysis. A, Representative Western blot showing the time course of CPA-induced changes in PP2a immunoreactivity. B, Quantified PP2a immunoreactivity, showing that PP2a increases within minutes in response to CPA treatment [control (n=7), 2 min (n=7), 5 min (n=7), 10 min (n=15) and 30 min (n=7)]. C, Representative Western blot showing that the CPA-induced increase in PP2a levels was blocked in the presence of DPCPX (500 nM). D, Quantification of C (n=8). E, Western blot showing that the p38 MAPK inhibitor SB239063 (15 μM) prevented CPA from increasing PP2a levels. F, Quantification of E (n=6). All data were normalized to β-actin immunoreactivity. Values are means ± SEM. Statistical significance compared with control was assessed using a Student t-test, where * denotes p < 0.05 and ** denotes p < 0.01.
A

Time of CPA treatment (min)

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B

PP2a immunoreactivity (% of control)

Time (minutes)

D

PP2a immunoreactivity (% of control)

F

PP2a immunoreactivity (% of control)

Control  CPA  CPA+DPCPX  Control  CPA  CPA+SB239063

Graphs showing changes in PP2a and β-actin immunoreactivity over time and under different conditions.
determined that CPA treatment increased PP2a immunoreactivity within minutes in memory fractions (Fig 3A,B). Specifically, PP2a immunoreactivity increased to 123 ± 10% (n=7; p < 0.05) of baseline after 2 min, 145 ± 13% (n=7; p < 0.05) after 5 min, 139 ± 13% (n=15; p < 0.05) after 10 min, and 121 ± 12% (n=7; p > 0.05) after 30 min. The response was maximal after 5 min of CPA exposure and returned partly back to baseline by 30 min.

In addition, we tested whether DPCPX prevented the increase in PP2a immunoreactivity in membrane fractions induced by treating slices with CPA. All slices were incubated in TTX (as above) and then exposed to either CPA (500 nM; 10 min) alone or in the presence of DPCPX (500 nM). CPA increased the amount of PP2a in the membrane fraction to 145 ± 21% (n=8; p < 0.05) of control (Fig. 3C,D). However, in the presence of DPCPX, the CPA-induced increase in PP2a was blocked (86 ± 16% of control; n=8; p > 0.30) (Fig. 3C,D). Finally, we also determined that p38 MAPK inhibition prevented the CPA-induced increase in PP2a immunoreactivity in membrane fractions (Fig. 3E,F). We incubated slices in either normal aCSF or the second generation p38 MAPK inhibitor SB239063 (15 μM) for 1 hour prior to CPA treatment (500 nM; 10 min). In slices treated with SB239063, CPA failed to increase levels of PP2a in the membrane fraction (91 ± 6% of control; n=6; p > 0.15). Together, these data suggest that p38 activation is required for the A1 receptor mediated translocation of PP2a to the membrane.
3.3.5. Adenosine-induced depression of synaptic transmission is mediated by the $A_1$ receptor subtype and is sensitive to p38 MAPK inhibition

To investigate the functional significance of $A_1$-receptor-mediated p38 MAPK activation in the brain, we used extracellular fEPSP recordings to monitor the effect of p38 MAPK inhibition on the action of adenosine in area CA1 of the rat hippocampus. We confirmed that adenosine-induced depression of synaptic transmission in area CA1 is mediated by the $A_1$-receptor subtype in area CA1 (Dunwiddie and Fredholm, 1989; Wu and Saggau, 1994; Johansson et al., 2001). In normal aCSF, perfusion of 20 μM adenosine onto the slice for 10 min decreased the mean normalized fEPSP slope to $33 \pm 4\%$ ($p < 0.01; n=8$) of baseline (Fig. 4A). Perfusion of 100 nM DPCPX, a specific $A_1$-receptor antagonist (Bruns et al., 1987; Haleen et al., 1987), onto the same slice for 20 min increased the fEPSP slope to $125 \pm 4\%$ ($p < 0.01; n=8$) of baseline, presumably due to removal of tonic $A_1$-receptor-mediated inhibition of glutamate release. Perfusion of 20 μM adenosine in the presence of 100 nM DPCPX had no effect on fEPSPs (98 ± 3% of baseline; $p > 0.15; n=8$), indicating that the $A_1$-receptor subtype mediates the inhibition of fEPSPs caused by adenosine in rat CA1.

Perfusion of the p38 inhibitor, SB203580 (25 μM; 40 min) slightly increased the mean fEPSP slope to $111 \pm 4.6\%$ of baseline ($p < 0.05; n=5$) (Fig. 4B). The magnitude of fEPSP depression induced by adenosine (10 μM) was attenuated by SB203580 (adenosine decreased the mean fEPSP slope by $55.0 \pm 3.8\%$ in normal aCSF but by only $27.3 \pm 2.6\%$ after SB203580 treatment; $n=5; p < 0.01$) (Fig. 4B,D). Because SB203580 was dissolved in DMSO, we ensured that 0.1% DMSO (Fig. 4C) had no effect on
Figure 3.4. Adenosine-induced depression of CA1 fEPSPs is mediated by the A1-receptor subtype and sensitive to p38 MAPK inhibition.

A-C Averaged sample traces and plot of the mean fEPSP slope (± SEM) over time normalized to baseline. fEPSPs (evoked every 30 sec) were recorded in CA1 stratum radiatum. A, Adenosine reversibly decreased the mean normalized fEPSP slope. After recovery to baseline, the A1-receptor antagonist DPCPX (100 nM) was bath applied, resulting in an increase in the mean fEPSP slope. A second perfusion of adenosine in the presence of 100 nM DPCPX had no effect on the mean normalized fEPSP slope (n=8).

B, Perfusion of SB20380 (25 μM) slightly increased baseline synaptic transmission and partially inhibited the ability of adenosine (10 μM) to decrease fEPSPs (n=5). C, The effect of adenosine (10 μM) was the same whether applied in the presence of 0.1% DMSO (50 min perfusion) or normal aCSF (n=10). D, Summary of A-C showing that SB203580 attenuates adenosine-induced depression whereas DMSO does not.

Calibration: 10msec, 1mV.

*Denotes statistical significance using a Student t-test (p < 0.01).
baseline synaptic transmission nor the magnitude of adenosine induced depression (adenosine decreased the mean fEPSP slope by 51.0 ± 2.4% in normal aCSF and 51.3 ± 3.5% after 50 min of DMSO treatment in the same slice; n=10; p > 0.30) (Fig. 4B,D).

3.3.6. **Neither the inactive analogue SB202474 nor the ERK 1/2 MAPK inhibitor PD98059 decreased adenosine-induced synaptic depression**

The health of hippocampal slices, as determined by the slope of evoked fEPSP slopes, was not adversely affected by prolonged exposure to SB203580 (over 50 min in the bath perfusate). Therefore in the next set of experiments we preincubated slices in SB203580 instead of prolonged bath perfusions. In all subsequent experiments matched slices from the same animal were either pre-incubated in SB203580 (25 μM; 1-2 hours) or normal aCSF. Perfusion of adenosine (10 μM; 10 min), depressed the mean normalized fEPSP slope by 61.7 ± 2.7% (n=5) in control, and only 30.6 ± 3.0% (n=6) in slices pre-incubated in SB203580 (p < 0.01) (Fig. 5A,D). Thus, the effects of SB203580 on adenosine-mediated synaptic depression were similar in both pre-incubation and perfusion experiments.

SB202474 is an inactive pyridinyl imidazole compound that has been used as a negative control in p38 MAPK studies (Lee et al., 1994; Yu et al., 2000). Slices were either pre-incubated with 25 μM SB202474 or with normal aCSF (control). There was no difference in the action of adenosine in slices treated with SB202474 compared with control slices (Fig. 5B,D). In control, perfusion of adenosine (10 μM; 10 min) depressed the mean fEPSP slope to 52.2 ± 6.4% (n=7). In slices that had been pre-treated with
Figure 3.5. Adenosine-induced synaptic depression was attenuated by SB203580, but not SB202474 (an inactive analogue) nor PD98059 (ERK 1/2 MAPK inhibitor).

A-C, Averaged sample traces and plot of the mean normalized fEPSP slope (± SEM) over time. A, Matched slices were either pre-treated with SB203580 for 1-2 hours (n=7) or normal aCSF (n=13). Adenosine-induced depression of fEPSPs was attenuated in the presence of SB203580. B, Matched slices were either pre-treated with SB202474 for 1-2 hours (n=5) or normal aCSF (n=7). SB202474 did not affect adenosine-induced depression of fEPSPs. C, Matched slices were either pre-treated with PD98059 (n=5) or normal aCSF (n=4). Adenosine-induced depression of fEPSPs was not attenuated by PD98059. D, Summary of A-C showing the mean maximal effect of adenosine expressed as percentage inhibition from baseline. Calibration: 10msec, 1mV. *Denotes statistical significance compared with control using a Student t-test (p < 0.01).
25 μM SB202474, adenosine depressed the mean fEPSP slope by 67.0 ± 3.4% (n=5), which was not statistically different from control (p > 0.10).

The extracellular signal-regulated kinase (ERK) 1/2 MAPK pathway is activated by synaptic activity and is required for the induction of long-term potentiation of synaptic transmission in area CA1 of the hippocampus (Impey et al., 1999; Bolshakov et al., 2000). The ERK 1/2 MAPK pathway is also required for metabotropic glutamate receptor-dependent long term depression (Gallagher et al., 2004). We tested whether ERK 1/2 MAPK also played a role in adenosine-mediated synaptic depression. Slices were either pre-treated with the selective ERK 1/2 MAPK inhibitor PD98059 (Pang et al., 1995) (50 μM) or with normal aCSF (control). In control, adenosine (10 μM; 10 min) depressed the mean fEPSP slope to by 52.5 ± 5.7% (n=4) of baseline (Fig. 5C,D). In slices that were pre-treated with 50 μM PD98059, adenosine depressed the mean fEPSP slope by 49.0 ± 3.0% (n=5) of baseline, which was not statistically different from control (p > 0.30). These data indicate that inhibiting ERK 1/2 MAPK does not modify adenosine-mediated synaptic depression in area CA1.

3.3.7. A₁-receptor-mediated synaptic depression was decreased by p38 MAPK inhibition

The physiological ligand adenosine, acts at multiple receptor subtypes, and is rapidly degraded by ecto-nucleotidases and/or removed from the extracellular space via nucleoside transporters (Dunwiddie and Masino, 2001). To avoid these issues, we next used the A₁-receptor agonist N⁶-cyclopentyladenosine (CPA). We tested whether synaptic depression induced by CPA was also attenuated by the p38 MAPK inhibitor
SB203580. Recordings were obtained from slices that were either incubated in 25 μM SB203580 (1-2 hours) or normal aCSF (control). In control, a 10 min perfusion of 40nM CPA decreased the mean fEPSP slope by 72.8 ± 2.1% (n=4) (Fig. 6A,B,E), confirming that selective A1-receptor activation decreases synaptic transmission. However, in SB203580, CPA perfusion only decreased the mean fEPSP slope by 10.4 ± 2.0% (n=5; p < 0.01) (Fig. 6A,B,E). These results provide additional evidence that p38 MAPK activity is necessary for A1-receptor-mediated synaptic depression.

To further confirm that p38 MAPK activity was required for A1-receptor-mediated depression of fEPSPs, we carried out an experiment using another pyridinyl imidazole compound, SB202190 (50 μM), which also blocks the activity of p38 MAPK (Davies et al., 2000). Recordings were obtained from slices that were either incubated in 50 μM SB202190 (1-2 hours) or normal aCSF (control). In control, a 15 min perfusion of CPA (40 nM) decreased the mean fEPSP slope by 74.3 ± 2.3% (n=5) (Fig. 6C,E). However, in SB202190, CPA perfusion decreased the mean fEPSP slope by only 25.0 ± 6.0% (n=5) of baseline (p < 0.01) (Fig. 6C,E).

To control for possible non-specific effects of the pyridinyl imidazole p38 MAPK inhibitors SB203580 and SB202190, we tested the effects of the inactive pyridinyl imidazole analogue SB202474 on the depression of CA1 fEPSPs induced by CPA. Recordings were obtained from slices that were either incubated in 25 μM SB202474 (1-2 hours) or normal aCSF (control). In control, a 15 min perfusion of 40nM CPA decreased the mean fEPSP slope 74.8 ± 3.4% (n=11) (Fig. 6C,E). In SB202474-incubated slices, a 15 min perfusion of 40nM CPA decreased the mean normalized fEPSP
Figure 3.6. CPA-mediated depression of CA1 fEPSPs was attenuated by p38 MAPK inhibition.

A, Representative fEPSP traces before (1) and after (2) CPA (40 nM) treatment in slices either in normal aCSF (top) or preincubated in 25 μM SB203580 (bottom). Calibration: 10 msec, 1 mV. B-D, Plots of the mean normalized initial fEPSP slope (± SEM) over time. B, Slices were either preincubated with SB203580 (n=5) or normal aCSF (n=4) prior to treatment with CPA for 10 min. C, Slices were either preincubated with the p38 MAPK inhibitor SB202190 (50 μM) (n=5) or normal aCSF (n=5) and then treated with CPA (10 min). D, Slices were either preincubated with the inactive analogue SB202474 (25 μM) (n=8) or normal aCSF (n=11) and then treated with CPA (10 min). E, Summary of B-D showing the magnitude of CPA-induced depression of fEPSP slope (± SEM) as a percentage decrease from baseline in normal aCSF, SB203580, SB202190, or SB202474. CPA-mediated depression of fEPSPs was attenuated by both p38 MAPK inhibitors (SB203580 and SB202190) but not by the inactive analogue (SB202474). *Denotes statistical significance using the Student t-test (p < 0.01).
amplitude to 70.6 ± 4.4% (n=8), which was not statistically different from control (p > 0.30) (Fig. 6D,E).

### 3.3.8. Hypoxia-induced synaptic depression was mediated by the A1-receptor and was attenuated by p38 MAPK inhibition

Hypoxia releases adenosine into the extracellular space (Fowler, 1993a; Dale et al., 2000), where it activates A1-receptors. During extended periods of hypoxia, adenosine release contributes to ~50% of the hypoxia-induced synaptic depression (Fowler, 1989). In contrast to long hypoxic insults, the synaptic depression induced by short hypoxic insults (e.g. 2–3 min) is almost completely mediated by the A1-receptor (Latini et al., 1999b; Sebastiao et al., 2000). In the present study, we found that the synaptic depression induced by a brief (5 min) hypoxic insult was virtually blocked in the presence of the A1-receptor antagonist DPCPX (100 nM) (Fig. 7A-C). Hypoxia was induced by perfusion of aCSF bubbled with 95%N2/5%CO2. In normal aCSF, 5 min perfusion of hypoxic solution decreased the mean fEPSP slope by 46.9 ± 4.3% (n=5) (Fig. 7A-C). In the presence of DPCPX, 5 min hypoxia did not have a significant effect, only decreasing the fEPSP slope by 5.6 ± 1.7% (n=5; p > 0.30). This was significantly less depression than occurred in normal aCSF (n=5; p < 0.01) (Fig. 7A-C).

To test the contribution of p38 MAPK activity to hypoxia-induced synaptic depression, we pre-treated slices with 25 μM SB203580 (1-2 hours) and exposed them to hypoxia for 5 min. In SB203580-treated slices, 5 min hypoxia decreased the mean fEPSP slope by 21.0 ± 1.8% (n=5), which was significantly less depression than occurred in matched control slices (p < 0.01) (Fig. 7A-C). Given that the synaptic depression induced
Figure 3.7. p38 MAPK inhibition attenuated hypoxia-mediated depression of CA1 fEPSPs.

A, Representative fEPSP traces before (1) and after (2) treatment with hypoxia (5 min). Hypoxia was induced by bath application of aCSF bubbled with 95%N2/5%CO2. Slices were either treated with 100 nM DPCPX, 25 µM SB203580, or normal aCSF prior to exposure to hypoxia. Calibration: 10 msec, 1 mV. B, Plot of the mean normalized initial fEPSP slope (± SEM) over time. A1-receptor inhibition with DPCPX (n=5) and p38 MAPK inhibition with SB203580 (n=5) attenuated hypoxia-induced synaptic depression versus matched controls (n=5). C, Summary of B showing the mean fEPSP slope (± SEM) as a percentage of baseline following 5 min hypoxia in normal aCSF, DPCPX, or SB203580. *Denotes statistical significance using a Student t-test (p < 0.01).
5 min hypoxia

A

B

C

Hypoxia

Mean EPSP Slope
(% of baseline)

Time (minutes)

% Inhibition

Control

DPCPX

SB203580

100 75 50

30 25

0

10 20 30 40

1

2

1+2

10 20 30 40

100 75 50 25

0

10 20 30 40

1

2

1+2

100 75 50 25

0

10 20 30 40

1

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1+2

100 75 50 25

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by 5 min hypoxia was almost entirely blocked by the A$_1$ antagonist DPCPX, this finding indicates that p38 MAPK contributes to the synaptic depression induced by A$_1$-receptor activation due to hypoxia. Thus, the neuroprotective action of adenosine released during oxygen deprivation may result from A$_1$-receptor-mediated activation of p38 MAPK.

### 3.4. Discussion

In this study, we discovered a novel interaction between the adenosine A$_1$-receptor and p38 MAPK in the central nervous system. We present biochemical and electrophysiological evidence showing that p38 MAPK is activated by A$_1$ receptor stimulation and that p38 MAPK inhibition significantly attenuates A$_1$-receptor mediated synaptic depression. Furthermore, co-immunoprecipitation revealed that phospho-p38 MAPK and the adenosine A$_1$ receptor are physically associated in the hippocampus. Our results suggest that A$_1$ receptors and p38 MAPK form a signaling complex, and that adenosine A$_1$ receptor-mediated synaptic depression is at least partly dependent on the activation of p38 MAPK in the rat hippocampus.

Although adenosine A$_1$-receptors and p38 MAPK are expressed both presynaptically (Maruyama et al., 2000) and postsynaptically (Bolshakov et al., 2000; Zhu et al., 2002a) in the hippocampus, the primary mechanism underlying adenosine-induced synaptic depression is inhibition of calcium influx into presynaptic nerve terminals (Dunwiddie and Masino, 2001). In area CA1 of the hippocampus, as well as other areas, the adenosine A$_1$-receptor subtype decreases neurotransmitter release from presynaptic nerve terminals primarily by inhibiting N-type calcium channels (Mogul et al., 1993; Yawo and Chuhma, 1993; Mynlieff and Beam, 1994; Wu and Saggau, 1994; Ambrosio et al., 1997; Zhang and Schmidt, 1999; Brown and Dale, 2000; Park et al., 2000).
2001; Sun et al., 2002; Wang et al., 2002b; Manita et al., 2004). Therefore, our study suggests that p38 MAPK activation is a necessary step for A1 receptor activation to decrease synaptic transmission by downregulating presynaptic voltage-dependent calcium channels. p38 MAPK activation is necessary for the depression of N-type calcium currents in neuroblastoma X glioma cells in response to bradykinin application (Wilk-Blaszczak et al., 1998). However, it is not yet determined that p38 MAPK activation modulates calcium channel function in the hippocampus.

A possible link between p38 MAPK and calcium channel modulation is our finding that A1-receptor stimulation induced PP2a translocation to the membrane requires p38 MAPK, which is in agreement with previous studies in cardiomyocytes (Liu and Hofmann, 2002, 2003). PP2a is also activated by p38 MAPK in the brain where it can modulate serotonin transporters (Zhu et al., 2005a). However, it is not known whether PP2a plays a role in presynaptic inhibition. PP2a binds to the C-terminus of alpha-1C calcium channels, and reverses PKA-mediated phosphorylation of serine 1928 located inside this C-terminal region (Davare et al., 2000). A recent report has also demonstrated direct interaction of the protein phosphatase 2Cα (PP2Cα) to the C-terminus of both N- and P/Q-type calcium channels, and that PP2Cα reverses phosphorylation of these neuronal calcium channels by PKC (Li et al., 2005). However, it is unclear whether PP2a also physically associates with and alters the function of presynaptic alpha 1B (N-type) calcium channels. In the present study, we demonstrated that CPA-mediated synaptic depression accompanied increased activation of p38 MAPK and PP2a. Future biochemical and functional studies are needed to address whether this decreased synaptic
transmission may be due, in part, to neuronal calcium channel inhibition by channel interaction with PP2a.

Other voltage-gated ion channels, including sodium channels and hyperpolarization-activated cyclic nucleotide (HCN)-gated channels, are newly discovered potential targets for the A₁ receptor-mediated p38 MAPK activation. A recent report demonstrated that the sodium channel Naᵥ₁.6 and p38 MAPK co-localized in rat cerebellar Purkinje cells and co-immunoprecipitated from rat brain (Wittmack et al., 2005). Activation of p38 MAPK decreased the sodium current density by phosphorylating a serine residue within the first cytoplasmic loop of this channel. Since the sodium channel Naᵥ₁.6 mRNA and proteins are expressed in the hippocampal presynaptic and postsynaptic sites (Schaller et al., 1995; Caldwell et al., 2000), it is possible that downregulation of Naᵥ₁.6 function by p38 MAPK activation contributes to decreased membrane excitability and leads to synaptic depression. Another possibility for p38 modulation is the HCN channel. p38 MAPK inhibition with SB203580 has recently been shown to decrease HCN current in hippocampal pyramidal neurons, which would be predicted to increase membrane excitability (Poolos et al., 2002; Poolos, 2005). Indeed we found that SB203580 caused a modest (10-20%) increase in field potential slopes (Figure 4B), which could be attributed, in part, to the modulation of HCN channels. It is interesting that this p38 MAPK inhibitor did not affect the Naᵥ₁.6 current density (Wittmack et al., 2005). It remains to be established whether adenosine A₁ receptor-mediated p38 MAPK activation accompanies Naᵥ₁.6 channel downregulation and/or HCN channel upregulation to play additional roles in decreasing membrane excitability and synaptic depression.
Canonical MAPK activation occurs via a cascade of three kinases in a module consisting of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. M KK3 and MKK6 are the MAPKKs that activate p38α MAPK. Pyridinal imidazoles such as SB203580 inhibit p38α MAPK by binding to both the active and inactive enzyme in an ATP-competitive manner, and thus prevent p38 MAPK from phosphorylating its downstream substrates (Kumar et al., 2003). SB203580 does not inhibit MKK3 or MKK6 (Kumar et al., 1999). Nonetheless, treatment with SB203580 results in the abolition of CPA-induced increases in p38 MAPK phosphorylation in the heart (Liu and Hofmann, 2003) and brain (Fig. 2; current study). SB203580 also prevents phosphorylation of p38α in a number of other experimental systems (Frantz et al., 1998; Galan et al., 2000; Matsuguchi et al., 2000; Zhuang et al., 2000). These observations imply that phosphorylation of p38 MAPK can occur due to intrinsic kinase activity. Indeed, it was reported that binding of TAB1 (transforming growth factor-b-activated protein kinase 1 (TAK1)-binding protein 1) to p38a causes autophosphorylation and subsequent activation of the kinase in a pathway independent of MKK3 and MKK6 (Ge et al., 2002). Moreover, TAB1-induced phosphorylation of p38a is sensitive to SB203580 both in vitro and in vivo, whereas TAB1-independent phosphorylation of p38a is not (Ge et al., 2002). Thus, the sensitivity of CPA-induced p38 MAPK phosphorylation to SB203580 may indicate that A1 receptor-dependent p38 MAPK activation occurs via autoactivation of p38 MAPK facilitated by interaction with a regulatory molecule (e.g. TAB1) in the hippocampus.

Excessive synaptic release of glutamate during trauma such as hypoxia/ischemia is a major mechanism underlying neuronal cell death (Kass and Lipton, 1982; Rothman,
Adenosine acts as an endogenous neuroprotectant during hypoxia/ischemia, likely by decreasing glutamate release in the brain via A₄ receptors (Dunwiddie and Masino, 2001). The importance of adenosine as an endogenous neuroprotective agent is exemplified by the observation that brief insults of hypoxia/ischemia are more deleterious when repeated (Tomida et al., 1987; Kato et al., 1989) likely because of adenosine depletion (Pearson et al., 2001b). However, a role for p38 MAPK in adenosine-mediated neuroprotection has not been described in the brain.

Previous studies have found that the p38 MAPK pathway is activated following hypoxia/ischemia in brain tissue (Walton et al., 1998; Zhu et al., 2002b). However, there is evidence that p38 MAPK activity is deleterious, mediating the cell death induced by hypoxia (Zhu et al., 2002b), oxygen glucose deprivation, magnesium withdrawal, and glutamate receptor agonist exposure (Legos et al., 2002). In contrast, in heart tissue, p38 MAPK plays an important role in cardioprotection during ischemia (Weinbrenner et al., 1997; Baines et al., 1998; Baines et al., 1999; Zhao et al., 2001a; Lasley et al., 2005). The results of the present study indicate that p38 MAPK could act as a neuroprotectant by mediating the actions of adenosine in the brain.

The apparent contradiction between our results showing that p38 MAPK activation may be neuroprotective, and previous findings showing that p38 MAPK inhibition may be neuroprotective (Barone et al., 2001; Legos et al., 2002) is probably attributable to differences in the time scales analyzed. We found that A₁ receptor stimulation phosphorylated p38 MAPK within minutes (Fig. 1A,B). However, most reports linking p38 MAPK activity and excitotoxic injury have analyzed cell survival in culture hours and/or days following trauma (Walton et al., 1998; Barone et al., 2001;
Legos et al., 2002; Zhu et al., 2002b). Our results suggest that, in contrast to its longer-term deleterious actions, p38 MAPK activation may have a short-term neuroprotective role during transient oxygen deprivation. Therefore, a viable therapeutic intervention to prevent ischemic brain damage might involve the use p38 MAPK inhibitors only after reperfusion.

We have shown previously that p38 MAPK is involved in presynaptic inhibition, as it mediates the potent and long-lasting synaptic depression induced by 2',3'-O-(4-benzoylbenzoyl)-ATP (Bz-ATP) at mossy fiber-CA3 synapses (Armstrong et al., 2002). However, in mossy fiber-CA3 synapses, there is no evidence that adenosine-induced synaptic depression is attenuated by SB203580 (Armstrong et al., 2002). Thus, although p38 MAPK is involved in presynaptic inhibition at both mossy fiber-CA3 and CA3-CA1 synapses, the pathway that activates p38 MAPK is different in these two distinct areas of the hippocampus.

It is unlikely that SB203580 exerts its effects on mossy fiber-CA3 synaptic depression by blocking adenosine transporters as previously suggested (Kukley et al., 2004). The inactive analogue SB202474 (Sweeney et al., 1999) is reported to have a similar effect on nucleoside transporters as SB203580 (Huang et al., 2002). Therefore to rule out the possibility that SB203580 and SB202190 were exerting their effects by blocking nucleoside transporters, we tested whether SB202474 affected adenosine- or CPA-induced synaptic depression. We found that SB202474 failed to affect adenosine A1-receptor-mediated synaptic depression in area CA1 (Fig 5B,D and 6D,E). In addition, inhibitors of nucleoside transporters in brain slices actually enhance the concentration of extracellular adenosine during hypoxia or electrical stimulation (Fredholm et al., 1994).
This indicates that the net effect of nucleoside transporters is the uptake of adenosine from the extracellular space (Latini and Pedata, 2001). Thus, blockade of nucleoside transporters would be expected to decrease synaptic transmission due to a build-up of excess adenosine in the extracellular space as has been reported (Dunwiddie and Diao, 2000). In contrast, we observed that SB203580 increased synaptic transmission (Fig. 4B).

In conclusion, we have shown in the present study that p38 MAPK physically associates with and is activated by A1-receptors within minutes and contributes to adenosine-induced synaptic depression in area CA1 of the hippocampus. Moreover, our results indicate that p38 MAPK activity contributes to the synaptic depression induced by the release of endogenous adenosine during hypoxia. Activation of p38 MAPK by adenosine A1-receptors may represent a novel mechanism underlying presynaptic inhibition of neurotransmission and ischemic preconditioning in the mammalian brain.

3.5. References


Chapter Four: $A_1$ Receptor-Mediated Synaptic Depression is mediated by sequential activation of p38 MAPK and C-Jun N-Terminal Kinase in the rat hippocampus

A version of this chapter was submitted for publication:

4.1. Introduction

Adenosine A₁ receptors are G protein-coupled receptors that rapidly cause potent depression of synaptic transmission in the central nervous system. The level of ambient adenosine in the extracellular space gates synaptic transmission and plasticity in the hippocampus by inhibiting glutamate release (de Mendonca et al., 1997; Nicoll and Schmitz, 2005). Because the concentration of adenosine in the extracellular space increases dramatically during brain trauma, such as stroke and seizure, it may act as an endogenous neuroprotectant by preventing excessive excitation (Dunwiddie and Masino, 2001). Despite the importance of A₁ receptor activation in mediating presynaptic inhibition, the detailed signalling pathway leading from A₁ receptor stimulation to decreased neurotransmitter release are unclear.

Mitogen-activated protein kinases (MAPKs) are a large family of serine/threonine kinases widely expressed in brain that regulate diverse physiological processes such as cell survival, synaptic plasticity, and inducible gene expression (Nozaki et al., 2001; Pearson et al., 2001a). There are three subfamilies of MAPKs: 1) extracellular signal-regulated kinases; 2) p38 MAPK; and 3) the c-Jun N-terminal kinase (JNK). p38 MAPK and JNK are activated when phosphorylated by MAPK kinases MKK3/6 and MKK4/7 respectively. This occurs in response to diverse cellular stresses such as ischemia, osmotic shock, cytokines, growth factors, ultraviolet light, and via activation of G protein-coupled receptors (Marinissen and Gutkind, 2001).

Numerous studies suggest that both p38 MAPK and JNK play roles in synaptic depression. For example, p38 MAPK mediates the long-term depression (LTD) induced by chemical stimulation of metabotropic glutamate receptors (Bolshakov et al., 2000), as
well as removal of AMPA receptors during LTD (Zhu et al., 2002a). JNK is involved in low frequency stimulation-dependent LTD in the dentate gyrus (Curran et al., 2003), and is required for the removal of synaptic AMPA receptors during depotentiation (Zhu et al., 2005b).

JNK activation may also depress synaptic transmission through a presynaptic mechanism. The inhibitory effect of interleukin-1β and the beta-amyloid peptide on long term potentiation (LTP) was reported to involve JNK and/or p38 MAPK activation (Vereker et al., 2000a; Costello and Herron, 2004), and increased JNK and p38 MAPK phosphorylation is associated with decreased glutamate release in the hippocampus (Vereker et al., 2000a). Together, these findings suggest that activation of endogenous JNK and p38 MAPK initiates signalling cascades that inhibit glutamate release from presynaptic terminals. In support of this hypothesis, we have recently described a role for p38 MAPK in adenosine A1 receptor-mediated depression of CA3-CA1 synaptic transmission in the rat hippocampus (Brust et al., 2005).

In the present study, we tested whether JNK activation also played a role in adenosine A1 receptor-mediated inhibition of neurotransmission. We recorded fEPSPs in area CA1 of rat hippocampal slices to monitor the effect of JNK inhibitors on adenosine-induced synaptic depression. Western blot analysis was used to quantify changes in the phosphorylation state (and thus activation) of JNK in hippocampal lysates. Co-immunoprecipitation was used to investigate an association between the A1 receptor and JNK. We report that the synaptic depression induced by selective A1 receptor activation was attenuated by JNK inhibition, and that A1 receptor stimulation induced a rapid and
transient phosphorylation of JNK through a mechanism that may involve the association of A<sub>1</sub> receptors and JNK in the plasma membrane.

4.2. Materials and methods

4.2.1. Hippocampal Slice Preparation

Sprague Dawley rats (p21-p28) were anaesthetized with halothane and decapitated according to protocols approved by the University of British Columbia committee on animal care. Brains were rapidly extracted and placed into ice-cold oxygenated dissection medium containing the following (in mM): 87 NaCl, 2.5 KCl, 2 NaH<sub>2</sub>PO<sub>4</sub>, 7 MgCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 0.5 CaCl<sub>2</sub>, 25 D-glucose, and 75 sucrose. Hippocampal slices (400µm thick) were cut using a vibrating tissue slicer (VT1000S, Leica, Nussloch, Germany) and maintained for 1-5 hours at 24°C in aCSF containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, and 10 D-glucose, and aerated with 95%O<sub>2</sub>/5% CO<sub>2</sub> (or 95%N<sub>2</sub>/5%CO<sub>2</sub> for hypoxia). For electrophysiological recordings, slices were transferred to a submerged recording chamber and allowed to equilibrate for at least 1 hour. The bath solution was perfused with aerated aCSF at a rate of 1.5 – 2 mL/min.
4.2.2. Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten-stimulating electrode. Glass micropipettes filled with aCSF (resistance 1-3MΩ) were used to measure CA1 fEPSPs in stratum radiatum. fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1–100 Hz, digitized at 10 kHz using a Digidata 1320A interface board (Axon Instruments, Foster City, CA), and transferred to a computer for analysis. Data were analyzed using Clampfit 9.0 (Axon Instruments).

Baseline synaptic responses were established by evoking fEPSPs every 30s (0.03Hz) for at least 20 min. The fEPSP slope was normalized to the mean of the 20 sweeps (10 min) immediately preceding drug perfusion. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the standard error of the mean (SEM). Sample traces are the average of 5 sweeps from a recording that was included in the plot of the mean normalized fEPSP slope. Input-Output curves were generated by systematically increasing the voltage delivered by the stimulating electrode (4V – 10V in increments of 1V), and measuring the resulting fEPSP slope. All bar graphs show the mean normalized percent inhibition from baseline ± SEM. Statistical significance was assessed using a Student t-test (p < 0.05).
4.2.3. Western Blot Analysis

For biochemical studies, rat hippocampal slices were first incubated with various treatments (see below) and then lysed in a solubilization buffer (30 min, 4 °C) that contained 1% NP-40, 20 mM MOPS (pH 7.0), 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM Na₃VO₄, 30 mM NaF, 40 mM β-glycerophosphate (pH 7.2), 20 mM sodium pyrophosphate, and 3 mM benzamidine. The tissue homogenates were then centrifuged at 13,000 x g (20 min, 4 °C) to remove cellular debris, then protein concentrations of the crude lysates were determined by performing a Bradford assay with the DC Protein Assay dye (Bio-Rad, Mississauga, ON, Canada). Membrane and cytosolic fractions from hippocampal slices were separated by centrifugation at 13,000 x g for 1 hr at 4°C by omitting the detergent from the solubilization buffer. The proteins from the particulate (membrane) fraction were resolved in normal solubilization buffer (as above) after removal of the cytosolic extract. Hippocampal homogenates were diluted with 1X Laemmili sample buffer and boiled for 5 min. The proteins were resolved in 10% polyacrylamide gel and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Cambridge, ON, Canada). The blots were blocked with 5% non-fat milk in TBST (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1 % Tween-20) for 1 hr, and the membranes were incubated overnight at 4 °C with primary antibody diluted in TBST containing 5% non-fat milk or 5% bovine serum albumin and 0.025% sodium azide. The antibody dilutions are as follows: polyclonal rabbit anti-A1 receptor (1:1000, Sigma), polyclonal rabbit anti-phospho JNK (1:1000, Cell Signalling), and polyclonal rabbit pan-
specific JNK antibody (1:1000, Cell Signalling). To normalize the protein bands from the membrane and cytosolic fractions, we used polyclonal rabbit anti-β-actin (1:8000, RBI). Following four washes with TBST, the membranes were incubated with a secondary antibody against rabbit IgG conjugated to horse radish peroxidase (1:3000, Santa Cruz) diluted in 5% non-fat milk (1 hr, room temperature). The membranes were then washed 3-4 X (15 min) with TBST, and proteins were visualized using enhanced chemiluminescence (ECL, Amersham Bioscience, Arlington Heights, IL) using Quantity One Fluor S-MAX imaging software (Bio-Rad, Hercules, CA). Densitometry analysis was carried out using ImageJ Software (National Institutes of Health, USA).

4.2.4. Immunoprecipitation and Co-Immunoprecipitation

To examine interactions between adenosine A₁ receptors and JNK and phosphorylated-JNK (phospho-JNK), co-immunoprecipitation was performed by first incubating 1 mg extract from hippocampal homogenates with rabbit IgG (1 hr, 4 °C). Next goat anti-rabbit IgG antibody covalently linked to agarose beads (Sigma) was added to the homogenates for a further 1 hr. The agarose beads were removed by pulse spinning at 6000 rpm for 5 s, and the supernatant was subsequently reacted with an immunoprecipitating antibody overnight at 4 °C. A₁ receptor was immunoprecipitated using a polyclonal rabbit anti-A₁ receptor antibody (5 µg, Sigma). JNK and phospho-JNK were immunoprecipitated with a polyclonal rabbit anti-pan JNK and anti-phospho JNK antibody respectively (5 µg, Cell Signalling, Beverly, MA). After overnight pre-incubation of lysates with the immunoprecipitating antibodies, the immune complexes were collected by incubating the lysates for >6 hrs at 4 °C with agarose beads conjugated to anti-rabbit IgG antibody. Agarose beads were then collected by pulse spins, and
washed 4 times with wash buffer (solubilization buffer containing 0.1 % NP-40). Proteins from the agarose beads were eluted with 60 μL of 1X Laemmli sample buffer (Bio-Rad), boiled for 5 min, resolved in 10% polyacrylamide gels, and subjected to Western blot analysis as described above.

4.2.5. Drugs

Adenosine, N⁶-cyclopentyladenosine (CPA), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel). SP600125, JNK inhibitor V, JNK inhibitor II negative control (N¹-methyl-1,9-pyrazoloanthrone), and SB239063 were obtained from Calbiochem (San Diego, CA, U.S.A.), and made up as stock in DMSO before being added to aCSF. The final concentration of DMSO was always ≤ 0.01%.
4.3. Results

4.3.1. JNK inhibitors attenuate the synaptic depression induced by adenosine, hypoxia, and CPA

First, we determined whether JNK activation contributed to synaptic depression induced by exogenously applied adenosine, endogenous adenosine released by hypoxia, or an A1 receptor agonist in area CA1 of the rat hippocampus. We tested the effects of the JNK inhibitor SP600125 (Shin et al., 2002) on the magnitude of fEPSPs depression in area CA1 of the rat hippocampus. Hippocampal slices were pre-treated with the JNK inhibitor SP600125 (20μM), which selectively blocks JNK activity at a concentration of 20μM in hippocampal slices (Vereker et al., 2000a; Curran et al., 2003) or with vehicle alone (0.01% DMSO) for at least 1 hour prior to activation of A1 receptors. After a stable fEPSP baseline was achieved, adenosine, CPA (an A1 receptor agonist), or hypoxic aCSF (which releases adenosine; Fowler, 1989; Zhu and Krnjevic, 1997) was perfused onto the slice to activate A1 receptors. We used 5 min exposures to hypoxic ACSF because, we have previously shown that the synaptic depression during this brief period of hypoxia is totally blocked by A1 receptor antagonism (Brust et al., 2005), which is in agreement
Figure 4.1. Synaptic depression induced by adenosine, hypoxia, and CPA is attenuated by JNK inhibition.

A, Sample traces and plot of the mean fEPSP slope (±SEM), normalized to baseline, showing that pre-incubation of slices in the JNK inhibitor SP600125 (20μM) decreases the magnitude of adenosine-induced synaptic depression. B, Sample traces and plot of the mean fEPSP slope (±SEM) showing that adenosine-induced synaptic depression was suppressed in slices pre-incubated in SP600125. C, Sample traces and plot of the mean fEPSP slope (±SEM) showing that synaptic depression induced by the adenosine A₁ receptor agonist CPA was attenuated in slices pre-incubated with SP600125. D, Summary of A-C showing that the maximal magnitude of depression of fEPSP slope (±SEM) induced by adenosine, hypoxia, and CPA was lower in slices incubated in SP600125 than slices incubated in vehicle. *Statistical significance assessed using the Student t-test, p < 0.01. Calibration: 10mV/10ms.
with other studies (Latini et al., 1999b; Sebastiao et al., 2000). Adenosine, CPA, and hypoxia all depressed the fEPSP slope (Fig. 1). When JNK was inhibited with SP600125, the reduction of the fEPSP slope by these agonists was significantly attenuated. Adenosine (+vehicle) decreased the fEPSP slope by 53.0 ± 1.7\% (n=6). In slices pretreated with SP600125 (Fig. 1A,D), however, adenosine only depressed the fEPSP slope by 19.7 ± 5.4\% (n=6), which was significantly less than the in vehicle alone (p < 0.01). JNK inhibition also decreased the magnitude of fEPSP depression induced by a brief hypoxic insult. In slices pretreated with SP600125, hypoxia decreased the fEPSP slope by only 18.3 ± 6.5\% (n=5; p < 0.01) (Fig. 1B,D), which was significantly less than in vehicle alone (53.6 ± 7.4\% depression of fEPSPs; n=5). We also tested whether synaptic depression induced by selective A₁ receptor activation (with CPA) was sensitive to JNK inhibition. CPA decreased the fEPSP slope by 67.6 ± 4.7\% (n=5) and this was reduced to 29.0 ± 7.8\% (n=6; p < 0.01) when slices were pretreated with SP600125 (Fig. 1C,D). These data suggest adenosine A₁ receptor-induced synaptic depression is mediated in part by JNK activation.

4.3.2. \emph{A₁-receptor-mediated synaptic depression is decreased by SP600125 and JNK Inhibitor V, but not by an inactive analogue}

To ensure that the effect of SP600125 on adenosine A₁-mediated synaptic depression was due specifically to JNK inhibition, we tested a second JNK inhibitor (JNK Inhibitor V), as well as an inactive analogue (N¹-methyl-1,9-pyrazoloanthrone), which has been used as a negative control for SP600125 at 5 \mu M (Shin et al., 2002). It is important to note that N¹-methyl-1,9-pyrazoloanthrone can inhibit JNK activity with an IC₅₀ of 24\mu M, but is ineffective at lower doses. Therefore, to compare the effects of the JNK inhibitors with
the negative control we used 5μM for all compounds. We recorded from matched slices (i.e. from the same animal) that were pre-incubated in either of 5μM SP600125 (instead of 20μM as in Fig. 1), JNK Inhibitor V (5μM), the negative control compound (5μM), or control aCSF. CPA (40nM; 10 min) was used to selectively activate A1 receptors. SP600125 at 5μM had a similar effect as SP600125 at 20μM (see above) in decreasing the magnitude of the CPA-induced depression of fEPSPs. In slices pre-treated with 5μM SP600125, CPA decreased the mean normalized fEPSP slope by 41.4 ± 2.7% (n=8), which was significantly less than CPA-alone in matched control slices (70.2 ± 1.0%; n=5; p < 0.01) (Fig. 2A,B,D). To ensure that SP600125 was exerting its effects by selectively blocking JNK activity, we also monitored CPA responses in slices that had been pre-incubated with an inactive analogue (5μM JNK Inhibitor II negative control compound, N1-methyl-1,9-pyrazoloanthrone). CPA similarly decreased the magnitude of the mean fEPSP slope in slices pre-incubated with the inactive analogue (63.0 ± 4.0%; n=9) and normal aCSF (70.2 ± 1.0%; p > 0.20) (Fig. 2A,B,D). We next tested a second JNK inhibitor (JNK inhibitor V), and found that like SP600125, JNK Inhibitor V attenuated CPA-induced synaptic depression. In slices pre-treated with 5μM JNK inhibitor V, CPA depressed the mean fEPSP slope by 28.6 ± 1.0% (n=8), which was significantly less than control (70.2 ± 1.0%; n=5; p < 0.01) (Fig. 2A,C,D). These results suggest that JNK activity contributes to A1-receptor-mediated synaptic depression.
Adenosine A\textsubscript{1} receptor-mediated synaptic depression is attenuated by JNK inhibition with SP600125 and JNK inhibitor V, but not an inactive analogue.

\textit{A}, Sample traces showing that decreases in fEPSP amplitude caused by 40nM CPA was reduced in slices pre-treated with 5\mu M SP600125 (JNK Inhibitor II) or 5\mu M JNK inhibitor V compared with control, but not in slices pre-treated with 5\mu M JNK Inhibitor II negative control. \textit{B}, Plot of the mean fEPSP slope (±SEM), normalized to baseline, showing that CPA-induced synaptic depression was attenuated in slices pre-treated with 5\mu M SP600125 compared with slices pre-treated with 5\mu M JNK Inhibitor II negative control or normal aCSF. \textit{C}, Plot of the mean fEPSP slope (±SEM) showing that pre-treating slices with 5\mu M JNK inhibitor V decreased the efficacy of CPA in decreasing synaptic transmission. \textit{D}, Summary bar chart of \textit{B} and \textit{C} showing the mean magnitude of CPA-induced depression of fEPSP slopes (±SEM). *Statistical significance assessed using the Student \textit{t}-test, \( p < 0.01 \). Calibration: 10mV/10ms.
4.3.3. **JNK inhibition increases the excitability of rat hippocampal slices**

It has been reported that the JNK inhibitor, SP600125 (20μM) increases baseline synaptic transmission in rat hippocampal slices (Costello and Herron, 2004), suggesting that tonic A1 receptor activation mediates inhibition of basal glutamate release. Consistent with this notion, inhibiting JNK by pre-treating slices with 5μM JNK Inhibitor V (n=8) led to increased excitability as indicated by an increase in the average magnitude fEPSPs compared to matched controls (n=11). This occurred at stimulus intensities ranging from 4 to 10 V without a change in the apparent threshold for activation (~6 V; Fig. 2E). However, the responses elicited by a given stimulus intensity were significantly larger in slices that had been pre-treated with JNK inhibitor V (5μM) (Fig 2E). On average, pre-treatment with JNK inhibitor V (5μM; 1-3 hours) increased the mean fEPSP slope by 89 ± 3.2% (p < 0.01) at suprathreshold stimuli. These data indicate that JNK inhibition increases fEPSP magnitude, possibly due to removal of tonic adenosine inhibition in the slice.

4.3.4. **A1 receptor stimulation increases JNK phosphorylation**

The sensitivity of adenosine A1 receptor-mediated synaptic depression to JNK inhibitors suggests that JNK activity is a necessary step in adenosine-induced inhibition of synaptic transmission. Therefore we tested whether A1 receptor activation increased JNK activity in hippocampal slices by quantifying the amount of activated (i.e. phosphorylated) JNK. Slices were treated with either CPA (500nM), to selectively activate A1 receptors, or with
normal aCSF for 2, 5, 10, or 30 min and the amount of phosphorylated JNK compared by Western blotting. Using centrifugation, we extracted the membrane fraction from hippocampal lysates. Western blot analysis was performed using a rabbit polyclonal antibody that only recognizes JNK when it is dually phosphorylated at threonine 183 and tyrosine 185. Of the three JNK isoforms encoded by different genes, JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is selectively expressed in the nervous system and heart (Gupta et al., 1996; Martin et al., 1996; Kuan et al., 1999). The antibody against phosphorylated JNK recognizes a band at 46 kDa which consists of all three JNK isoforms (JNK1, JNK2, and JNK3), and a band at 54 kDa which consists of only the JNK2 and JNK3 isoforms (Kuan et al., 2003). Densitometry analysis of the 54 kDa band (representing JNK2 and JNK3), normalized to B-actin, revealed that A1 receptor stimulation with CPA increased JNK phosphorylation to $154 \pm 14.3\%$ of baseline within 2 minutes ($n=6; p < 0.01$; Fig 3A). Phospho-JNK immunoreactivity remained significantly elevated after CPA treatment for 5 min ($144 \pm 7.0\%$ of baseline; $n=6; p < 0.05$) and 10 min ($165 \pm 19.4\%$ of baseline; $n=5; p < 0.01$). By 30 min of CPA treatment, JNK phosphorylation had returned towards baseline levels ($121 \pm 17.9\%; n=6; p > 0.20$) (Fig 3A). Densitometry analysis of the 46 kDa band, which contains all three JNK isoforms (Fig. 3B) revealed that JNK phosphorylation significantly increased after 2 min of CPA treatment ($134 \pm 12.3\%; n=6; p < 0.05$). Although the mean phospho-JNK immunoreactivity trended higher than control after 5 min ($121.7 \pm 11.9\%; n=6; p = 0.09$) and 10 min ($137 \pm 16.6\%; n=9; p = 0.09$) of CPA treatment, this trend failed to reach statistical significance. By 30 min, which was the longest time point tested, phospho-JNK immunoreactivity had returned to control levels ($102 \pm 12.0\%$ of baseline; $n=5$).
Figure 4.3. Stimulation of adenosine A<sub>1</sub> receptors, which are physically associated with JNK, increases JNK phosphorylation.

Hippocampal slices were exposed to either normal aCSF or CPA (500 nM) for 2, 5, 10, or 30 min. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation for Western blot analysis. A (upper panel), Representative Western blots showing phosphorylated JNK 2/3 (phospho-JNK 2/3) and β-actin in the membrane fraction at time points ranging from 0 to 30 min. Lower panel, Quantitative representation of multiple Western blots showing phospho-JNK 2/3 immunoreactivity (mean ± SEM) in the membrane fraction at time 0 min (n=6), 2 min (n=6), 5 min (n=6), 10 min (n=5) and 30 min (n=5). Data were normalized to the level of protein phosphorylation at time 0 after CPA treatment (control), and β-actin immunoreactivity was used as a loading control. B (upper panel), Representative Western blots showing phosphorylated JNK 1/2/3 and β-actin immunoreactivity in the membrane fraction at time points ranging from 0 to 30 min. Lower panel, Quantitative representation of multiple Western blots showing phospho-JNK 1/2/3 immunoreactivity (mean ± SEM) in the membrane fraction at time 0 min (n=6), 2 min (n=6), 5 min (n=6), 10 min (n=5) and 30 min (n=5). C, Representative Western blots and mean immunoreactivity (normalized to β-actin; ± SEM) using a pan-specific JNK antibody showing that CPA treatment does not change the total amount of JNK immunoreactivity in the membrane fraction (n=6). D, Western blot showing that immunoprecipitation with a pan-specific JNK antibody pulls down the A<sub>1</sub> receptor (lane 1). Lane 2 shows the presence of the A<sub>1</sub> receptor in whole-cell hippocampal lysate. E, Western blot showing that immunoprecipitation with a phospho-JNK antibody does not pull down the A<sub>1</sub> receptor (lane 1). Lane 2 shows the
presence of the A₁ receptor in whole-cell hippocampal lysate. Statistical significance assessed using the Student t-test, where * denotes $p < 0.05$ and ** denotes $p < 0.01$. 

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**A**

Phospho-JNK 2/3 (54 kDa)

<table>
<thead>
<tr>
<th>Time in CPA (minutes)</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>48</th>
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<tr>
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</table>

Phospho-JNK 2/3 (54 kDa) percentage of control over time of CPA treatment (minutes).

**B**

Phospho-JNK 1/2/3 (46 kDa)

<table>
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<tr>
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<th>2</th>
<th>5</th>
<th>10</th>
<th>30</th>
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</tbody>
</table>

Phospho-JNK 1/2/3 (46 kDa) percentage of control over time of CPA treatment (minutes).

**C**

Total JNK 2/3

<table>
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<tr>
<th></th>
<th>Control</th>
<th>CPA</th>
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<tbody>
<tr>
<td>β-actin</td>
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</table>

Total JNK 2/3 percentage of control over time of CPA treatment (minutes).

**D**

WB: A₁ Receptor

IP: JNK Lysate

**E**

WB: A₁ Receptor

IP: Phospho-JNK Lysate
We next determined if A$_1$ receptor activation changed the amount of JNK associated with the plasma membrane. Using a pan-specific antibody, we determined that total JNK immunoreactivity in the membrane fraction did not change in response to CPA treatment ($105 \pm 8.0\%$ of baseline; $n=6$; $p > 0.50$) (Fig. 3C). Thus, western blot analysis of phospho-JNK immunoreactivity suggests that A$_1$ receptor stimulation increased the activity of JNK in the hippocampus. This finding is consistent with our electrophysiological data (see above) which show that blocking JNK activity reduced the ability of A$_1$ receptors to inhibit synaptic transmission.

### 4.3.5. The A$_1$ receptor is physically associated with JNK, but not phospho-JNK

We performed co-immunoprecipitation experiments to determine whether the adenosine A$_1$ receptor exists in the same signalling complex as JNK or phospho-JNK. The adenosine A$_1$ receptor corresponds to a band at approximately 37 kDa in the rat hippocampus (Rebola et al., 2003). Immunoprecipitation with the JNK antibody (probed with a polyclonal rabbit anti-A$_1$ receptor antibody) revealed the A$_1$ receptor (Fig. 3D, lane 1). Lane 2 shows the presence of the same band in whole-cell lysates. Immunoprecipitation with the anti-phospho-JNK antibody failed to pull down the A$_1$ receptor (Fig. 3E, lane 1). Lane 2 shows that the A$_1$ receptor was detectable in whole-cell lysates. These data suggest that the adenosine A$_1$ receptor is physically associated with JNK, but not phospho-JNK, in the hippocampus.
4.3.6. **CPA-dependent increases in JNK phosphorylation are blocked by $A_1$ receptor antagonism**

To determine whether the increase in JNK phosphorylation that we observed in response to CPA treatment (see above) was specifically mediated by $A_1$ receptor stimulation, we tested whether the $A_1$ receptor antagonist DPCPX blocked the CPA-induced increases in phospho-JNK immunoreactivity. As reported above, the 54 kDa band showed sustained significant increase in the level of phospho-JNK after 10 min of CPA treatment, but the 46 kDa band was not significantly increased at this time point. We therefore focused our analysis on the 54 kDa band. Because DPCPX has been reported to increase glutamate release and evoke seizure discharges (Sehmisch et al., 2001; Marcoli et al., 2003) which could alter JNK phosphorylation, we pre-treated slices with 1.2μM tetrodotoxin (TTX) for 20 min. CPA effectively increased phospho-JNK in the presence of TTX (177 ± 17.8% of control; n=10; $p < 0.01$) (Fig. 4A). Slices were incubated in DPCPX (500nM; 20 min) or normal aCSF and then treated with CPA (500nM; 10 min). We observed that the CPA-induced increase in JNK phosphorylation was attenuated in the presence of DPCPX (Fig. 4A) to 125 ± 21% (n=10; $p > 0.20$) of the control level (+TTX). These findings indicate that $A_1$ receptor stimulation selectively increases JNK phosphorylation in the rat hippocampus.

4.3.7. **CPA-dependent increases in JNK phosphorylation are blocked by inhibition of JNK and p38 MAPK**

In a previous study, we found that p38 MAPK activity contributes to adenosine $A_1$-receptor mediated synaptic depression (Brust et al., 2005). The p38 MAPK inhibitor
Figure 4.4. The increase in JNK phosphorylation induced by CPA was blocked by A₁ receptor antagonism.

Hippocampal slices were pre-incubated in normal aCSF or DPCPX (500nM; 20 min) and then exposed to CPA (500nM; 10 min). The membrane fraction was separated by centrifugation for Western blot analysis. *Upper panel*, Representative Western blots showing phosphorylated JNK (phospho-JNK) and β-actin immunoreactivity in response to CPA treatment in the presence and absence of DPCPX. *Lower panel*, Quantified phospho-JNK immunoreactivity (mean ± SEM) for control (n=10), CPA (n=10), and CPA + DPCPX (n=10). Data were normalized to β-actin immunoreactivity. Statistical significance compared with control was assessed using a Student t-test, where * denotes $p < 0.05$ and ** denotes $p < 0.01$. 
SB203580 attenuated CPA-induced depression of fEPSPs, and CPA treatment increased the phosphorylation of p38 MAPK in membrane fractions. In the present study, we tested whether SP600125 and SB206393 (a second generation p38 MAPK inhibitor) affected CPA-induced increases in JNK phosphorylation. We also investigated whether the A1 induced activation of p38 MAPK and JNK was through parallel or serial pathways. Slices were incubated in control aCSF (n=9), 20µM SP600125 (n=4), or 5µM SB206393 (n=4) for one hour prior to CPA treatment (500nM; 10 min). As expected, CPA increased phospho-JNK immunoreactivity in normal aCSF (in this case to 152 ± 14.0% of control; n=9; p < 0.01) (Fig 5B, lane 2). We found that CPA failed to increase phospho-JNK in the presence of SP600125 (Fig. 4B, lane 3). In slices pre-treated with SP600125, phospho-JNK levels were 93 ± 12.0% of baseline (n=4), which was significantly less than the increase to 152% observed in slices exposed to CPA in control conditions (p < 0.05).

Surprisingly, the p38 MAPK inhibitor SB206393 also prevented increases in JNK phosphorylation due to CPA (Fig. 4B, lane 4). In slices pre-incubated in SB206393 and treated with CPA (500nM; 10 min), phospho-JNK immunoreactivity was reduced to 68 ± 13.0% of baseline (n=5). The level of phospho-JNK immunoreactivity observed in slices pre-incubated with SB206393 was significantly less than both slices exposed to normal aCSF (p < 0.01) and slices exposed to normal aCSF plus CPA (p < 0.01). Together these data show that inhibiting JNK and/or p38 MAPK activity prevents CPA-induced increases in JNK phosphorylation.
4.3.8. CPA-dependent increases in p38 MAPK phosphorylation are blocked by p38 MAPK inhibition but not JNK inhibition

The above data show that p38 MAPK and JNK activation are both activated by A1 receptor stimulation, and suggest that p38 MAPK activity may be required for JNK activation. To gain further insight into whether p38 MAPK and JNK are activated in parallel or sequential pathways, the JNK inhibitor SP600125 was tested for its ability to affect CPA-induced p38 MAPK phosphorylation. Slices were incubated in control aCSF, 20μM SP600125, or 5μM SB206393 for one hour prior to CPA treatment (500nM; 10 min). As in our previous study (Brust et al., 2005), activation of A1 receptors with CPA increased p38 MAPK phosphorylation in membrane fractions of rat hippocampal lysates (in this case to 131 ± 11.8% of control; n=5; p < 0.05; Fig. 5A). Incubating slices in SP600125 did not prevent p38 MAPK phosphorylation, which increased to 138 ± 18.5% of control (n=5; p < 0.05; Fig. 5B). However, SB206393 completely abolished the CPA-induced increase in p38 phosphorylation, and actually decreased phospho-p38 immunoreactivity to 71 ± 10.5% of baseline (n=4; p < 0.05). To summarize, JNK phosphorylation is prevented by both p38 MAPK inhibition (with SB206393) and JNK inhibition (with SP600125) (see above), whereas p38 MAPK phosphorylation is prevented by SB206393 but not SP600125. Therefore, p38 MAPK phosphorylation appears to be necessary for JNK phosphorylation. Our data supports a model where p38 MAPK and JNK are activated serially instead of in parallel following A1 receptor stimulation.
Figure 4.5. Adenosine A₁ receptor stimulation sequentially activates p38 MAPK and JNK.

Hippocampal slices were either pre-incubated in normal aCSF, SP600125 (20μM; 1 hour), or SB206393 (5μM; 1 hour) and then exposed to CPA (500nM; 10 min). The membrane fraction was separated by centrifugation for Western blot analysis. A (upper panel), Representative Western blots showing phosphorylated JNK (phospho-JNK) and β-actin immunoreactivity in response to CPA treatment in the presence and absence of SP600125. SP600125 and SB206393 prevent CPA-induced JNK phosphorylation. Lower panel, Quantified phospho-JNK immunoreactivity (mean ± SEM) for control (n=9), CPA (n=9), and CPA + SP600125 (n=4). Data were normalized to β-actin immunoreactivity. B (upper panel), Representative Western blots showing phosphorylated p38 MAPK (phospho-p38) and β-actin immunoreactivity in response to CPA treatment in the presence and absence of SB206393. SB206393 prevents CPA-induced p38 MAPK phosphorylation, but SP600125 does not. Lower panel, Quantified phospho-p38 immunoreactivity (mean ± SEM) for control (n=5), CPA (n=5), and CPA + SB206393 (n=4). Data were normalized to β-actin immunoreactivity. Statistical significance compared with control was assessed using a Student t-test, where * denotes \( p < 0.05 \) and ** denotes \( p < 0.01 \).
4.4. Discussion

In the present study, we provide evidence that A₁ receptor activation stimulates JNK activity in hippocampal slices, and that JNK activation is necessary for A₁ receptor-mediated synaptic depression. We demonstrated that adenosine, hypoxia, and CPA decreased fEPSP slopes in a pathway requiring JNK activity. Inhibition of JNK with both SP600125 and JNK inhibitor V, but not an inactive analogue, attenuated A₁ receptor-dependent synaptic depression. Western blot analysis confirmed that A₁ receptor stimulation phosphorylated membrane associated JNK in a time course consistent with CPA-induced attenuation of fEPSPs.

In our western blot analysis, we observed that the phospho-JNK antibody recognized two bands: a 46 kDa band composed of all three JNK isoforms, and a band at 54 kDa band, which comprises only JNK2 and the neuronal specific JNK3 isoform (Kuan et al., 2003). The 54 kDa band contained much less total immunoreactivity than the 46 kDa band, probably because JNK1, which is responsible for the high level of basal JNK activity in the brain (Yang et al., 1997), exists primarily as p46JNK (Kuan et al., 2003). Both JNK2 and JNK3 exist as p54JNK and p46JNK (Kuan et al., 2003). Although we observed that CPA initially (within 2 min) increased the amount of phospho-JNK immunoreactivity in both bands, CPA-induced phosphorylation was proportionally greater in the JNK2/3 54 kDa band than the JNK1/2/3 46 kDa band. This suggests that there is a correlation between A₁ receptor activation and the specific phosphorylation of neuronal JNK3, although effects on JNK1 and JNK2 cannot be ruled out.

Co-Immunoprecipitation revealed that JNK, but not phospho-JNK, is physically associated with A₁ receptors. The differential binding of JNK and phospho-JNK supports
a model in which A1 receptors and JNK exist in a signalling complex, and that A1 receptor activation phosphorylates JNK causing it to dissociate from the complex. Such a model would imply that the A1 receptor-JNK complex is in constant dynamic equilibrium, with JNK continually cycling into the complex to replace dissociated phospho-JNK. Presumably, the phosphorylated JNK then mediates inhibition of neurotransmission.

Although the precise mechanism of JNK-induced inhibition of neurotransmission has yet to be determined, there is tantalizing evidence that JNK activation is intimately linked to decreased glutamate release in the hippocampus (Vereker et al., 2000b; Vereker et al., 2000a; Lynch and Lynch, 2002; Curran et al., 2003; Costello and Herron, 2004). JNK inhibition with SP600125 increases baseline synaptic transmission and reduces paired-pulse facilitation, which is consistent with enhanced neurotransmitter release (Costello and Herron, 2004). Moreover, glutamate release is inhibited in synaptosomes exhibiting elevated JNK phosphorylation (induced by applying a tetanus in slices obtained from interleukin-1β-treated rats), providing a further indication that endogenous JNK activity regulates neurotransmitter release (Vereker et al., 2000a). In agreement with the above studies, we also found that inhibition of JNK increased excitability in hippocampal slices. Because ambient adenosine in the extracellular space is a tonic suppressor of glutamate release under basal conditions, the increase in synaptic transmission due to SP600125 or JNK Inhibitor V may occur because JNK inhibition removes tonic A1-receptor-mediated depression of neurotransmission.

We have previously demonstrated that p38 MAPK activation is necessary for adenosine A1 receptor-mediated synaptic depression (Brust et al., 2005), as well as Bz-
ATP-induced depression of mossy fiber-CA3 synaptic transmission (Armstrong et al., 2002). Inhibition of p38 MAPK activity almost completely blocks the depression of evoked CA1 fEPSPs induced by the A1 receptor agonist CPA, adenosine, or brief hypoxia. Moreover, CPA phosphorylates p38 MAPK within minutes in hippocampal slices. The link between adenosine A1 receptors and p38 MAPK is well established in other cells, as A1 receptor stimulation activates p38 MAPK in transfected Chinese hamster ovary cells (Robinson and Dickenson, 2001). A1 receptors also activate p38 MAPK in the perfused rat heart (Zhao et al., 2001b; Schulte et al., 2004), where A1-p38 MAPK signalling mediates ischemic preconditioning. Here, we show that CPA-induced JNK phosphorylation is blocked by p38 MAPK inhibition but CPA-induced p38 phosphorylation is not blocked by JNK inhibition, revealing a sequential activation of p38 MAPK then JNK upon A1 receptor stimulation. Our previous results (Brust et al., 2005) also demonstrated that p38 MAPK inhibition with SB203580 greatly attenuated CPA-induced inhibition of synaptic transmission (i.e., in the presence of SB203580, CPA only produced <10% inhibition of synaptic transmission compared with ~70% inhibition in control). In contrast, CPA was still able to depress synaptic transmission by about 30% in slices pre-incubated with JNK inhibitors (SP600125 or JNK inhibitor V, present study). Our data thus indicate that p38 MAPK activation may be necessary for JNK activation following A1 receptor stimulation, and that both p38 MAPK and JNK contribute to inhibition of synaptic transmission.

The mechanism by which JNK is activated following p38 MAPK is unclear at present, and may involve multiple substrates. For example, adenosine A1 receptor activation leads to protein-phosphatase 2a translocation in both cardiac tissue (Liu and
Hofmann, 2003) and hippocampal tissue (Brust et al., 2005) in a pathway requiring p38 MAPK activation. Inhibiting PP2a activity (with okadaic acid) also blocks JNK phosphorylation in response to TNF-α, suggesting that PP2a regulates activation of JNK (Ray et al., 2005). Therefore, p38 MAPK may activate JNK through PP2a translocation and activation. Alternatively CK2 (formerly casein kinase II) may function as a mediator of p38 MAPK-JNK signalling. CK2 is directly activated by p38 MAP kinase (Sayed et al., 2000), and also phosphorylates JNK (Min et al., 2003). However, determining whether JNK is a downstream target of p38 MAPK in the hippocampus will require further studies.

The primary mechanism underlying A1 receptor-mediated inhibition of neurotransmission is thought to reflect a G protein-coupled inhibition of calcium influx in nerve terminals (Dunwiddie and Masino, 2001). This is not the only possibility however, because adenosine also decreases excitability by activating G protein-gated inwardly rectifying potassium channels (GIRKs) in the postsynaptic membrane (Luscher et al., 1997). Although JNK is involved in the activation of voltage-gated K+ channels (Gao et al., 2004), whether JNK modulates GIRKs following A1 receptor stimulation remains to be established.

During trauma such as oxygen deprivation, adenosine increases to concentrations of 10μM or more in the extracellular space where it acts as an endogenous neuroprotectant by inhibiting potentially toxic glutamate release (Rudolphi et al., 1992; Von Lubitz, 1999; Latini and Pedata, 2001; Marcoli et al., 2003; Boeck et al., 2005). JNK phosphorylation rapidly increases (within minutes) following cerebral ischemia (Comerford et al., 2004; Gao et al., 2005), possibly as a result of adenosine release and
subsequent A₁ receptor-mediated JNK activation. However, whether JNK activation is
neuroprotective or neurodegenerative is controversial. JNK mediates neurodegeneration
by activating genetic programs through phosphorylation of the nuclear transcription
factor c-Jun/AP-1, the release of cytochrome c or the pro-inflammatory actions of
microglia (Manning and Davis, 2003). Indeed ischemic injury and excitotoxicity is
dramatically reduced in the brains of mice lacking the JNK3 gene (Kuan et al., 2003) or
treated with a JNK-inhibitor (Borsello et al., 2003; Gao et al., 2005) in the days following
an ischemic insult. Although these findings support role for JNK in neuronal death after
ischemia/reperfusion injury, the long-term effects of JNK activation may differ
significantly from the effect of JNK activation during an ischemic insult, when it may
contribute to adenosine-mediated neuroprotection.

In contrast to its role in apoptosis during ischemia, JNK activation promotes cell
survival by phosphorylating Akt/PKB in ventricular myocytes after hypoxia-
reoxygenation (Shao et al., 2006). The dichotomous actions of JNK could result from
differential availability and preferential site-specific phosphorylation of a given JNK
substrate depending on the context of JNK activation. Alternatively, analysis of the
consequences of JNK activation at different time points during and following ischemic
insults may yield different results. In the hours and days post-ischemia, JNK activation
may indeed lead to apoptosis (Manning and Davis, 2003), whereas immediately during an
ischemic insult JNK activation may confer protection from excitotoxicity by inhibiting
glutamate release in response to A₁ receptor stimulation. Because JNK inhibitors are
potential therapeutics for stroke, it will be valuable to ensure that the timing of their use
does not impede the neuroprotective power of endogenous adenosine.
The present study demonstrates that JNK activation is necessary for adenosine A<sub>1</sub> receptor-mediated depression of neurotransmission at CA3-CA1 synapses in the rat hippocampus. Adenosine A<sub>1</sub> receptor mediated JNK signalling may represent a novel mechanism underlying neuroprotection and inhibition of neurotransmitter release in the central nervous system.

4.5. References


Lynch AM, Lynch MA (2002) The age-related increase in IL-1 type I receptor in rat hippocampus is coupled with an increase in caspase-3 activation. Eur J Neurosci 15:1779-1788.


Chapter Five: General Discussion
5.1. Introduction

The research described here tests the hypothesis that MAPKs are a component of purinergic signalling in the central nervous system. A breadth of biochemical, pharmacological, and electrophysiological techniques provides evidence that the synaptic inhibition caused by purinoceptor stimulation requires the activation of p38 MAPK and/or JNK in the hippocampus. The data suggests that P2X$_7$ receptors, located in mossy fiber terminals, decrease glutamate release through activation of p38 MAPK. Further studies in the CA1 region suggest that sequential activation of p38 MAPK and JNK is required for A1 receptor-mediated inhibition of neurotransmission. A working hypothesis that emerges from these observations is that A$_1$ receptors exist in a complex with p38 MAPK and JNK and that this complex functions to decrease Ca$^{2+}$ influx through presynaptic neuronal calcium channels upon A$_1$ receptor-dependent phosphorylation of p38 MAPK and JNK. Although the evidence presented in the previous three chapters provides a compelling case for such a notion, some potential limitations are discussed below, and alternate interpretations of the data are identified. In addition, the findings presented here are integrated and evaluated in the context of current knowledge. Finally, implications to the field of study as well as ideas for future experiments are proposed.

5.2. P2X$_7$ receptor-mediated synaptic depression

In our study investigating the role of P2X$_7$ receptor-mediated synaptic depression of mossy fiber-CA3 synaptic transmission, we used an antibody directed towards the C-
terminus of the P2X<sub>7</sub> receptor protein (Cterm-ab) to show P2X<sub>7</sub> receptor immunoreactivity in mossy fiber terminals (Armstrong et al., 2002). We observed labelling of small-terminal-like puncta throughout the hippocampus, a finding that has since been replicated in another study using the same antibody (Sim et al., 2004). P2X<sub>7</sub> immunoreactivity was particularly dense in the termination zones of hippocampal mossy fibers, where it was colocalized with the presynaptic marker syntaxin 1A/B but not the dendritic marker MAP-2. Based on this immunohistochemical data, we concluded that P2X<sub>7</sub> are abundant in mossy fiber terminals. We used electrophysiology and pharmacology to confirm that selective P2X<sub>7</sub> receptor activation had functional consequences in area CA3 of the hippocampus. The P2X<sub>7</sub> receptor agonist Bz-ATP induced a rapid and long-lasting synaptic depression that was inhibited by the P2X<sub>7</sub> receptor antagonist oxidized periodate-ATP, but not the P2Y receptor antagonist reactive blue 2 or the P2X<sub>1-3,5,6</sub> receptor antagonist PPADS. Together, our data indicated that P2X<sub>7</sub> receptors are present in mossy fiber presynaptic terminals where they are functionally important in depressing mossy fiber-CA3 synaptic transmission.

P2X<sub>7</sub> receptors are only activated by high concentrations of ATP (North, 2002), such as would occur during a tetanus or seizure. Thus, P2X<sub>7</sub> receptor-dependent inhibition of neurotransmission may be an important neuroprotective mechanism in the brain because of its potential to dampen potentially toxic release of excitatory neurotransmitters (i.e. glutamate).

The P2X<sub>7</sub> receptor is a particularly intriguing membrane receptor because it can not only function as a non-selective cation channel, but also as a mediator of cell membrane permeabilization (North, 2002). This unique property is due to a longer
intracellular C-terminus than other P2X members, conferring the ability to form a large permeabilizing pore. In addition, the P2X$_7$ receptor is Ca$^{2+}$ permeable, which could help explain the diverse effects of P2X$_7$ receptor stimulation on processes relating to immune responses, cell survival, release of interleukin-1b, activation of NFkB and NFAT, and the induction of both necrotic and apoptotic cell death (North, 2002). The mechanism by which P2X$_7$ receptors initiate second messenger cascades is not known in nearly as much detail as prototypical P1 or P2Y G protein-dependent signalling.

5.2.1. Possible limitations

In addition to the P2X7 receptor Cterm-ab that we used in our study (Alamone Laboratories), two other antibodies have been used to examine P2X$_7$ localization in rat brain (EctoAb-1 and EctoAb-2). Unfortunately, each of these three antibodies show different patterns of P2X$_7$ immunoreactivity (Sim et al., 2004), making it difficult to determine which pattern reliably indicates P2X$_7$ receptor expression in rat brain. It should be noted that the Cterm-ab that we used in our P2X$_7$ receptor study is the most specific of the three antibodies, recognizing a single band at the appropriate molecular weight in HEK cells (Sim et al., 2004). In an attempt to determine which pattern of immunoreactivity is an accurate representation of P2X$_7$ protein expression, Annmarie Surprenant and colleagues (Sim et al., 2004) examined the patterns of immunoreactivity of each of these antibodies in two types of P2X$_7$ knock-out mice (Pfizer P2X$_7^{-/-}$ and Glaxo P2X$_7^{-/-}$).

Surprisingly, there was no difference in the pattern of P2X$_7$ immunoreactivity between wild-type mice and either of the two knockout mice in the hippocampus (Sim et al., 2004). In contrast, the robust immunoreactivity detected by the Cterm-ab and the
EctoAb-1 in the lung and submandibular gland was completely absent in both Pfizer P2X<sup>−/−</sup> and Glaxo P2X<sup>−/−</sup> mice. Moreover, Western blot analysis using these antibodies revealed the presence of a band at the appropriate weight to represent P2X<sub>7</sub> receptor protein (75 kDa) in the brain, lung, and submandibular gland of wild-type mice that was absent in the lung and submandibular gland, but not brain, of both P2X<sub>7</sub> knockout mice. Surprenant et al. (2004) argue that ourselves (Armstrong et al., 2002) and others who have described P2X<sub>7</sub> receptor expression and function in neurons (Deuchars et al., 2001; Atkinson et al., 2002; Lundy et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003; Cavaliere et al., 2004) have been "fooled" by the presence of a brain-specific protein that happens to be the same molecular weight as the P2X<sub>7</sub> receptor and that happens to be recognized by all three P2X<sub>7</sub> receptor antibodies. The authors conclude that "the only reasonable explanation for all of these results is that P2X<sub>7</sub> receptor protein is either not expressed in neurons of the normal adult rodent brain, or that levels are too low to be detected by any method currently available."

An alternate explanation is that a slightly different isoform of the P2X<sub>7</sub> receptor protein exists in the brain, and that this neuronal isoform is not recognized by the P2X<sub>7</sub> receptor antibodies in wild-type or knockout mice, whereas the isoform expressed in peripheral tissue is recognized by these antibodies. It may seem unlikely that a different isoform of the P2X<sub>7</sub> receptor protein that does not contain the appropriate epitopes is expressed in brain and not the periphery. However, seven additional variants of the human P2X<sub>7</sub> receptor resulting from alternative splicing have recently been reported, with differential expression patterns in different tissues (Cheewatrakoolpong et al., 2005). One variant lacked the entire cytoplasmic tail, yet Ca<sup>2+</sup> flux was largely unaffected. This
finding supports the idea that different isoforms are expressed in different tissues. The Pfizer P2X$_7^{-/-}$ knockout mouse was generated by deletion of the amino acids from Cys$^{506}$ to Pro$^{532}$ in the carboxyl-terminal domain of the P2X$_7$ receptor gene product (Solle et al., 2001). Therefore, the discovery of a functional P2X$_7$ variant lacking the cytoplasmic tail supports the notion that genetic deletion of the cytoplasmic tail (as in the Pfizer P2X$_7^{-/-}$ knockout mice) may not actually functionally inhibit P2X$_7$ receptor channels. Real-time PCR revealed that the alternatively spliced (and presumably functional) P2X$_7$ variant lacking the cytoplasmic tail was abundantly expressed in brain tissue (Cheewatrakoolpong et al., 2005). Clearly, it will take further investigation to determine whether P2X$_7$ receptors truly are absent in hippocampal neurons.

Another study that casts doubt on the hypothesis that P2X$_7$ receptors are localized in presynaptic nerve terminals and function to decrease neurotransmitter release was recently conducted by Dirk Dietrich and colleagues (Kukley et al., 2004). Although the authors confirmed that Bz-ATP produces a pronounced inhibition of mossy fiber-CA3 synaptic transmission, they suggest this is caused by activation of adenosine receptors following enzymatic conversion of Bz-ATP to Bz-adenosine and heteroexchange with cellular adenosine via nucleoside transporters.

Bz-ATP mimics both the mossy fiber-CA3 synaptic depression and the activation of GIRK channels induced by A$_1$ receptor activation (Luscher et al., 1997). Furthermore, all effects of Bz-ATP could be reversed by the selective A$_1$ receptor antagonist DPCPX. Therefore, the authors conclude that Bz-ATP application onto hippocampal slices leads to A$_1$ receptor activation, and that Bz-ATP induced synaptic depression is actually A$_1$ receptor-mediated.
Because Bz-Adenosine, the hydrolyzed product of Bz-ATP, is unlikely to be an effective \( A_1 \) receptor agonist (Klotz, 2000; Kukley et al., 2004), the authors conducted further experiments to determine the mechanism by which Bz-ATP ultimately stimulates \( A_1 \) receptors. They found that inhibition of either ecto-nucleotidases (with ARL 67156, ITP, and concanavalin A) or nucleoside transporters (with excess inosine) blocks Bz-ATP-induced currents, suggesting that Bz-ATP is converted to adenosine by a combined action of ecto-nucleotidases and nucleoside transporters.

In our P2X\(_7\) receptor study, we found that Bz-ATP-induced depression of mossy fiber-CA3 synaptic transmission was blocked by p38 MAPK inhibition with SB203580, but adenosine-induced synaptic depression was not. The Dietrich group (2004) attempts to account for the difference in the effect of SB203580 on Bz-ATP- versus adenosine-induced synaptic depression by proposing that SB203580 is actually blocking nucleoside transporters and preventing the exchange of Bz-adenosine for adenosine. The net effect of SB203580 in hippocampal slices, they argue, is blockade of adenosine release into the extracellular space where it can act on \( A_1 \) receptors. The evidence they present in support of this hypothesis is that the activity of Bz-ATP was potently suppressed by treating slices with SB203580 (in agreement with our results), whereas the current evoked by ATP was not. Since ATP is rapidly degraded to adenosine by ecto-nucleotidases (Zimmermann and Braun, 1996; Cunha et al., 1998), the authors have in effect simply replicated our results without any new evidence to support their interpretation over ours.

Furthermore, the net effect of nucleoside transporters is the uptake of adenosine from the extracellular space (Fredholm et al., 1994; Latini and Pedata, 2001). Therefore, if SB203580 truly were blocking nucleoside transporters, then treating slices with
SB203580 would be expected to increase ambient adenosine in the extracellular space and cause a decrease in baseline synaptic transmission, as has been reported (Dunwiddie and Diao, 2000). This is clearly not the case, as we have demonstrated that perfusion of SB203580 onto slices actually increases the amplitude of fEPSPs (Fig. 3.4), which is not consistent with an increase in adenosine tone due to the inhibition of nucleoside transporters.

Perhaps the main problem with the study Kukely et al. (2004) is that most of their experiments use postsynaptic K\textsuperscript{+} currents as an assay for the effects of Bz-ATP and SB203580. It is difficult to extrapolate such data to presynaptic terminals, where the signalling pathway linking P2X\textsubscript{7} or A\textsubscript{1} receptors to decreased neurotransmission may be significantly different.

One question that remains is why adenosine-induced synaptic depression was not attenuated by SB203580 in mossy fiber synapses (Armstrong et al., 2002), whereas in the CA1 region it was inhibited by ~50% (Figs. 3.4 & 3.5). Mossy fiber-CA3 synapses have distinct properties from other central synapses such as the CA3-CA1 synapse (Nicoll and Schmitz, 2005). For example, they can exhibit larger paired-pulse facilitation (with a ratio of > 3) and can undergo frequency facilitation (changing the frequency of stimulation from low to high dramatically increases synaptic strength). In addition, the induction of LTP is independent of NMDA receptor activation. Given the distinct functional properties of mossy fiber synapses, it is perhaps not surprising that mossy fiber terminals also have markedly different expression of voltage-dependent calcium channels, exhibiting predominantly P-type calcium-dependent evoked release of neurotransmitter (Castillo et al., 1994). Therefore, a possible explanation for the
differential effect of p38 MAPK on adenosine-mediated inhibition of neurotransmitter release could be that A<sub>1</sub>-p38 MAPK signalling specifically inhibits the N-type calcium channel subtype.

The role of P2X<sub>7</sub> receptors in inhibiting presynaptic glutamate release was further challenged by studies showing that Bz-ATP actually facilitated glutamate release in the rat hippocampus (Sperlagh et al., 2002) and spinal cord (Deuchars et al., 2001). However, the question of whether P2X<sub>7</sub> receptor activation mediates presynaptic inhibition or facilitation may be moot if P2X<sub>7</sub> receptors are not present in the brain at all. In support of this notion, Rodrigo Cunha and colleagues (2005) confirmed that Bz-ATP evoked release of glutamate in purified nerve terminals, but they showed that the facilitation of glutamate release was blocked by suramin and not by the P2X<sub>7</sub> receptor antagonist brilliant blue G. This pharmacological profile indicates the involvement of P2X<sub>1</sub> receptors and not P2X<sub>7</sub> receptors because P2X<sub>7</sub> receptors, particularly in the rat, are sensitive to brilliant blue G and not suramin (North, 2002).

5.2.2. Future Directions

Clearly more studies will be required to determine whether P2X<sub>7</sub> receptors are expressed in neurons, glia, or both in the central nervous system. Approaches using techniques in molecular biology are probably the most appropriate. One such approach may be to identify the protein from SDS-PAGE gels prepared from hippocampal lysates or mossy fiber synaptosomes using mass spectrometry. In such an experiment, the excised gel slice containing the protein of interest is subjected to proteolytic digestion followed by mass spectrometric analysis of the resulting peptides. Until there is a definitive answer as
to whether P2X<sub>7</sub> receptors are expressed in neurons, it is hard to justify further functional studies.

### 5.3. Adenosine A<sub>1</sub> receptor signalling through p38 MAPK

This study is an overdue investigation into the molecular mechanisms underlying the inhibition of glutamate release by adenosine, a powerful and ubiquitous inhibitory modulator of excitatory synaptic transmission in the mammalian central nervous system. Although the inhibitory actions of adenosine have been known for some time, the signalling by which this occurs has not been studied in detail.

A number of independent lines of evidence suggest that p38 mitogen-activated protein kinase (MAPK) may play an important role in A<sub>1</sub>-mediated modulation of the CA3-CA1 synapse. First, there is a precedent for A<sub>1</sub> receptor activation of p38 MAPK in a smooth muscle cell line (Robinson & Dickenson, 2001). Second, there is a growing body of evidence supporting a role for p38 MAPK in synaptic plasticity in the hippocampus (see Introduction). Third, there is evidence, from studies of NG108-15 cells, that N-type current can be modulated by a pathway that involves p38 MAPK (Wilk-Blaszczak et al., 1998). An obvious hypothesis that emerges from this is that A<sub>1</sub> receptor presynaptic inhibition at the CA3-CA1 synapse is due to a p38 MAPK-dependent suppression glutamate release.

This study uses a variety of techniques to put the above hypothesis to the test. First, adenosine, acting via the inhibitory A<sub>1</sub> receptor, activates p38 MAPK and this activation is responsible for the inhibition of glutamate release, perhaps ultimately via an action on voltage-gated Ca<sup>2+</sup> channels. Second, the A<sub>1</sub> agonist CPA phosphorylates p38
MAPK, and this is blocked by an A₁ antagonist and a p38 MAPK inhibitor. Third, the A₁ receptor and p38 MAPK co-immunoprecipitate. Fourth, a downstream substrate of p38 MAPK, PP2a, is also activated by adenosine A₁ receptors. Finally, the synaptic depression mediated by exogenous adenosine, endogenous adenosine released during hypoxia, or the agonist CPA is blocked by p38 MAPK inhibitors, providing an important physiological context for the above observations.

A₁ receptor signalling through p38 MAPK could represent a novel mechanism underlying neuroprotection and presynaptic inhibition in the mammalian brain. This study extends the findings of our previous work showing that Bz-ATP-mediated depression of mossy fiber-CA3 synaptic transmission required activation of p38 MAPK. We have now established that p38 MAPK functions in synaptic depression at two distinct synapses following activation of two distinct purinoceptors.

5.3.1. Possible limitations

One potential limitation of the study is that it depends entirely on the specificity of the p38 MAPK inhibitors. Although it is reassuring that consistent results were obtained with two different inhibitors and not when an inactive analogue was used, a critic could easily propose that the p38 MAPK inhibitors interfere directly with A₁ receptor activation, especially given the demonstrated constitutive association of phospho-p38 MAPK and A₁ receptors. In support of this view, there is evidence that, in addition to classical adenosine receptor ligands, enzyme inhibitors commonly used in transduction research also bind adenosine receptors. For example, the protein kinase C inhibitor chelerythr ine and the tyrosine kinase inhibitor genistein bind adenosine receptors and compete with adenosine receptor ligands for binding (Okajima et al., 1994; Ji et al., 1996; Schulte and
Fredholm, 2002). Appropriate control experiments must be used in studies employing such substances, as both chelerythrine and genistein bind adenosine A_1 receptors at concentrations in which they are often used for inhibiting intracellular signalling pathways. However this is not the case for the p38 MAPK inhibitor SB203580. A binding assay revealed that 20µM SB203580 (which is the concentration used in our study) does not compete with A_1 receptor antagonist DPCPX binding in a rat membrane preparation (Schulte and Fredholm, 2003; see Figure 1).

5.3.2. Future Directions

An obvious hypothesis that emerges from the research presented here is that p38 MAPK modulates neuronal calcium channels. It would be interesting to test whether calcium transients in central nerve terminals are sensitive to p38 MAPK inhibition.

Simultaneously recording from presynaptic and postsynaptic neurons is possible in the Calyx of Held. It would thus be possible to test whether SB203580 prevents adenosine-induced decreases in Ca^{2+} influx in Calyx terminals as well the effects of SB203580 on transmitter release.

Regulation of calcium channels by p38 MAPK could be either direct or indirect. One avenue of future research that may be worth pursuing is determining whether p38 MAPK directly binds to either the α1 or β subunit of Cav2.2 (N-type calcium channel), and what functional consequence on calcium currents such an interaction may have. Such experiments could be conducted by expressing channels in a heterologous expression system such as COS-7 or HEK 293 cells. If p38 MAPK does directly interact with Cav2.2 α1 or β-subunits, further experiments using mutant channels could help
define the structural components of the channel that are functionally important for this interaction.

5.4. Adenosine A\textsubscript{1} receptor signalling through JNK

Having already shown that p38 MAPK, but not ERK, is activated by A\textsubscript{1} receptors to depress synaptic transmission, and knowing that p38 MAPK and JNK are often activated by similar stimuli, we next tested whether A\textsubscript{1} receptors activated JNK and whether A\textsubscript{1}-JNK signalling played a role in synaptic depression. We found that JNK and p38 MAPK were similarly activated by the A\textsubscript{1} agonist CPA. Like CPA-induced p38 MAPK activation, CPA-induced JNK activation was blocked by the A\textsubscript{1} receptor antagonist DPCPX. Moreover, like p38 MAPK, JNK activity was required for adenosine and CPA depression of fEPSPs in the CA1 region. Unlike phospho-p38, phospho-JNK did not co-immunoprecipitate with the A\textsubscript{1} receptor. However, using a pan-specific JNK antibody, we found that JNK did co-immunoprecipitate with the A\textsubscript{1} receptor. Based on the above evidence, we proposed that A\textsubscript{1} receptors and JNK exist in a membrane-associated complex. Stimulation of A\textsubscript{1} receptors within this complex leads to phosphorylation of JNK and dissociation of phospho-JNK, which leads to a decrease in glutamate release.

We also proposed that p38 MAPK activation is upstream of JNK activation in the signalling cascade initiated by A\textsubscript{1} receptor simulation. This idea is based on pharmacological evidence showing that inhibition of p38 MAPK blocked CPA-induced JNK phosphorylation whereas inhibition of JNK had no effect on CPA-induced p38 MAPK activation. With this study we have extended the findings of our previous work by describing a potential mechanism underlying presynaptic inhibition, whereby
activation of both p38 MAPK and JNK following A₁ receptor stimulation is a major component of adenosine mediated decreases in neurotransmitter release in the hippocampus, possibly by regulating N-type calcium channel phosphorylation through PP2a (Fig. 5.1). The evidence presented in this study suggests that JNK is a downstream target of p38 MAPK activation, and that both p38 MAPK and JNK are mediators of A₁-receptor-dependent synaptic depression.

### 5.4.1. Possible limitations

Like the A₁-p38 MAPK study, the A₁-JNK study depends heavily on the specificity of the JNK inhibitors. In an attempt to ensure that the observed effects were due to specific inhibition of JNK, we used two distinct JNK inhibitors as well as an inactive analogue. Moreover, we found that SP600125 attenuated CPA-induced synaptic depression at doses of 20μM and 5μM, the latter concentration being one quarter of the concentration commonly used to inhibit JNK in hippocampal slices (Curran et al., 2003; Costello and Herron, 2004). It would be difficult to determine the concentration of SP600125 within the neurons of hippocampal slices incubated with SP600125. In any case, the intracellular concentration of SP600125 is almost certainly less than the concentration of SP600125 in the bath solution.

There was a dose dependent effect of SP600125 on the magnitude of CPA-induced fEPSP depression. In the presence of 20μM SP600125 CPA depressed fEPSPs by 29.0 ± 7.8% (n=6), whereas in 5μM SP600125 CPA was able to depress fEPSPs by 41.4 ± 7.7% (n=8; p < 0.05). (CPA depression of fEPSPs in control conditions is ~70%). Although it is possible that the increased potency of SP600125 at the higher dose reflects an increase in non-specific inhibition of another kinase, this is unlikely considering that
Figure 5.1. Model of adenosine $A_1$ receptor-MAPK signalling in the nerve terminal.

Adenosine $A_1$ receptors and p38 MAPK are physically associated in presynaptic terminals. Stimulation of $A_1$ receptors leads to p38 MAPK phosphorylation, activation of JNK, and translocation of PP2a to the membrane. PP2a activation could then modulate neuronal N-type calcium channel function, leading to a decrease in neurotransmitter release.
we obtained the same results for SP600125 at 20μM (29.0 ± 7.8% CPA-depression; n=6) as we did for JNK Inhibitor V at 5μM (28.6 ± 1.0%; n=8), whilst preincubation in 5μM of the inactive analogue had no effect on CPA-induced depression. Rather, the increased ability of SP600125 to block JNK activity at higher doses is probably simply a reflection of increased specific inhibition of the JNK signalling pathway. JNK inhibition due to SP600125 is thus most likely submaximal at a concentration of 5μM.

5.4.2. Future Directions

The results presented herein indirectly indicate that A1 receptor stimulation is selectively activating the JNK3 isoform, as CPA-induced phosphorylation of p54-JNK (which consists of JNK2/3) is proportionally greater than that of p46-JNK (consisting of JNK1/2/3). The high baseline level of JNK phosphorylation in the brain is due to JNK1. The greater increase in p54-JNK vs p46-JNK phosphorylation upon CPA treatment is suggestive that A1 receptors specifically activate the neuronal-specific JNK3 isoform. A powerful test of this hypothesis could be accomplished using transgenic mice in which the JNK3 gene has been deleted (Kuan et al., 2003). It would be expected that both CPA-induced synaptic depression and JNK phosphorylation would be attenuated in JNK3 knockout mice. Another possibility would be to interfere with the translation of JNK3 protein using RNA interference.

Another aspect of the current research that lends itself to further study is whether A1 receptors mediate some of their effects by directing the recruitment, activation, and scaffolding of cytoplasmic signalling complexes via β-arrestins. The classic role of β-arrestins, based on studies of their interaction with B2-adrenergic receptors, was to
desensitize seven transmembrane receptors. The mechanism of β-arrestin desensitization is as follows: the activated (agonist-bound) form of the receptor is rapidly phosphorylated by G protein-coupled receptor kinases, which leads to recruitment of β-arrestins and results in receptor desensitization (Lefkowitz and Shenoy, 2005). However, in addition to desensitization of G protein-signalling, recent evidence indicates that β-arrestins can also act as signal transducers themselves. For example, β-arrestins can function as endocytic adaptors, linking receptors to the clathrin-coated pit machinery, and thus facilitate endocytosis of G protein-coupled receptors (Lefkowitz and Shenoy, 2005).

Of particular interest is the finding that JNK3, as well as its upstream activators ASK1 and MKK4, exist in a complex with β-arrestin 2 in the mouse brain, where it acts as a scaffold to bind the components of the JNK module (McDonald et al., 2000). The presence of β-arrestin 2 causes cytosolic retention of JNK3 and enhances ASK1-dependent JNK3 phosphorylation. In addition, stimulation of the angiotensin II type 1a receptor activates JNK3 and induces colocalization of β-arrestin 2 and active JNK3 to intracellular vesicles. Through its actions as a scaffold, β-arrestin is able to couple a specific MAPK pathway to a particular G protein-coupled receptor.

Does β-arrestin signalling mediate the effects of A1 receptor stimulation in the hippocampus? We tested this hypothesis by attempting to co-immunoprecipitate β-arrestin 2 and the A1 receptor. We found that β-arrestin 2 and the A1 receptor did not co-immunoprecipitate (data not shown). However, two additional experiments suggest that the A1 receptor may by associated with β-arrestin after all: 1) the A1 receptor and the endocytic protein endophilin co-immunoprecipitate; and 2) endophilin and β-arrestin 2 co-immunoprecipitate (data not shown). We thus have indirect evidence that the A1
receptor and β-arrestin can exist in a complex, as the A1 receptor is associated with endophilin which is in turn associated with β-arrestin 2. However, further experiments will be needed to determine whether β-arrestin plays a functional role in A1-JNK signalling in the hippocampus.

Finally, we also have evidence that selective A1 receptor stimulation leads to a long lasting (> 4 hours) synaptic depression that, in contrast to short-term (i.e. 10 min) depression, is independent of JNK inhibition (Fig. 5.2). One explanation is that the short-term depression caused by A1 receptor stimulation is due to JNK-dependent changes in release probability, whereas A1 receptor-mediated long-term depression (LTD) is caused by internalization of postsynaptic AMPA receptors in a mechanism similar to classic frequency-dependent LTD. Two major predictions arise from this hypothesis: 1) Prolonged exposure to CPA will decrease GluR2 surface expression; and 2) Preventing AMPA receptor internalization will block CPA-LTD. The first prediction could be tested using a biotinylation assay of GluR2 surface expression. A test of the second prediction could be accomplished using a peptide interference strategy. For example, LTD can be abolished by infusion of a peptide through the recording pipette that specifically binds to AP2, and thus prevents recruitment of AP2 to AMPA receptors, assembly of the clathrin coat, and endocytosis of AMPA receptors (Lee et al., 2002).

We have results from biotinylation experiments showing that prolonged stimulation of A1 receptors (10 - 30 min) leads to a decrease in receptor surface
Figure 5.2. The $A_1$ receptor agonist CPA induces a form of long-term depression that is insensitive to JNK inhibition.

Plot of the normalized fEPSP slope over time. Slices were either preincubated in 5µM SP600125 (JNK Inhibitor II) or normal aCSF. CPA was applied for 10 min.
expression. This is consistent with agonist-induced internalization of postsynaptic A₁ receptors. A₁ receptors are known to be physically associated with NMDA receptors. Whether A₁ receptor stimulation causes LTD by internalizing glutamate receptors is an open question. Adenosine A₁ receptor-dependent internalization of glutamate receptors would be a novel mechanism underlying G protein-dependent modulation of synaptic plasticity in the brain and would be expected to have a wide array of functional consequences.

5.5. The physiological relevance of ambient adenosine

Basal transmitter release in the hippocampus is depressed by ambient adenosine (adenosine tone). Removing the inhibitory action of adenosine using an A₁ receptor antagonist (e.g. DPCPX) typically increases baseline synaptic transmission at Schaffer collateral-CA1 synapses by 20-30% (Dunwiddie and Diao, 1994; Brundege and Dunwiddie, 1996; de Mendonca et al., 1997; Latini et al., 1999a; Bon and Garthwaite, 2002).

The role of ambient adenosine in modulating baseline synaptic transmission and synaptic plasticity is controversial. It was recently reported that a number of forms of plasticity unique to mossy fiber synapses (i.e. large paired-pulse ratio, capacity to undergo frequency facilitation, large posttetanic potentiation, and presynaptic expression of LTP) were specifically due to the tonic presynaptic inhibition by a local, high concentration of ambient adenosine (Moore et al., 2003). These forms of synaptic plasticity are distinct to mossy fiber synapses because of the extremely low basal release probability of mossy fiber terminals. Previously, the low-release probability of mossy fibers was attributed to intrinsic properties of the synapse, such as high endogenous Ca²⁺
buffering in terminals, release-incompetent presynaptic calcium channels, and strongly inactivating presynaptic K\textsuperscript{+} channels. However, Moore et al. (2003) suggest the low-release probability is in fact imposed on mossy fiber synapses by tonic activation of presynaptic A\textsubscript{1} receptors. The authors came to this conclusion based on their findings that blockade of A\textsubscript{1} receptors (with DPCPX), enzymatic degradation of extracellular adenosine, and the genetic deletion of the A\textsubscript{1} receptor all caused a dramatic (~ 5-fold) increase of baseline mossy fiber-CA3 synaptic transmission, whilst both short-term plasticity (paired-pulse facilitation and frequency facilitation) and long-term potentiation were almost absent under these conditions.

The results of the study conducted by Moore et al. (2003) were recently called into question by the Dietrich group (Kukley et al., 2005), who found no evidence that ambient adenosine regulates plasticity at mossy fiber synapses. In stark contrast with the ~500% increase in baseline synaptic transmission caused by DPCPX reported by Moore et al. (2003), Kukley et al. (2005) found that blockade of A\textsubscript{1} receptors caused only a modest 20-30% increase in mossy fiber responses. Furthermore, the magnitude of posttetanic potentiation, paired-pulse facilitation, and frequency facilitation were all unchanged when A\textsubscript{1} receptors were either genetically deleted or antagonized by DPCPX. The authors suggest that release probability of mossy fiber terminals is low enough to spawn these forms of synaptic plasticity without the requirement for potent inhibitory adenosine tone.

According to Kukley et al. (2005), the most likely reason for the differences in adenosine tone observed in their study versus that of Moore et al. (2003) is that Moore et al. (2003) used a submerged recording chamber that led to hypoxia in their slices.
However, a difference in the extracellular adenosine concentration does not explain why Kukely et al. (2005) and Moore et al. (2003) achieved vastly different results for frequency facilitation (~180% vs ~400%) and posttetanic stimulation (~280% vs ~520%) when A1 receptors were antagonized by DPCPX or genetically deleted.

In the hippocampal slice preparation, the concentration of extracellular adenosine is dependent upon the temperature of slice incubation. The concentration of extracellular adenosine at 21°C is approximately double that of slices incubated at 32°C (Dunwiddie and Diao, 2000). The increase in extracellular adenosine at lower temperatures is due to diminished activity of dipyridamole-sensitive (ei) nucleoside transporter. Given the effect of temperature on adenosine tone, it is surprising that Kukley et al. (2005) did not discuss, let alone test, whether temperature differences could account for some of the discrepancies between their results and those obtained by Moore et al. (2003); especially considering Moore et al. (2003) conducted all experiments at room temperature and Kukley et al. (2005) conducted their experiments at 30°C. The larger effects of DPCPX observed by Moore et al. (2003) may be attributable to increased adenosine tone resulting from decreased adenosine uptake by ei transporters.

In support of a physiological role for adenosine tone in regulating baseline synaptic transmission and plasticity, Pascual et al. (2005) recently reported that purinergic release from astrocytes enhances adenosine tone and modulates synaptic plasticity. Blockade of astrocytic ATP release (and thus accumulation of extracellular adenosine) was accomplished using inducible transgenic mice expressing a dominant-negative SNARE domain selectively in astrocytes. Mice with impaired astrocytic purinergic signalling exhibited increased baseline synaptic transmission and reduced
LTP. Moreover, activity-dependent heterosynaptic depression was completely abolished. Therefore, tonic suppression of excitatory transmission mediated by astrocytes regulates the degree to which a synapse may be plastic. In addition, activation of an astrocyte to release ATP leads to widespread coordination of synaptic networks, as astrocytes-derived adenosine can depress distant synapses.

An interesting observation is that activation of metabotropic glutamate receptors with DHPG, which leads to a form of LTD, appears to abolish adenosine tone in the slice (data not shown). Perfusing the A₁ receptor agonist DPCPX onto slices after induction of DHPG-LTD does not increase the slope of fEPSPs. This is in contrast to CPA-LTD, which is still sensitive to DPCPX administration. Manipulation of adenosine tone by receptor-dependent processes could be an important mechanism underlying metaplasticity (i.e. the phenomenon whereby the history of synaptic activity influences the capacity of the synapse to undergo future plasticity).

Another fascinating observation is that the inhibition of CK2 (formerly casein kinase II) with 4,5,6,7-Tetrabromo-2-azabenimidazole (TBB) (Sarno et al., 2005) completely abolishes Schaffer-collateral fEPSPs, and this is reversible by DPCPX (data not shown), suggesting that inhibiting CK2 activity leads to a huge increase in adenosine tone. TBB is likely exerting its effects by interfering with CK2-dependent regulation of equilibrative nucleoside transporters (Stolk et al., 2005), although this has not yet been demonstrated in nervous tissue. Nonetheless, it would appear that CK2-dependent modulation of nucleoside transporters is a novel mechanism underlying regulation of adenosine tone in the central nervous system. Such a mechanism could have important clinical implications in anaesthesia and neuroprotection.
5.6. A revised role for p38 MAPK and JNK in neuroprotection

One of the major contributions of the research presented in this thesis to the field of neuroscience may be a greater understanding of the mechanisms underlying neuroprotection. There is a great deal of controversy over whether or not p38 MAPK and JNK exert neuroprotective or neurodegenerative influences when the brain undergoes trauma such as an ischemic insult. I believe that both scenarios are likely true under specific circumstances, and that the research presented here provides a framework for determining when p38 MAPK and JNK activation is likely to be neuroprotective and when it is likely to be a harbinger of cell death.

5.6.1. Whether p38 MAPK and JNK activation is neuroprotective or neurodegenerative may be a matter of timing.

There is extensive evidence that adenosine, acting at A₁ receptors, attenuates ischemic injury in the heart and brain (see introduction). The data presented in this thesis establish that p38 MAPK and JNK are activated by A₁ receptor stimulation, and are necessary for A₁ receptor-dependent inhibition of neurotransmission, which is the primary means by which adenosine protects cells from excitotoxicity. Therefore one would predict that, like adenosine, p38 MAPK and JNK are also involved in protecting cardiac and/or neuronal tissue during an ischemic insult. Previous studies have found that the p38 MAPK pathway (Irving et al., 2000; Barone et al., 2001) and the JNK pathway (Comerford et al., 2004; Gao et al., 2005) are activated within minutes following hypoxia/ischemia in brain tissue. It is reasonable to speculate that the increase in p38 MAPK and JNK activity following hypoxia/ischemia is attributable to increased...
activation of A1 receptors resulting from increased release of endogenous adenosine
during the ischemic insult. However, whether p38 MAPK and JNK activation is
neuroprotective is controversial.

There is a body of evidence that would seem to disprove the hypothesis that JNK
and p38 MAPK activation underlie the neuroprotective effects of adenosine in the
hippocampus. JNK and p38 MAPK are well-established mediators of cell death in the
brain, where they are activated to execute apoptotic cell death in vulnerable brain areas
after hypoxic/ischemic injury (Herdegen et al., 1998; Ozawa et al., 1999; Irving et al.,
2000; Legos et al., 2002; Zhu et al., 2002b). JNK mediates neurodegeneration by
activating genetic programs through phosphorylation of the nuclear transcription factor c-
Jun/AP-1, the release of cytochrome c or the pro-inflammatory actions of microglia
(Manning and Davis, 2003). Similarly, p38 MAPK is activated by a number of stimuli
that cause apoptosis (Xia et al., 1995; Juo et al., 1997), and is thought to function
downstream of caspase activation in mediating apoptotic cell death (Fernandes-Alnemri
et al., 1996; Huang et al., 1997).

In support of a deleterious role for JNK in cerebral ischemia, systematic
administration of the JNK inhibitor SP600125 (Gao et al., 2005) or a peptide inhibitor of
JNK (Borsello et al., 2003) protects against ischemic injury. SP600125 attenuates the
mitochondrial apoptosis signaling pathway by preventing ischemia-induced translocation
of Bax and Bim, release of cytochrome C and Smac, and activation of caspase-9 and
caspase-3. The JNK inhibiting peptide, even when injected 6 hours post-reperfusion,
reduces lesion volumes by more than 90% and confers significant behavioural
improvement on a task measuring locomotor performance as long as 14 days post-
ischemia. In addition, disruption of the gene encoding specifically the neuronal JNK3 isoform in mice prevents apoptosis of hippocampal neurons and decreases seizure activity in response to the excitotoxic glutamate-receptor agonist kainic acid (Yang et al., 1997). Moreover, stress-induced JNK activity and brain injury is reduced in JNK3 knockout mice after cerebral ischemia/hypoxia (Kuan et al., 2003). These data indicate that extinction of the JNK3 signalling pathway leads to neuroprotection and that JNK3 activation is an important component in the pathogenesis of glutamate neurotoxicity.

There is also evidence that p38 MAPK activation is deleterious during cerebral ischemia. The p38 MAPK inhibitor SB203580 protects against neuronal death after NMDA receptor overstimulation (Kawasaki et al., 1997) or hypoxia (Zhu et al., 2002b). A second generation p38 MAPK inhibitor, SB206393, also reduces brain injury (Legos et al., 2002) and neurological deficits (Barone et al., 2001) due to cerebral ischemia.

It is important to note that most of the neuroprotective effects of p38 MAPK and JNK inhibition have been reported in studies that examined ischemic injury or neurological deficits many hours, and often days, post-ischemia. Moreover, the most dramatic neuroprotective effects have often been described in studies where the p38 MAPK or JNK inhibitor was administered for many hours beginning at some time point after reperfusion. For example, Barone et al. (2001) administered SB239063 continuously for 6 hours post-middle cerebral artery occlusion (MCAO) starting 15 min following the onset of ischemia. Borsello et al. (2003) achieved their greatest reduction in infarct size by injecting a peptide inhibitor of JNK 6 hours post-reperfusion. Because JNK and p38 activity increases within minutes of the onset of ischemia (Sugino et al.,
2000), early activation of JNK and p38 is clearly not responsible for the ischemic injury observed in these studies.

Early activation of p38 MAPK and JNK due to ischemia is almost certainly mediating different physiological processes than activation of these kinases hours or days post-ischemia. In support of this notion an experiment was conducted where rats were given SB203580 or vehicle prior to transient middle cerebral artery occlusion and serial magnetic resonance imaging was used to evaluate the extent of the resulting lesion (Lennmyr et al., 2003). After 1 day, lesions were significantly larger in the SB203580 group compared with vehicle, suggesting that p38 MAPK may aggravate ischemic brain injury when present during the ischemic insult, which is remarkably similar to the deleterious effect of A1 receptor antagonism (Rudolphi et al., 1992; Zhou et al., 1994). These data make it clear that not only is inhibition of p38 MAPK or JNK during transient ischemia unnecessary to achieve neuroprotection, but inhibiting these kinases during an ischemic insult may actually exacerbate ischemic injury.

The results of the research presented in this thesis may offer an explanation for why late activation of p38 MAPK and JNK is helpful whereas early activation may be harmful. Based on our discovery that adenosine inhibits neurotransmission by activating p38 MAPK and JNK through A1 receptor stimulation, early activation is likely mediating the neuroprotective effects of endogenous adenosine acting at A1 receptors, whereas late activation is involved in completely separate processes that mediate cell death. Evidence that early activation of p38 MAPK and JNK is beneficial has long been supported by studies of ischemic injury in the heart. In heart tissue, adenosine activates both p38 MAPK and JNK (Haq et al., 1998), and both p38 MAPK and JNK mediate
cardioprotection in response to ischemia (Weinbrenner et al., 1997; Baines et al., 1998; Baines et al., 1999; Barancik et al., 1999; Fryer et al., 2001c). Ischemic preconditioning (or ischemic tolerance) is a phenomenon in which a brief sublethal ischemic insult induces long-term resistance to the effects of a subsequent more severe ischemic insult that depends on A1-receptor activation (Thornton et al., 1992; Tsuchida et al., 1993) in a pathway requiring p38 MAPK (Zhao et al., 2001b; Schulte et al., 2004). Recent studies have confirmed that A1 receptor-mediated delayed preconditioning against myocardial infarction is dependent on p38 MAPK in vivo (Lasley et al., 2005).

The role of p38 MAPK and JNK in mediating ischemic preconditioning has not been studied as extensively in the brain as it has in the heart. Nonetheless, there is a growing body of evidence, albeit indirect, that supports the hypothesis that p38 MAPK and JNK mediate A1 receptor-dependent ischemic tolerance in the brain. As in the heart, the adenosine A1 receptor is thought to trigger ischemic tolerance in the gerbil hippocampus (Kawahara et al., 1998). Evidence that p38 MAPK contributes to ischemic tolerance in the same system came from the finding that SB203580, administered 30 min before a 2-minute sublethal ischemic insult, increases neuronal cell death in CA1 neurons in response to a second 5 minute ischemic insult 48 hours after reperfusion (Nishimura et al., 2003).

Perhaps the most compelling evidence that A1 receptors can activate p38 MAPK in the brain with functional consequences, aside from the research presented in this thesis, comes from integrating the findings of a number of studies on isoflurane-induced tolerance for focal cerebral ischemia. Isoflurane preconditions neurons to improve tolerance for subsequent ischemic insults in both in vitro and in vivo models (Kapinya et
al., 2002; Zhao and Zuo, 2004). In the heart, isoflurane-induced tolerance shares a number of cellular mechanisms with ischemic preconditioning (Aizawa et al., 2004), including the requirement for an A₁-receptor mediated pathway (Roscoe et al., 2000). Recently, it was reported that isoflurane tolerance against focal cerebral ischemia is dependent on an A₁ receptor-mediated pathway as well (Liu et al., 2006). This is a particularly interesting finding because isoflurane tolerance against cerebral ischemia requires the activation of p38 MAPK in vivo (Zheng and Zuo, 2004). The above data demonstrate that a physiological process (isoflurane tolerance) in the brain depends on both A₁ receptor stimulation and p38 MAPK activation. It would be interesting to directly test whether A₁ receptor antagonism prevents isoflurane-mediated increases in p38 MAPK phosphorylation. A1 receptor-dependent activation of p38 MAPK in response to isoflurane may represent a novel neuroprotective mechanism in the mammalian brain.

The apparent dichotomy between the neuroprotective and neurodegenerative roles of p38 MAPK and JNK in ischemia will likely disappear with more precise knowledge of the timing and context of their activation. Obviously, p38 MAPK and JNK activation will have a different function in presynaptic nerve terminals during an ischemic insult than in microglia surrounding an infarct a week after a stroke. Because both p38 MAPK and JNK inhibitors have been put forward as potential therapeutics for stroke, it will be valuable to test the hypothesis that inhibition of p38 MAPK and JNK is harmful during an ischemic insult but beneficial after reperfusion. Ultimately, ischemic injury might best be decreased by enhancing p38 MAPK and JNK activity during hypoxia/ischemia and decreasing p38 MAPK and JNK activity beginning in the hours post-ischemia.
5.7. Presynaptic inhibition revisited

Another major contribution of the research presented in this thesis is that it proposes a novel mechanism underlying presynaptic inhibition, namely p38 MAPK/JNK-dependent inhibition of neurotransmitter release following A₁ receptor stimulation. Regulation of neurotransmitter release can occur via direct interaction with proteins associated with vesicular release, or by modulation of presynaptic neuronal calcium channels.

Modulation of neuronal calcium channels by G protein-coupled receptors has been studied extensively, and a number of canonical mechanisms by which activated G proteins regulate calcium channel function in presynaptic terminals have been described. A discussion of these classical pathways is presented here in an attempt to reconcile what is known about G protein-dependent modulation of neuronal calcium channels and the somewhat radical idea proposed here that p38 MAPK and JNK play a role in G protein-dependent presynaptic inhibition.

5.7.1. Classic G protein modulation of neuronal calcium channels

Calcium channels are divided into low-threshold (T-types) and high threshold (L-, N-, P/Q-, and R-types) and have been classified according to both their electrophysiological and pharmacological properties (Catterall, 2000). A prototypical high-threshold neuronal calcium channel is a heterotrimeric complex that consists of a pore-forming α₁-subunit, a β-subunit, and an α₂σ-subunit. The α₁ subunit contains four conserved structural domains (domain I-IV) linked by cytoplasmic hydrophobic linkers. The diversity of α₁-subunits accounts for all known voltage-gated calcium currents. Most neurons express multiple types of α₁- and β-subunits, although the subcellular localization of individual
subtypes is more prescribed. For example, the α1_E (Cav2.3) R-type and α1_C (Cav1.2) and α1_D (Cav1.4) L-types are localized to cell bodies and dendrites, whereas the α1_B (Cav2.2) N type and α1_A (Cav2.1) P/Q type subunits are concentrated in large numbers at presynaptic terminals, where they trigger neurotransmitter release (Catterall, 2000; Elmslie, 2003).

Activation of G protein-coupled receptors, including opioid, cannabinoid, neuropeptide Y, and adenosine receptors, inhibits N-type current by releasing Gβγ from the trimeric G-αβγ complex allowing it to directly bind to presynaptic N-type calcium channels in a 1:1 stoichiometry (Elmslie, 2003). Binding of Gβγ to the I-II linker of the N-type calcium channel stabilizes the channel in the closed state, and thus decreases neurotransmitter release in response to subsequent action potentials arriving at the terminal. This Gβγ-mediated inhibition is strongly voltage dependent, is relieved by action potential trains, and is affected by the type of β-subunit (Cavβ1 vs Cavβ2a), associated with the N-type channel complex (Snutch, 2005). Moreover, PKC phosphorylation of the N-type channel can also inhibit the Gβγ-N-type channel interaction (Zamponi and Snutch, 1998).

There is evidence that in addition to Gβγ-dependent inhibition, G protein-coupled receptors modulate neuronal calcium channels via parallel Gβγ-independent pathways as well. For example, retinal ganglion cells possess baclofen-sensitive GABA receptors that, when activated, can either directly inhibit voltage-dependent calcium channels via Gβγ binding, or indirectly inhibit these channels through an independent parallel PKA-dependent pathway (Zhang et al., 1997).
Protein kinases play an important role in the regulation of neuronal calcium channels, and the role of PKA, PKC, and CaMKII as N-type calcium channel substrates is particularly well documented (Nastainczyk et al., 1987; Hell et al., 1994; Zamponi et al., 1997).

### 5.7.2. MAPK modulation of neuronal calcium channels

There is a growing body of evidence that N-type calcium channels are modulated by members of the MAPK family in addition to PKA, PKC, and CaMKII. For example, the inhibition of N-type calcium current by bradykinin, which requires the sequential activation of two G-proteins, heterotrimeric G13 and monomeric Rac1/Cdc42, also requires the activation of p38 MAPK in NG108-15 cells (Wilk-Blaszczak et al., 1998). However, the mechanism by which p38 MAPK inhibits N-type channels is not well understood.

In contrast, ERK modulation of N-type calcium channels has been studied in greater detail (Fitzgerald, 2000, 2002; Martin et al., 2006). The Ras/ERK signaling is responsible for tonic up-regulation of sensory neuronal N-type calcium channels (Collin et al., 1990; Fitzgerald and Dolphin, 1997; Lei et al., 1998). ERK modulation of N-type calcium channels is thought to reflect a direct phosphorylation of consensus sites within the intracellular linker between transmembrane domains I and II (I-II linker) (Martin et al., 2006). The Cavβ subunit binds to the Cav2.2-subunit within a conserved motif in the I-II linker called the α1-interaction domain (Pragnell et al., 1994). This binding readily occurs because all Cavβ subunits have a complimentary α1-interaction domain binding pocket (De Waard et al., 1994; Opatowsky et al., 2004). The presence of the Cavβ subunit is required for ERK-dependent modulation of Cav2.2 (Fitzgerald, 2002).
Because ERK-dependent modulation of Cav2.2 occurs regardless of which of the four neuronal Cavβ isoforms is co-expressed (Fitzgerald, 2002), it is likely that a universal mechanism of interaction exists at the channel level. The Cavβ subunit also has two putative ERK consensus sites that that fulfill the minimum requirement for ERK phosphorylation (Ser-Pro) that are conserved on all four Cavβ subtypes and these sites are functionally important for ERK-mediated Cav2.2 modulation (Martin et al., 2006). These two Cavβ sites, plus phosphorylation of Ser-447 on the Cav2.2 I-II linker, are both necessary and sufficient for ERK-dependent modulation of Cav2.2 channels (Martin et al., 2006).

The biophysical mechanism by which ERK-mediated phosphorylation modulates channel activity probably reflects altered channel gating mediated by conformational or electrostatic changes caused by phosphorylation of the I-II linker. Binding of the Cavβ subunit, which is required for ERK-dependent modulation of Cav2.2, may allow previously inaccessible sites on the Cav2.2 I-II linker to become targets for ERK phosphorylation (Martin et al., 2006). Phosphorylation of the conserved Ser-Pro consensus sites on Cavβ subunits may also influence channel function, as they are present within the functional core of the Cavβ protein. Indeed, one of these sites (Ser-448) is found within the α1 domain interaction binding pocket, and therefore even modest phosphorylation would be expected influence Cavβ-Cav2.2 interaction.

ERK phosphorylation of Cavβ subunits could potentially regulate Ca\textsuperscript{2+} influx in a variety of ways depending on which Cavβ is co-expressed with Cav2.2. For example, all Cavb subunits alter channel conductance, Cavβ2a and Cavb4 alter activation/inactivation kinetics, and Cavβ2a shifts voltage dependence (Wittemann et al., 2000). Therefore,
depending on which Cavβ subunit is expressed with Cav2.2, ERK signaling could potentially allow channels expressed in different regions or cell types to be differentially modulated by the same neurotransmitter.

Whether p38 MAPK and/or JNK directly interact with Cav2.2 in a similar fashion as ERK remains to be determined. The ERK signaling pathway often opposes the p38 MAPK and JNK signaling pathways. Therefore, it would not be surprising if p38 MAPK or JNK activity suppressed Ca\(^{2+}\) influx through Cav2.2 and thus functioned in opposition to Ras/ERK-dependent enhancement of Ca\(^{2+}\) influx.

One possible mechanism for p38 MAPK and/or JNK to oppose ERK phosphorylation of Cav2.2 is through activation of a serine/threonine protein phosphatase. The PP1, PP2a, PP2b, and PP2c families of Ser/Thr protein phosphatases are expressed in the brain, and play important roles in regulating the activity of neuronal calcium channels (Price and Mumby, 1999). For example, all four protein phosphatases dephosphorylate sites on both the I-II linker and the II-III linker the of the α1 subunit after phosphorylation by PKCε, although PP2c was the most effective (Li et al., 2005). Moreover, PP2c and Cav2.2 channels form a functional signaling complex in neurons that is responsible for the rapid dephosphorylation of the channel after its phosphorylation by PKC.

The results presented in this thesis show that A₁ receptor stimulation leads to translocation (and presumably activation) of PP2a. As has previously been reported in cardiac tissue (Liu and Hofmann, 2003), the results presented here show that translocation of PP2a to the plasma membrane due to A₁ receptor activation is dependent on p38 MAPK activity in the hippocampus (Fig. 3.3). p38 MAPK-dependent activation
of PP2a is a potential mechanism by which A$_1$ receptor activation could modulate neuronal calcium channel function. In support of this hypothesis, PP2a is known to be functionally associated with Cav1.2, and this interaction is responsible for reversing PKA phosphorylation of serine 1928 (Hall et al., 2006). It would be interesting to test whether PP2a is also associated with Cav2.2 and whether A$_1$-p38 MAPK-PP2a signaling is in fact a viable pathway mediating inhibition of neuronal calcium channels.

It should be noted that prolonged exposure (> 2 hours) to the PP2a inhibitor Okadaic acid results in a complete run down of AMPA and NMDA responses in hippocampal slices (data not shown). Even a dramatic increase in stimulus intensity fails to elicit fEPSPs after prolonged Okadaic acid treatment. This observation suggests that Okadaic acid results in the death of the slice. Therefore, it is difficult to pharmacologically test whether PP2a activation contributes to synaptic depression.

MAPK pathways are extremely complex and subject to regulation by numerous upstream activators. In contrast, direct inhibition of calcium channels through G$\beta\gamma$ is an all-or-none process with significantly less opportunity for subtle modulation. It is therefore not surprising that parallel protein kinase-dependent mechanisms have evolved for modulating neuronal calcium channels given their enormous structural and functional diversity. Indeed, modulation of neuronal calcium channels by MAPK has virtually unlimited potential for fine-tuning neurotransmitter release and thus synaptic transmission in the central nervous system.

### 5.8. Conclusion

The body of work presented here gives insight into the mechanism by which purinergic signalling modulates neurotransmission in the central nervous system. Stimulation of
purinoceptors at both mossy fiber and Schaffer collateral synapses leads to decreased transmitter release in a pathway requiring MAPK activation (Fig. 5.3). Whether P2X7 receptors are in fact expressed in mossy fiber terminals awaits confirmation using molecular biology. However, it is reassuring that the results of this study have been reliably replicated by other labs and it is only the interpretation of the results that is still an open question. Future experiments will also be required to determine the precise molecular mechanism underlying MAPK modulation of neuronal calcium channels. Nonetheless, a working model has emerged from this work whereby p38 MAPK and JNK exist in a complex with A1 receptors in the CA1 region of the hippocampus where they are activated sequentially following A1 receptor stimulation (see Fig. 5.1, page 182). Such a signalling complex could modulate neuronal calcium channels by regulating the activity of the serine/threonine phosphatase PP2a. A1-p38-JNK signalling is a novel pathway mediating presynaptic inhibition. Because purinergic signalling is of such importance to the functioning of the nervous system under both normal and pathophysiological conditions, the results herein are expected to be of general interest to the field of neuroscience.
Figure 5.3. Summary of purinergic signalling through MAPKs in the hippocampus.

In area CA1, p38 MAPK and JNK are activated sequentially following A1 receptor stimulation. The activity of p38 MAPK and/or JNK may lead to an increase in the activity of PP2a which could in turn result in decreased calcium influx through N-type calcium channels and hence a decrease in neurotransmitter release. In area CA3, presynaptic P2X7 receptors lead to a decrease in neurotransmitter release in a pathway requiring p38 MAPK, possibly involving a decrease in calcium influx through P-type calcium channels.
5.9. References


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