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Uncovering the Molecular Mechanisms of Ischemic Tolerance – the Role of ATP in Depolarization-induced Neuroprotection

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Uncovering the molecular mechanisms of ischemic tolerance – the role of ATP in depolarization-induced neuroprotection

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This thesis is submitted as a partial fulfillment of the Ph.D. program in Neuroscience

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2. McKee SC, Thompson SC, Sabourin LA, Hakim AM. Regulation of expression of early growth response transcription factors in rat primary cortical neurons by extracellular ATP. Brain Research. 2006; 1088: 1-11.

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1. Gidday JM. Cerebral preconditioning and ischaemic tolerance. Nature Reviews Neuroscience. 2006; 7(6): 437-448.

Abstract

Cortical spreading depression (CSD) is a slowly propagating wave of neuronal depolarization which causes alterations in ion homeostasis, blood flow, and energy metabolism without causing irreversible damage to brain tissue. One interesting effect of CSD is that it can provide protection against a subsequent ischemic attack. However, the molecular mechanisms of this neuroprotection are still unclear. The present study was undertaken to further elucidate the molecular mechanisms of the induction of ischemic tolerance by CSD. Using a combination of molecular techniques we have investigated the role of adenosine triphosphate (ATP) in the induction of ischemic tolerance and have identified receptors, signaling pathways, channels and a transcription factor involved in the onset of protection following CSD. One of the genes known to be upregulated following CSD as well as other preconditioning stimuli is early growth response factor-1 (Egr-1). Egr-1 is a transcription factor that may control the overall genomic response to CSD. Therefore, we characterized Egr-1 expression in rat primary cortical neurons in response to conditions thought to occur during CSD. It was found that Egr-1 was upregulated following exposure to depolarization, glutamate, and, to the greatest extent, ATP. Through reverse transcription polymerase chain reaction (RT-PCR) the P2Y purinergic receptors and the protein kinase A (PKA), protein kinase C (PKC) and phospholipase C (PLC) signal transduction pathways were shown to be involved in the upregulation of Egr-1 by extracellular ATP. Rat primary cortical cultures were then exposed to extracellular ATP as a preconditioning stimulus. These cultures developed tolerance to subsequent oxygen glucose deprivation (OGD) as well as a chemical form of ischemia potassium cyanide (KCN). We also found that the P2Y purinergic receptors as

well as multiple signal transduction pathways mediate the tolerance induced by ATP with the PKA and PLC pathways contributing the most. Finally, we determined that exposing cultures to potassium chloride (KCl) also elicits protection against OGD and KCN. This protection requires the release of ATP through connexin channels, specifically connexin 36 hemichannels. Through the course of this study we have found that CSD releases ATP into the extracellular space through connexin hemichannels. ATP activates P2Y purinergic receptors that induce signal transduction pathways and lead to the upregulation of transcription factors such as Egr-1, which may control the overall genomic response to preconditioning.

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List of Abbreviations

A1	Adenosine Receptor
A23187	Calcium ionophore
ACh	Acetylcholine
Act	Actinomycin D
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
ATP	Adenosine 5'-triphosphate
BDNF	Brain derived neurotrophic factor
bHLH-PAS	Basic helix-loop-helix-PAS protein
BIM IV	bisindolylmaleimide IV
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
campto	Camptothecin
CBX	Carbenoxolone
cdk5	Cyclin-dependent kinase 5
cDNA	Complimentary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CGN	Cerebellar granular neurons
CHX	Cycloheximide

Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide
COO^-	Carboxylate
CSD	Cortical spreading depression
Cx	Connexin
CY3	Cyanine 3
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DC	Direct current
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
dNTP	Deoxynucleosides
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DTT	Dithiothreitol
E15/16	Embryonic day 15/16
EC_{50}	Half maximal effective concentration
ECL	Enhanced chemiluminescence
EEG	Electroencephalography
Egr	Early growth response factor

ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FFA	Flufenamic acid
fmol	Fentomolar
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
GPCR	G protein-coupled receptor
h	Hour
H_2O_2	Hydrogen peroxide
HBSS	Hank's Basic Salt Solution
HI	Hypoxic preconditioning
HOBIT	human osteoblast-like cell line
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IL-1-β	Interleukin-1-beta
IP_3	Inositol triphosphate
IPC	Ischemic Preconditioning
K^+	Potassium
KCl	Potassium chloride
KCN	Potassium cyanide

kD	KiloDalton
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
LMO4	LIM only domain 4
M	Molar
MCAO	Middle cerebral artery occlusion
MEK1/2	Mitogen-activated protein kinase 1/2
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
msec	Millisecond
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Millivolts
na	Nanoampere
Na^+	Sodium
NAB	NGFI-A binding protein
NaCl	Sodium chloride
NaF	Sodium Fluoride
NeuN	Neuronal nuclei
ng	Nanograms

NGF	Nerve growth factor
NGFI	Nerve growth factor induced
nm	Nanometers
nM	Nanomolar
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
OGD	Oxygen glucose deprivation
PBS	Phosphate buffered saline
PC12	Pheochromocytoma Cells
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PGDF	Platelet-derived growth factor
PI3K	Phosphoinositol 3 kinase
PIP_2	Phosphatidylinositol bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
pmol	Picomolar
PMSF	Phenylmethylsulfonyl fluoride
PPADS	Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate
PTX	Pertussis toxin
Quin	Quinine

RB2	Reactive blue 2
RLU	Relative light units
RNA	Ribonucleic acid
Rp-cAMPs	Rp-diastereomer of adenosine 3',5'-phosphorothioate
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SD	Spreading depression
SDS	Sodium dodecyl sulfate
Ser	Serine
SIM2	Single minded 2 protein
siRNA	Small interfering RNA
TBST	Tris-buffered saline tween-20
Thr	Threonine
Tm	Melting temperature
tPA	Tissue plasminogen activator
TTX	Tetrodotoxin
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
UDP	Uridine diphosphate
µg	Micrograms
µl	Microliters
µM	Micromolar
µm	Micrometer

UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor
WT-1	Wilms' tumor suppressor gene

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Chapter 1: General Introduction

1.1 Stroke

Stroke is the fourth leading cause of death in Canada (Heart and Stroke Foundation Website, 2007). A stroke or ischemic brain injury occurs when there is a depletion of both oxygen and glucose to the brain. A stroke may either occur from a hemorrhage or occlusion, which is due to the blockage of a blood vessel. After the blood supply is blocked or significantly reduced there is a breakdown of metabolic processes, ion homeostasis, cellular energy supply and a loss of cellular integrity. Within minutes cell death occurs and cell and tissue necrosis evolve due to the energy failure caused by a drastic decrease in cellular ATP and glucose levels (Figure 1). There is a wave of depolarization of neurons and glia that is induced by the energy deficit which results in the activation of voltage-gated calcium channels and leads to the release of extracellular signalling molecules. Massive glutamate release activates glutamate receptors such as NMDA, AMPA, and metabotrophic receptors which leads to activation of downstream mechanisms such as calcium influx (Love 1999) and cell swelling and eventually rupturing of the cell. Reperfusion after ischemia causes free radical stress, which accelerates glutamate release and excitotoxicity (Behl and Moosmann 2002). The cells in the boundary zone, known as the penumbra, begin the apoptotic process that starts hours after ischemia and can last for days (Figure 1). Apoptosis following ischemia is triggered by variety of death signals such as production of free radicals and tumor necrosis factor, deficiency of growth factor and neurotrophins, DNA damage and p53 induction, cytochrome *c* release during mitochondrial injury, etc. (MacManus and Linnik, 1997; Chan, 2001). The penumbra can account for up to 50% of the volume that later progresses to infarction (Mergenthaler et al., 2004). As of now there are two compounds

approved for use in Canada for acute stroke treatment; aspirin and the thrombolytic agent, tissue plasminogen activator (tPA). However, aspirin is not highly effective and only a small percentage of stroke patients benefit from tPA. Therefore, there is still a need to uncover other possible therapeutic targets with the potential to combat the deleterious effects of stroke.

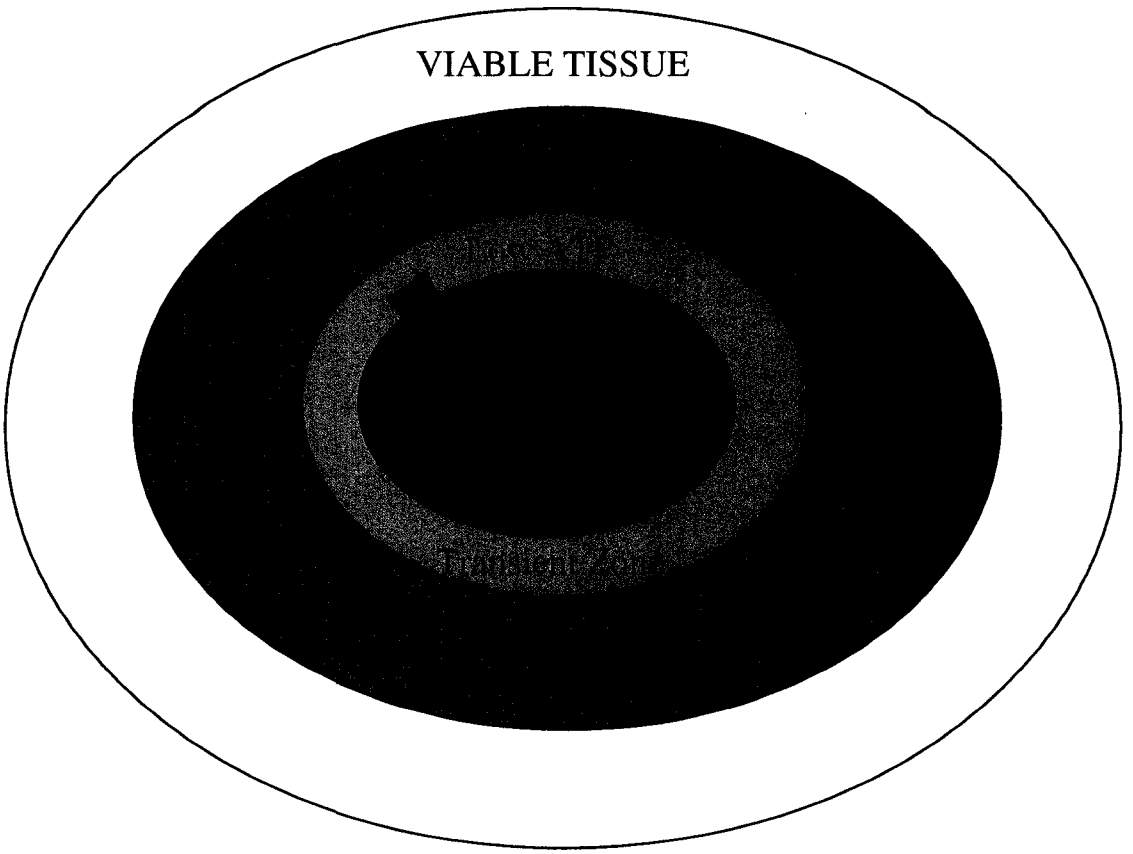

Figure 1. Areas of cell death occurring during ischemia. Cells within the ischemic core die by way of necrosis within minutes where ATP has been depleted. In the penumbra, cells die by way of programmed cell death, also known as apoptosis.

1.2 Preconditioning and Neuroprotection

During stroke, not only pathways of damage are induced, but also endogenous protective mechanisms are initiated. Extensive research has been directed at harnessing

the protective events of ischemic injury. The first description of brain tolerance was described by Kitagawa et al., (1990; 1991), where it was found that preconditioning with a brief period of global ischemia protected hippocampal neurons from cell damage induced by severe subsequent global ischemia. However, they found that the preconditioning stimulus had to last no less than 2 min and the lethal insult had to be applied not earlier than 24 h, after the preconditioning stimulus.

The principle of preconditioning is to achieve a protected state of a cell, tissue, or a whole organism by exposure to a harmful stimulus, such as hypoxia (Kirino, 2002; Gidday et al., 1994), reactive free oxygen radicals (Perez-Pinzon et al., 2005), spreading depression (Kobayashi et al., 1995; Matsushima et al., 1995; 1996), inflammation (Dirnagl et al., 2003), etc, which is applied below but close to the level where it induces damage. The stimulus induces a protective state against insults otherwise lethal. There are two different time windows in which protection can occur. An early one within minutes after the stimulus that is a result of changes in ion channel permeabilities, protein phosphorylation, and other post-translational modifications (Gidday, 2006). The second takes one to several days to fully manifest; this type of preconditioning is composed of three stages. The first stage is where the expression of molecular sensors and transducers such as receptors, channels and regulators are upregulated by transcription factors. In the next phase, protein kinases, transcription factors and para- and autocrine mediators such as growth factors, augment the signal and prepare for the final phase. In this phase, proteins with a direct protective effect, such as antiapoptotic, anti-inflammatory and antioxidative actions, are upregulated (Nandagopal, et al. 2001) (Figure 2). Although many specific cellular and molecular elements of the protective cascade have been

identified, most researchers agree that there is not one specific cell type or one specific mechanism of neuroprotection in ischemic preconditioning; rather a combination of pro-survival genes is activated and, in turn, encodes proteins that serve to enhance the brain's resistance to ischemia.

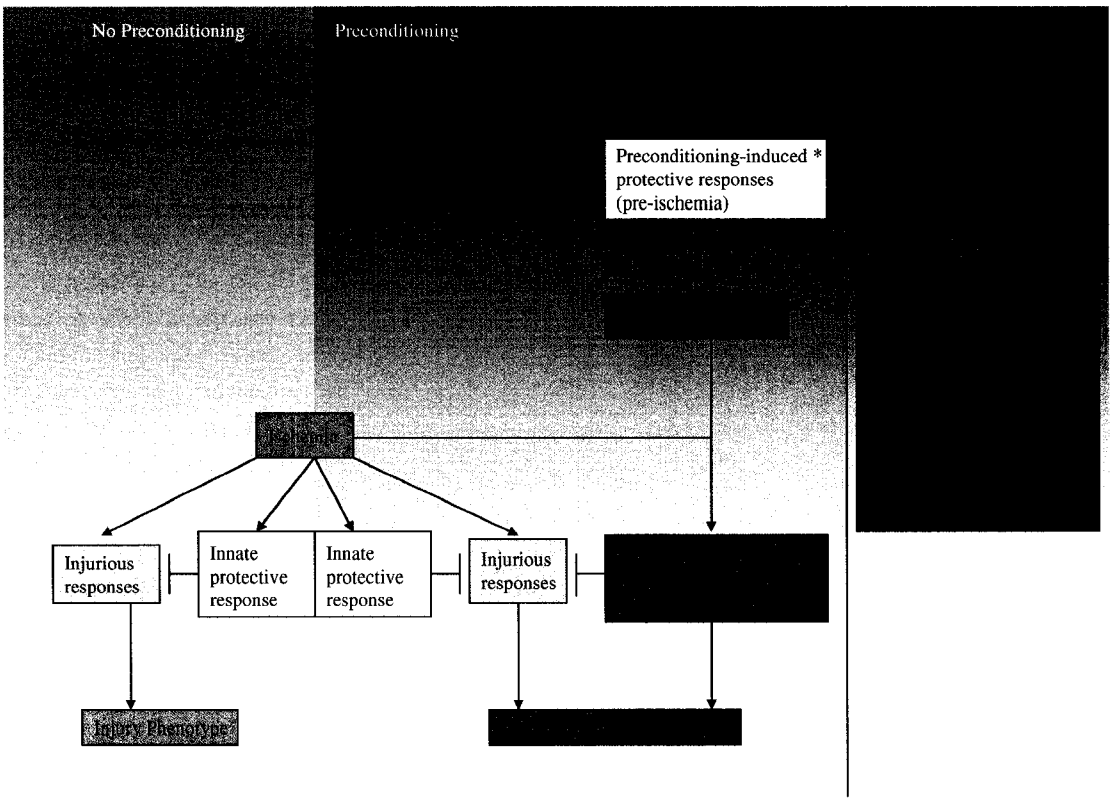

Figure 2. Ischemic tolerance induced by preconditioning. Protection induced by preconditioning requires the activation of cascades that, through a variety of effector molecules and transcription factors (see inset) provoke an ischemia-tolerant phenotype. In the non-preconditioned brain, ischemia-induced responses (red arrows) are opposed by innate protective responses that are also activated by ischemia. However, the tolerant phenotype is a result of pre- and post-ischemic protective responses induced by preconditioning. (Adapted from Gidday, 2006).

Preconditioning is known to reduce the deleterious inflammatory response, to cause a shift in metabolism from oxidative phosphorylation to glycolysis, as well as enhance neuroprotective mechanisms on various other levels (Dirnagl, et al. 2003). More

specifically, it has been shown that ischemic tolerance reprograms the response to ischemia, leading to a protected state (Stenzel-Poore, et al. 2003). Without another preconditioning stimulus to sustain it, the window for protection recedes within days. Understanding these mechanisms may allow us in the future to induce or enhance endogenous protection in patients as a novel strategy to safeguard the brain against ischemia.

1.3 Cortical Spreading Depression

One form of preconditioning that has been extensively studied is cortical spreading depression (CSD) (Matsushima, et al. 1998). CSD is a slowly propagating wave of depolarized neurons and astrocytes that causes depressed synaptic activity but is not associated with injury in the normoxic brain (Leao 1944; Higashida, et al. 1974; Sugaya, et al. 1975; Nedergaard and Hansen, 1988). The induction of CSD has been shown to occur *in vivo* due to epileptic seizures (Kager, et al. 2000), hypoxia (Buresh, et al. 1999), traumatic brain injury (Mayevsky, et al. 1996), focal ischemia (Dietrich, et al. 1994) and migraine (Parsons, 2004). Experimental induction of CSD is accomplished with the use of mechanical trauma, electrical stimulation, or by application of high potassium or a variety of other chemical agents (Somjen 2001).

Spreading depression has been shown to occur in almost all grey matter regions of the central nervous system (CNS). CSD causes a dramatic change in the distribution of ions between the extracellular and intracellular spaces. Potassium (K^+) along with glutamate are released from the cells while, calcium (Ca^{2+}), sodium (Na^+), and chloride (Cl^-) enter along with water that causes cell swelling and the extracellular compartment to

be reduced (Somjen, 2001). Accumulated K^+ in the restricted interstitial space can cause further neuronal depolarisation. Changes in gap junctional conductance also play a role in the propagation of CSD since studies have found that junctional blockers such as heptanol or hexanol completely block propagation of SD events in the CA1 region of the hippocampus (Largo et al., 1997). CSD is also known to produce changes in regional cerebral blood flow (Lauritzen et al., 1982), and release a variety of neurotransmitters such as ATP (Schock et al., 2007), glutamate (Bradley et al., 2002), acetylcholine (ACh; Shimizu et al., 2002) and nitric oxide (Read et al., 1997). CSD is accompanied by an increase in glucose consumption and oxygen utilization; recovery from CSD is dependent on energy metabolism.

Kawahara, et al., (1995) were the first to describe SD as being neuroprotective in hippocampal CA1 neurons. Since then many studies have shown the protective effects of CSD induced preconditioning against various forms of ischemic insults in cortical neurons (Matsushima et al., 1996, 1998; Horiguchi, et al., 2005; Kawahara, et al., 1997; Kobayashi et al., 1995; Taga et al., 1997; Yanamoto et al., 1998, 2000; Otori et al., 2003). Ischemic tolerance has been shown to develop over time and induce a long-lasting protection (Kobayshi et al., 1995, 1998; Taga et al., 1997; Yanamoto et al., 2004).

Many genes have been shown to be up- or down-regulated following CSD. For example the SIM2-related bHLH-PAS protein Nxf is rapidly and transiently expressed in cortical neurons following CSD (Hester, et al., 2007). Also, CSD induces alterations in the levels of BDNF and mice deficient in this gene show a reduction in ischemic tolerance compared to controls when preconditioned with CSD (Yanamoto et al., 2004; Kawahara et al., 1997; Matsushima et al., 1998). Other studies have shown changes in

expression of immediate early genes such as c-Fos, c-Jun, and the early growth response factor gene family, as well as heat shock proteins and cyclooxygenase-2 (cox-2) (Herdegen et al., 1993; Caggiano et al., 1996; Plumier et al., 1997; Koistinaho et al., 1999; McKee et al., 2006; Yokota et al., 2003; Urbach et al., 2006). As well, the ERK1/2 kinases have been shown to be transiently activated during CSD and have been implicated in mediating the preconditioning response elicited by CSD (Chow et al., 2002). However, isoforms of protein kinase C have been noted to be downregulated following CSD (Osten et al., 1996). Although there are many studies into the basis of CSD induced preconditioning the mechanisms of this neuroprotection are still unclear and uncovering the molecular mechanisms elicited by CSD may lead to novel therapeutic targets to combat stroke.

1.4 Early growth response factor-1

Egr-1, also known as KROX-24, NGFI-A, zif268, TIS8 and ZENK, is one member of the early growth response family of zinc finger transcription factors, as stimulation with many environmental signals strongly and rapidly induces Egr-1 gene expression. The Egr protein contains activation, repression and DNA-binding domains (Figure 3). The binding domain contains three zinc fingers that allow Egr to bind to DNA and alter gene transcription through mechanisms dependent on both co-activators and co-repressors (Silverman and Collins, 1999). All members of the Egr family recognize the consensus motif GCG(G/T)GGGCG (Patwardhan et al., 1991; Crosby et al., 1991). The repression domain contains a binding site for two transcriptional co-factors termed NGFI-A binding proteins 1 and 2 (NAB1, NAB2) (Russo et al., 1995;

Svaren et al., 1996). It has also been shown that the expression of the NAB2 gene is controlled by Egr-1 (Ehrengruber et al., 2000), indicating that Egr-1 controls it's activity in a negative feedback loop via synthesis of NAB2. The activation domain of Egr-1 is, in contrast to the binding domain, not as well characterized.

Figure 3. Schematic diagram of the Egr-1 transcription factor. Depicts the three domains of the gene: the activation domain, the repression domain and the DNA-binding domain containing the three zinc fingers.

Egr-1 was first isolated by differential hybridization from NGF-treated rat pheochromocytoma (PC12) cells (Milbrandt, 1987). Since then, other members of the Egr family have been identified and include Egr-2, 3, 4, and Wilm's tumor gene (WT-1). In the brain, Egr proteins have a basal level of expression that varies among different structures and cell types however they are localized to the nucleus excluding the nucleoli (Waters et al., 1990). Egr-1 mRNA can be found in the neocortex, cerebellar cortex and the hippocampus (Schlingensiepen et al., 1991). A high basal level of expression of Egr-1 protein in rat brain is found in the neurons of the cerebral cortex, hippocampus and thalamus (Mack et al., 1990). A wide variety of signals can stimulate Egr expression, such as serum, growth factors, as well as physiological stimuli such as injury, tactile stimulation of whiskers and seizure activity (Gashler and Sukhatme, 1995; Beckmann and Wilce, 1997). Neuronal activation via glutamate receptors such as the NMDA receptor can increase Egr-1 expression (Vaccarino et al., 1992). Injection of NMDA in

vivo dose-dependently elevated the protein levels of Egr-1 in the rat cerebral cortex (Beckmann et al., 1997). Ligands that bind and activate G-protein coupled receptors are also potent inducers of Egr-1 biosynthesis. Distinct intracellular signalling cascades connect GPCR-stimulation with enhanced Egr-1 transcription.

Various forms of in vivo ischemia can also induce the expression of Egr-1. For example, focal brain injury increases Egr-1 mRNA expression in the cortex and also alters the levels of Egr-2 and Egr-4 (Honkaniemi et al., 1995). Transient focal ischemia induced by middle cerebral artery occlusion (MCAO) leads to increased Egr-1 mRNA expression in the cerebral cortex (Abe et al., 1991) with the highest levels expressed in the penumbral area (Collaco-Moraes et al., 1994). Some studies have implicated a detrimental role for Egr-1 in the onset of cell death during ischemia. However, one study suggested that a short-term antagonism of Egr-1 might actually diminish some of the injurious effects elicited by ischemia (Yan et al, 2000).

Gene expression of members of the Egr family is induced by CSD (Jacobs et al., 1994) as well as other preconditioning stimuli (Carmel et al., 2004; Kawahara et al., 2004) in neurons. Egr-1 expression has been associated with apoptosis in neuronal cells (Catania et al., 1999) as well as differentiation, neuronal stress, wound repair, neurite outgrowth, cell growth and long-term potentiation (Beckmann et al., 1997; Herdegen et al., 1993; Herdegen and Leah, 1998; O'Donovan et al., 1999; Slade et al., 2002; Cantor and Orkin, 2002; Nieto, 2002; Harada et al., 2001; Khachigian et al., 1996). Studies have shown that synapsin I (Thiel et al., 1994), synapsin II (Petersohn et al., 1995) and the cdk5-regulator p35, an anti-apoptotic protein, (Harada et al., 2001) are regulated by Egr-1 in neurons. As well Egr-1 has been shown to increase the expression of platelet-derived

growth factor (PGDF; Zuo et al., 2006), which has previously been shown to protect neurons from glutamate-induced excitotoxicity in vitro (Tseng and Dichter, 2005). Egr-1 can also enhance the expression of Bcl-2, another anti-apoptotic protein (Bettini et al., 2002). During ischemia Egr-1 has been shown to upregulate the expression of many proinflammatory molecules such as IL-1-β, ICAM-1, and VEGF (Yan et al., 2000). However, little is known about genes targeted specifically by Egr-1 during preconditioning models.

1.5 Objectives and Hypothesis - Chapter 2

Cortical spreading depression (CSD) induces altered gene expression in the brain. Egr-1 gene expression is increased in response to CSD as well as other preconditioning stimuli. Since Egr-1 is a transcription factor, it may serve to regulate the overall genomic response to preconditioning. **Therefore, it was our main hypothesis that extracellular nucleotides released during CSD regulate the expression of Egr-1, through activation of various receptors and signal transduction pathways.**

Specifically, our objectives were:

- To determine which ligands upregulate the expression of Egr-1 at the mRNA level using real time RT-PCR and if this upregulation is due to transcriptional activation
- To determine if other members of the Egr family are upregulated by extracellular ATP
- To determine which purinergic receptors extracellular ATP is binding to induce the expression of Egr-1

- To determine which signal transduction pathways are activated in response to extracellular ATP to induce the expression of Egr-1
- To determine the requirement for intracellular and/or extracellular calcium in the induction of Egr-1 by extracellular ATP

1.6 Adenosine 5'- Triphosphate (ATP)

ATP is used in living cells as an energy substrate for nearly all cellular activities and is known to function as an extracellular signalling molecule in the brain (Zimmermann, 1994). ATP may be stored in vesicles in nerve terminals along with other neurotransmitters and act as a co-transmitter during synaptic transmission (Burnstock, 2004). ATP may be released from both neuronal and non-neuronal cells by way of ruptured cell membranes or a variety of channels including connexin hemichannels (Cotrina et al., 1998), pannexin hemichannels (Bao et al., 2004), voltage-dependent anion channels (Milbrandt, 1987), stretch activated channels (Boudreault and Grygorczyk, 2002) and the cystic fibrosis transmembrane conductance regulator, CFTR (Reigada and Mitchell, 2004).

Following release into the extracellular space, ATP is quickly degraded by ecto-ATPases and ectonucleotidases (Wang and Guidotti, 1996; Zimmerman and Braun, 1999). By the hydrolytic action of these enzymes, the extracellular concentration of ATP is kept in the nanomolar rage (Schwiebert, 2000; Franke et al., 2006). Since the intracellular concentration of ATP is thought to be in the millimolar range (Gordon, 1986), during cell injury there is a significant outflow of ATP that is rapidly catabolized to adenosine in the traumatic tissue (Deuticke et al., 1966).

ATP acts on two families of purinergic receptors; ligand-gated cationic channels (P2X receptors) and G-protein-coupled receptors (P2Y receptors) with seven transmembrane domains (Figure 4). Up until now seven mammalian P2X receptor subtypes and eight mammalian P2Y receptor subtypes have been cloned and functionally characterized (Abbracchio et al., 2003). The P2X receptors are comprised of intracellular C and N termini and two transmembrane domains. The extracellular loop contains the ATP binding site (Ennion et al., 2000; Jiang et al., 2000) and sites for antagonists and modulators (Buell et al., 1996; Garcia-Guzman et al., 1997). All isoforms of the P2X receptors identified are expressed in the CNS and have been shown to be expressed in neurons both pre- and post-synaptically (Illes and Ribeiro, 2004). The P2X receptors allow the rapid non-selective passage of cations across the cell membrane, resulting in an increase in the intracellular calcium concentration and a depolarization of the cell membrane (Norenberg and Illes, 2000; Burnstock, 2006). The P2Y receptor-mediated responses develop at a slower speed due to the generation of second messenger molecules (Franke et al., 2006). The P2Y receptor family is activated by extracellular nucleotides and is coupled via G proteins to the phospholipase C signal transduction pathway (PLC). This pathway causes the formation of diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate, which causes the release of intracellular calcium from endoplasmic reticulum (ER) stores. DAG is also able to stimulate different subtypes of protein kinase C (PKC), triggering a number of different PCK-activated enzyme cascades.

Once ATP is released it can act as a trophic factor in both development or growth and regeneration or proliferation of different cell types (Franke and Illes, 2006). It may also mediate other effects such as neurotransmission, modulation of the effects of other

neurotransmitters and growth factors, trophic actions and cytotoxicity (Amadio et al., 2002; Dhodda et al., 2004). In conditions like trauma, ischemia, hypoxia, and inflammation, extracellular ATP is released from damaged cells, activated astrocytes, neurons, microglia, and endothelial cells (Bodin and Burnstock, 1998; Dubyak and el-Moatassim, 1993). ATP is also involved in the stimulation of survival and neurite regeneration (D'Ambrosi et al., 2001) and can be a moderator of inflammatory processes mediated by microglial cells (Ferrari et al., 1997).

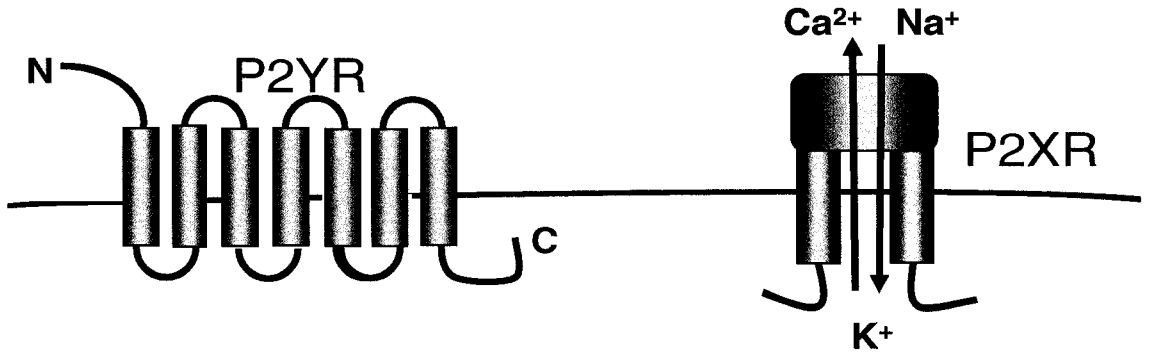

Figure 4. Categories of P2 purinergic receptors. The P2Y receptors are G-protein coupled metabotropic receptors that are coupled to the activation of PLC and IP3 and the release of calcium from intracellular stores. The P2X receptors are ligand-gated cationic receptors with permeability to sodium, potassium and calcium.

After ischemia, ATP is released into the extracellular space within the first few minutes (Phillis et al., 1993). This increase in ATP concentration elicits an excitotoxic effect on surrounding cells. During oxygen glucose deprivation (OGD), $P2X_2$ and $P2X_4$ receptors are found to be upregulated, with expression not only on microglia but also on astrocytes and neurons (Franke et al., 2004). The $P2X_7$ receptor is a unique member of the P2X family, because it forms a pore in response to ligand stimulation, regulating cell permeability, cytokine release and apoptosis (Surprenant et al., 1996). In other in vitro

studies high extracellular levels of ATP were demonstrated to be toxic for primary cortical neuronal cultures, inducing both necrotic and apoptotic cell loss (Amadio et al., 2002). However, studies have shown that activation of $P2Y_2$ receptors in astrocytic cells promotes cell survival mechanisms (Burgos et al., 2007), suggesting that the $P2Y_2$ receptor subtype can play an important role in survival and neuroprotective mechanisms under pathological conditions. Other evidence is present for the neuroprotective function of P2Y purinergic receptors. For example, leukemia inhibitory factor (LIF) is a cytokine involved in the survival and differentiation of neuronal cells. ATP induces the LIF mRNA in cultured astrocytes mediated by P2Y receptors and helps to prevent cell death (Yamakuni et al., 2002). $P2Y_2$ receptors have also been shown to upregulate the expression of genes for neurotrophins, neuropeptides, and growth factors known to regulate neuroprotection (Chorna et al., 2004). In cultured astrocytes, ATP, but not adenosine, protects against cell death induced by hydrogen peroxide (H_2O_2), and P2 receptor antagonists inhibited protection. It has also been reported that ATP inhibits excess neuronal excitation by inhibiting the release of glutamate and is has been shown to protect neurons against excitotoxicity (Chen et al 2007; Koizumi and Inoue, 1997). As well, it has been shown that antioxidants exhibit a protection for cells by inhibiting the decrease in ATP levels therefore causing an inhibition of glutamate release (Hurtado et al., 2003).

Extracellular ATP has also been reported to induce the expression of a variety of genes (Tsim et al., 2003; Choi et al., 2003; D'ambrosi et al., 2004; Priller et al., 1995; McKee et al., 2006). For example, extracellular ATP stimulates a calcium-dependent protein kinase C pathway, ultimately leading to the activation of the transcription factor

Egr-1 (Pines et al., 2003). It was also found that in ROS 17/2.8 cells that lack P2Y receptors there is also a lack of basal Egr-1 binding activity, this suggests that purinergic receptors are required for maintaining a basal level of Egr-1 activity.

1.7 Objectives and Hypothesis – Chapter 3

CSD and ATP induce the expression of Egr-1 mRNA. CSD induces a degree of tolerance on the brain to a subsequent ischemic attack. **Therefore our main hypothesis is that extracellular ATP induces a degree of neuroprotection in neurons *in vitro* and during CSD, waves of depolarization cause the release ATP from neurons and/or glia.**

Specifically our objectives were:

- To directly measure ATP release into the extracellular space during CSD
- To determine the concentration and time length needed for ATP to induce protection and to see if ATP is protective against various types of insults
- To determine if ATP is protective both pre and post-insult and if neurons are permanently rescued by extracellular ATP
- To determine if extracellular glutamate and adenosine are playing a role in the protection seen by ATP
- To determine the purinergic receptors and signal transduction pathways activated by extracellular ATP to induce protection
- To determine the role of Egr-1 in the induction of tolerance by extracellular ATP

1.8 Gap Junction Channels

Gap junctions are specialized cell-cell contacts that provide direct intercellular communication. They often occur as junctional plaques that contain up to thousands of single gap junction channels (Kumar and Gilula 1996) and they allow the passive diffusion of molecules of up to 1,000 Daltons. Such molecules can include nutrients, metabolites, second messengers, cations, and anions (Evans and Martin 2002). Each channel consists of two hemichannels, each of which is composed of subunit proteins. The main gap junction proteins are the connexins, however, it was recently discovered that pannexin proteins are also capable of forming gap junctions (Bruzzone, et al. 2003). Previous studies have shown that gap junction blockers reduce the infarct volume *in vivo* (Rawanduzy, et al. 1997) and neuronal death *in vitro* (Frantseva, et al. 2002). Gap junctions are involved in the control of cytoplasmic sodium and calcium levels. The spread of calcium depolarizes a large number of neurons and glial cells. This can lead to excitotoxic glutamate release that will promote damage expansion. This may help explain why blocking gap junctions could be neuroprotective in these conditions where spreading depression takes place.

1.9 Connexin Hemichannels

Connexins are a family of transmembrane proteins with at least 19 and 20 members in the mouse and human, respectively (Willecke et al., 2002). All connexins have four transmembrane domains, with N- and C-termini, a cytoplasmic loop between the second and third transmembrane domains, and two extracellular loops (Figure 5). The extracellular loops of almost all connexins contain three cystine residues that are

believed to form disulfide bonds essential for tertiary structure. The four transmembrane domains are largely α-helical, the two extracellular loops are largely β-sheet, and the cytoplasmic domains are largely unstructured (Duffy et al., 2006). The carboxyl terminus and cytoplasmic loop contain domains that participate in both intra- and inter-molecular binding, inducing sites that are presumably responsible for gating of gap junction channels. Newly synthesized connexins are assembled, depending on the connexin type, in the endoplasmic reticulum (ER) or the golgi apparatus to form hexamers known as hemichannels or connexons (George et al., 1999) which make up one half of a gap junction. After assembly, hemichannels are transported to the cell surface membrane. The half-life of several rodent gap junctions has been found to be between two and five hours (Saez et al., 2003). The hemichannels are then internalized and degraded by both proteosomes and lysosomes (Laing et al., 1997). Hemichannels can be gated by various mechanisms including voltage, calcium, certain lipophilic agents and protein phosphorylation (Bennett and Verselis, 1992).

In the brain, expression of different connexins varies according to region, cell type, and developmental stage. Cx36 and Cx45 have been identified as the predominant connexins expressed in adult neurons (Rash et al., 2000; Sohl et al., 1998; Maxeiner, 2003) and deletion of either of the adult neuronal connexin genes results in deficiencies in the signalling and transmission properties of neurons (Maxeiner et al., 2005; Deans et al., 2002). Astrocytes have been shown to express Cx43 and lower levels of Cx26, Cx30, Cx40 and Cx45 (Nagy et al., 1997; Dermietzel et al., 1991). Oligodendrocytes express Cx29, Cx32, and Cx47 (Rash et al., 2001).

When hemichannels are open, they connect the cell's interior with the

extracellular space, unlike that of gap junctions that connect the interior of one cell to the interior of another. Hemichannels have been shown to be functional under both physiological and pathological conditions (Bennett et al., 2003). Low extracellular calcium causes hemichannel opening (DeVries and Schwartz, 1992) as well as membrane depolarization, mechanical stimulation (Stout et al., 2002) and pharmacological agents (Plotkin et al., 2002; Malchow et al., 1994). Connexin hemichannels are blocked by hyperpolarization of the plasma membrane (Trexler et al., 1996), and low extracellular pH (Bennett et al., 2004), as well as gap junctional blockers (Eskandari et al., 2002).

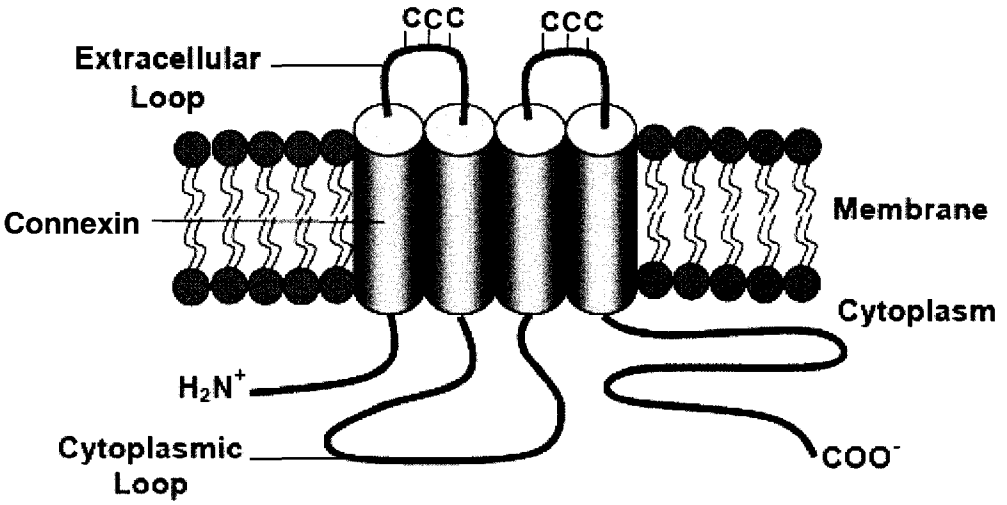

Figure 5. Connexin structure. The cylinders represent the transmembrane domains and there are three conserved extracellular cystine residues.

Prolonged opening of connexin hemichannels is thought to induce metabolic stress and cell death. The first study of open hemichannels was in Xenopus oocytes expressing Cx46 and it showed the induction of a non-selective inward current, cell swelling and death (Paul et al., 1991). In fact, studies have shown that gap junction

blockers reduce the infarct volume *in vivo* (Rawanduzy et al., 1997) and neuronal death *in vitro* (Frantseva et al., 2002). Thompson et al., (2006) suggested that the opening of hemichannels could mediate the dysregulation of ionic fluxes and anoxic depolarization, as well as cause the energy depleting efflux of glucose and ATP characteristic of ischemic neurons. However, some researchers have shown that if you block or knockout individual connexins, such as Cx43, during ischemia, there is actually an expansion of the infarct area (Siushansian et al., 2001; Nakase et al., 2003).

Cx43 hemichannels in astrocytes as well as connexins in other cell types have been suggested to mediate the ATP release triggered by mechanical stimulation (Cotrina et al., 1998; Guthrie et al., 1999; Stout et al., 2002). Recently, it was shown that release of IP_3 induces opening of Cx43 hemichannels and release of ATP into the extracellular space (Braet et al., 2003). Hemichannel opening has also been implicated in the ATP-induced ATP release, a process known to mediate calcium wave propagation (Locovei et al., 2006) and the addition of apyrase, an enzyme that catalyses the hydrolysis ATP to AMP, reduces the degree of calcium propagation (Stout et al., 2002). Previous studies have also shown that ATP can induce neurite outgrowth in PC12 cells mediated by connexin hemichannels (Belliveau et al., 2006; Gysbers et al., 2000). The Cx36 channel blockers quinine, quinidine and mefloquine have been shown to inhibit cortical spreading depression (Margineanu and Klitgaard, 2006) and application of other connexin blockers such as carbenoxolone and flufenamic acid have also been shown to abolish the spontaneous wave propagation (Weissman et al., 2004).

1.10 Objectives and Hypothesis – Chapter 4

During cortical spreading depression ATP is being released into the extracellular space. Connexin hemichannels are known to release the ATP required for the propagation of intracellular calcium waves that accompany CSD. In vitro, ATP provides a degree of neuroprotection on neurons against various forms of ischemia. **Therefore, it was our main hypothesis that in vitro KCl depolarization is leading to a release of ATP through connexin hemichannels, which elicits the induction of ischemic tolerance.**

Specifically, our objectives were:

- To determine if KCl induced depolarization is protective against various forms of in vitro ischemia
- To determine the involvement of ATP in KCl induced tolerance with the use of inhibitors
- To determine the purinergic receptors as well as the signal transduction pathways activated by KCl in the induction of tolerance
- To measure the release of ATP from neurons in response to KCl induced depolarization in vitro
- To determine the involvement of connexin hemichannels in the release of ATP from neurons in response to KCl depolarization
- Use siRNA to knock-down expression of connexin 36 to determine if this is the connexin channel releasing ATP in response to KCl depolarization

Chapter 2:

Regulation of expression of early growth response transcription factors in rat primary cortical neurons by extracellular ATP

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2.1 Author Contribution

Sarah C. McKee: Unless otherwise indicated all experiments, including preparation of reagents and cell culture, were performed by the first author under the supervision of Dr. Hakim, Dr. Thompson and Dr. Sabourin. The manuscript was co-written by Dr. Thompson with the first author.

Charlie S. Thompson: All immunohistochemistry experiments in this study were performed by Dr. Thompson. The manuscript was co-written by Dr. Thompson with the first author.

Luc A. Sabourin: This author was the co-supervisor of the first author and contributed ideas and reagents to this paper.

Antoine M. Hakim: This author supervised the work on this paper, edited the manuscript and provided financial support.

2.2 Abstract

The zinc finger transcription factor early growth response-1 (Egr-1, NGFI-A, zif268, Krox 24, TIS8, ZENK) is upregulated immediately in the brain by cortical spreading depression (CSD) and other preconditioning stimuli and thus might participate in regulation of the overall genomic response to preconditioning. In the present study, the induction of expression of Egr-1 and other early growth response family members was characterized in rat primary cortical neuronal cultures. In neuronal cultures in vitro, depolarization or exposure to extracellular glutamate caused a 4-fold increase in *egr-1* mRNA while exposure to extracellular ATP caused a 10-fold increase. The presence of mRNA encoding for multiple types of purinergic receptors was confirmed by RT-PCR. A number of nucleotide agonists proved effective in eliciting an increase in *egr-1* mRNA. Over a limited range of concentration, the most effective agonists were ATP > ADP > α, β-methylene ATP > UTP > cAMP > UDP > AMP > adenosine. Pertussis toxin, suramin, reactive blue 2, PPADS, DPCPX and inhibitors of Protein Kinase C, Protein Kinase A and PI3 kinase significantly reduced the upregulation of *egr-1* by exposure to extracellular ATP. These findings suggest that neuronal metabotropic purinergic receptor activation contributes to the induction of early growth response transcription factors and may provide a target that can be manipulated to increase ischemic tolerance of the brain in vivo.

2.3 Introduction

The early growth response family of zinc finger transcription factors comprises *Egr-1* (also known as *Krox*-24, *NGFI-A*, *zif*268, *TIS*8 and *ZENK*), *Egr-2* (*Krox*-20), *Egr-3*, *Egr-4* (*NGFI-C*, *pAT*133) and Wilm's tumor gene WT-1. Egr proteins are immediate early genes in that they are rapidly activated in the absence of de novo protein synthesis (Lechner et al., 2004 and Lemaire et al., 1988). *Egr-1* is the most well studied member of the family and is known to be induced in numerous cell types by a variety of extracellular stimuli and stresses and be involved in many processes related to cell growth, differentiation and injury repair. In the present study, the expression of members of the early growth response (*egr*) family of zinc finger transcription factors was characterized in cultured rat cortical neurons. This family of genes was selected because some members are induced immediately by cortical spreading depression (CSD; Hanley et al., 2004 and Jacobs et al., 1994) and other preconditioning stimuli (Carmel et al., 2004 and Kawahara et al., 2004) and, as transcription factors, might serve to regulate the overall genomic response to preconditioning and the induction of ischemic tolerance in vivo. In the brain, Egr proteins have a basal level of expression that varies among different structures and cell types and show a rapid induction by a variety of physiological stimuli, including light, tactile stimulation restraint stress, injury, ischemia, nerve transection and seizure activity (Beckmann and Wilce, 1997). *Egr-1* expression has also been associated with apoptosis in neuronal cells (Catania et al., 1999 and Nakata et al., 2004) and with synaptic plasticity in the hippocampus (Izquierdo and Cammarota, 2004). It has been proposed that egr-1 expression in normal adult brain is determined in large part by ongoing synaptic activity and should be considered in the context of neuronal activity

that is associated with neuronal plasticity (Knapska and Kaczmarek, 2004).

ATP and other nucleotides are known to serve as extracellular signaling molecules in the brain (Di Virgilio, 2000 and Zimmermann, 1994). ATP may be stored in vesicles in nerve terminals along with other neurotransmitters and act as a co-transmitter during synaptic transmission (Burnstock, 2004) and may also be released from neuronal and non-neuronal cells by way of ruptured cell membranes or a variety of channels including connexin hemichannels (Cotrina et al., 1998), pannexin channels (Bao et al., 2004), voltage-dependent anion channels (Milbrandt, 1987) and the cystic fibrosis transmembrane conductance regulator, CFTR (Reigada and Mitchell, 2004). Extracellular nucleotide receptors may act as either ligand-gated cationic channels (P2X receptors) or G-protein-coupled receptors (P2Y receptors) and both of these families of receptors have a wide distribution in the central nervous system (Illes and Alexandre Ribeiro, 2004). Once released into the extracellular medium, ATP may bind to receptors and mediate a wide range of effects including neurotransmission, modulation of the effects of other neurotransmitters and growth factors, trophic actions and cytotoxicity (Amadio et al., 2002, Dhodda et al., 2004 and Salim et al., 2002). In the present study, we show that extracellular ATP upregulates the expression of *egr-1*, *egr-2* and *egr-3* in cultured rat primary cortical neurons.

2.4 Results

2.4.1 Egr-1 expression in cultured primary cortical neurons

At days 12–14 in vitro, the majority of cells in the cultures that were positive for GFAP (astrocytes) or NeuN (neurons) expressed Egr-1 protein (Fig. 1).

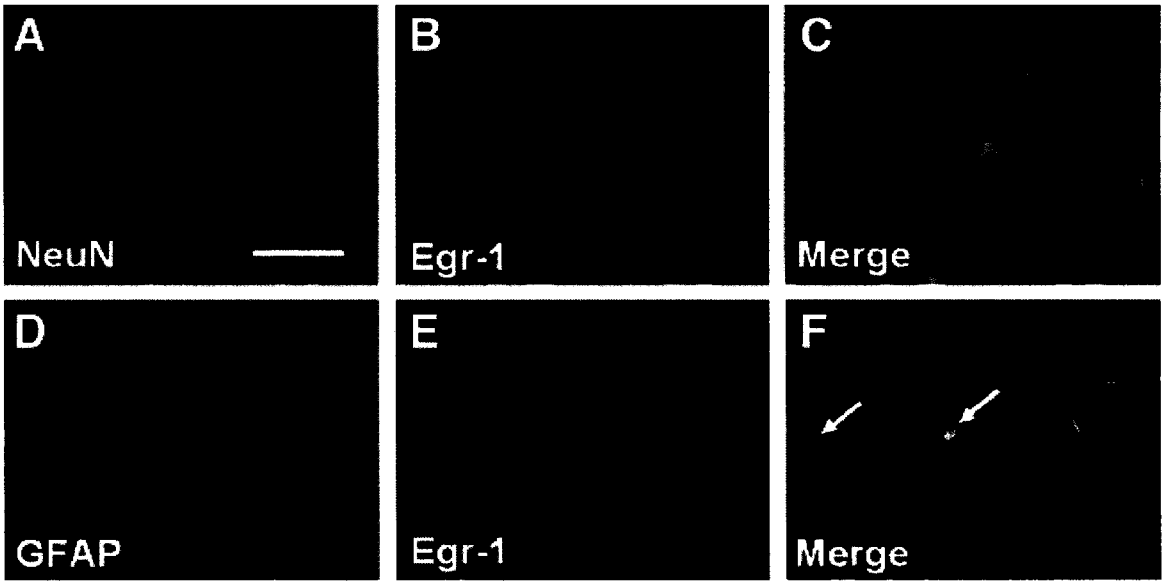

Figure 1. Both neurons and astrocytes in primary cortical cultures express Egr-1 protein. (A) NeuN positive cells (green) express Egr-1 (B, red). (C) Panels A and B merged. (D) GFAP positive cells (green) express Egr-1 (E, red). (F) Panels C and E merged. Egr-1 immunoreactivity is found mainly in the nuclei (arrows). Scale bar in panel A is 10 μm

Cultures were exposed to depolarization with high potassium medium, extracellular glutamate, extracellular acetylcholine or extracellular nucleotides, all conditions likely to occur in vivo during CSD, and the level of *egr-1* mRNA was assessed after 15 min by real-time RT-PCR (Fig. 2B). Neurons in untreated cultures were found to express *egr-1*, *egr-2*, *egr-3* and *egr-4* but *wt-1* was not detected (Fig. 2E). Acetylcholine (10 μM) did not significantly affect the level of expression while glutamate (10 μM) and KCl

(50 mM) caused a significant ($P < 0.001$) increase of 3.48 ± 0.85-fold and 4.19 ± 0.64-fold, respectively. ATP (10 μM) elicited an increase of 10.37 ± 1.03-fold.

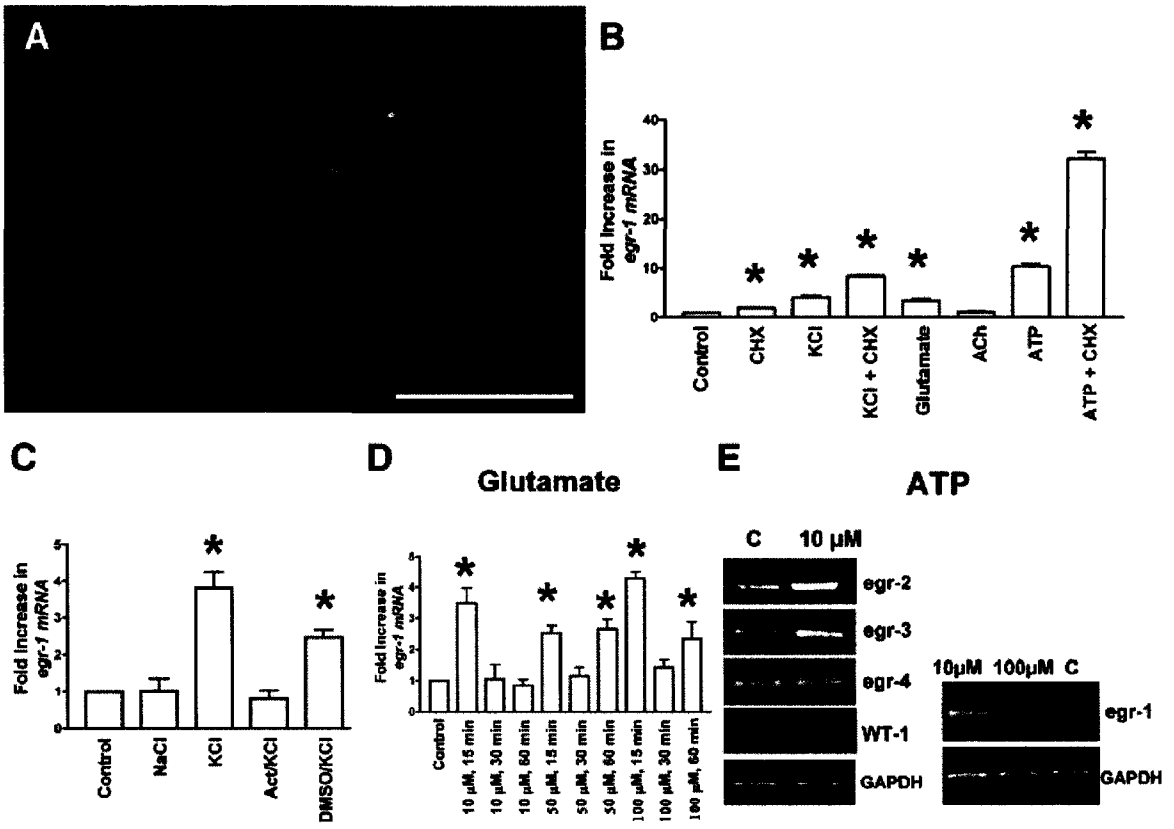

Figure 2. egr-1 expression in primary cortical cell cultures. (A) The cultures consisted of a majority of MAP2 positive cells (neurons, red) and GFAP positive cells (astrocytes, green). Scale bar = 250 μm. (B) Levels of egr-1 expression under control conditions and following exposure to high potassium, glutamate, acetylcholine and ATP. KCl was elevated by 50 mM. Glutamate, ACh, ATP and the protein synthesis inhibitor cycloheximide (CHX) were added to a final concentration of 10 μM. All cultures were treated for 15 min before RNA extraction. (C) The increase in egr-1 expression induced by exposure to high potassium medium is blocked by the RNA synthesis inhibitor actinomycin D (Act). Note that the vehicle for Act (DMSO) itself caused a significant reduction in egr-1 mRNA levels. (D) The effect of glutamate on egr-1 expression is dependent upon concentration and time of exposure. (E) Exposure to extracellular ATP upregulates expression of egr-1, egr-2 andegr-3 but not egr-4 mRNA. WT-1 mRNA was not detected in treated or control (C) cultures. * indicates a statistically significant difference between the means of the treated and control cultures (P, 0.05) in this and all subsequent figures.

When cultures were exposed to the protein synthesis inhibitor cycloheximide (10 μM) for 1 h prior to and during exposure to KCl or ATP, the elevation in *egr-1* mRNA was greatly enhanced. When cultures were exposed to cycloheximide alone, there was a significant increase in egr-1 mRNA of 2.06 + 0.1-fold ($P < 0.001$). In a separate trial, the increase in *egr-1* expression elicited by exposure to high potassium medium was completely blocked by the RNA synthesis inhibitor actinomycin D (10 μM), indicating that the response is mediated by an increase in transcription (Fig. 2C).

The degree to which *egr-1* expression was affected by various treatments was found to vary with time of exposure and concentration. The response to a given concentration of glutamate, for example, could vary significantly depending on the duration of exposure (Fig. 2D). Exposure of cultures to extracellular ATP also elevated the expression of *egr-2* and *egr-3* mRNA, but not of *egr-4* or *WT-1* (Fig. 2E).

2.4.2. mRNA encoding P2X and P2Y receptors is expressed by cultured primary cortical neurons

Because cultured primary cortical neurons respond to extracellular ATP, cultures were screened for the presence of purinergic receptor mRNA by RT-PCR with primers designed specifically for various cloned members of the P2X and P2Y families of receptors (Table 1). Bands corresponding to mRNA encoding P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 and P2Y14 were present, but not for P2Y12 or P2Y13 (Fig. 3A). mRNA for P2X2, P2X4, P2X5, P2X6 and P2X7 were present as well but not to mRNA for P2X1 or P2X3 (Fig. 3B). These primers did not generate additional bands. As a positive control for detection of P2Y12, P2Y13 and P2X3 mRNA, total RNA was extracted from cultures

of undifferentiated PC12 cells, which are known to express mRNA for these receptors (Lechner et al., 2004 and D'Ambrosi et al., 2001), and analyzed by RT-PCR (Fig. 3C). RNA was extracted from rat kidney to provide a positive control for P2X1 receptors (Fig. 3C, Turner et al., 2003).

Table 1. The sense and antisense sequences, annealing temperatures, sources and product lengths for primers used to detect P2 receptor mRNA in primary cortical neuronal cultures

	Tm (°C)	Source	cDNA (bp)
P2X primers			
P2X1 (S) 5′-TGG ATG ACA AGA TCC CAA GC-3′	60	Norway rat vas deferens X80477	418
(A) 5′-ACG GTT TGT CCC ATT CTG CA-3′			
P2X2 (S) 5′-ATG GAA CTT CTG ACA ACC AT-3′	60	Norway rat caeballum Y10473	418
(A) 5′-AGT GGT GGT AGT GCC GTT TA-3′			
P2X3 (S) 5′-CAA CTT CAG GTT TGC CAA A-3′	55	Arslan et al. (2000)	519
(A) 5′-TGA ACA GTG AGG GCC TAG AT-3′			
P2X4 (S) 5′-ACG ACG TTG GCG TGC CAA CG-3′	60	Norway rat brain U32497	411
(A) 5′-GCG CTG CTC TTT GCC GGC CA-3′			
P2X5 (S) 5′-CAA AGT CCA TGC CAA CGG AT-3′	55	Norway rat heart X97328	421
(A) 5′-ACG GAA CTC TAC CCC ATT AG-3′			
P2X6 (S) 5′-GTA GTG CTG TGC CCA GGA AA-3′	60	Norway rat brain X92070	418
(A) 5′-GGA CTC CAC GCC TGA GGC TG-3′			
P2X7 (S) 5′-AAG GGA AAG AAG CCC CAC GG-3′	60	Norway rat macrophage X95822	418
(A) 5′-CCG CTT TTC CAT GCC ATT TT-3′			
P2Y primers			
P2Y1 (S) 5′-CCT GCG AAG TTA TTT CAT CTA-3′	51	*Ruttus norvegius* U22830	318
(A) 5′-GTT GAG ACT TGC TAG ACC TCT-3′			
P2Y2 (S) 5′-GCA GCA TCC TCT TCC TCA CCT-3′	61	Norway *rat strain* U56839	499
(A) 5′-CAT GTT GAT GGC GTT GAG GGT-3′			
P2Y4 (S) 5′-GGC ATT GTC AGA CAC CTT GTA-3′	58	Norway rat Y14705	550
(A) 5′-AAG GCA CGA AGC AGA CAG CAA-3′			
P2Y6 (S) 5′-CGC TTC CTC TTC TAT GCC AA-3′	60	Norway rat aorta D63665	481
(A) 5′-GTA GGC TGT CTT GGT GAT GTG-3′			
P2Y11 (S) 5′-CTG GTG GTT GAG TTC CTG GT-3′	60	Elia et al., 2003	205
(A) 5′-GTT GCA GGT GAA GAG GAA GC-3′			
P2Y12 (S) 5′-TCC CAT TGC TCT ACA CTG TC-3′	53	Norway rat AF313450	895
(A) 5′-TGT CCT TTC TTC TTA TTT GC-3′			
P2Y13 (S) 5′-CAG GGA CAC TCG GAT GAC A-3′	60	Norway rat AY639875	600
(A) 5′-TGT TCG GCA GGG AGA TGA-3′			
P2Y14 (S) 5′-TGT CTG CCG TGA TCT TCT-3′	55	*Ruttus Norvegicus* U76206	589
(A) 5′-GGG TCC AGA CAC ACA TTG-3′			

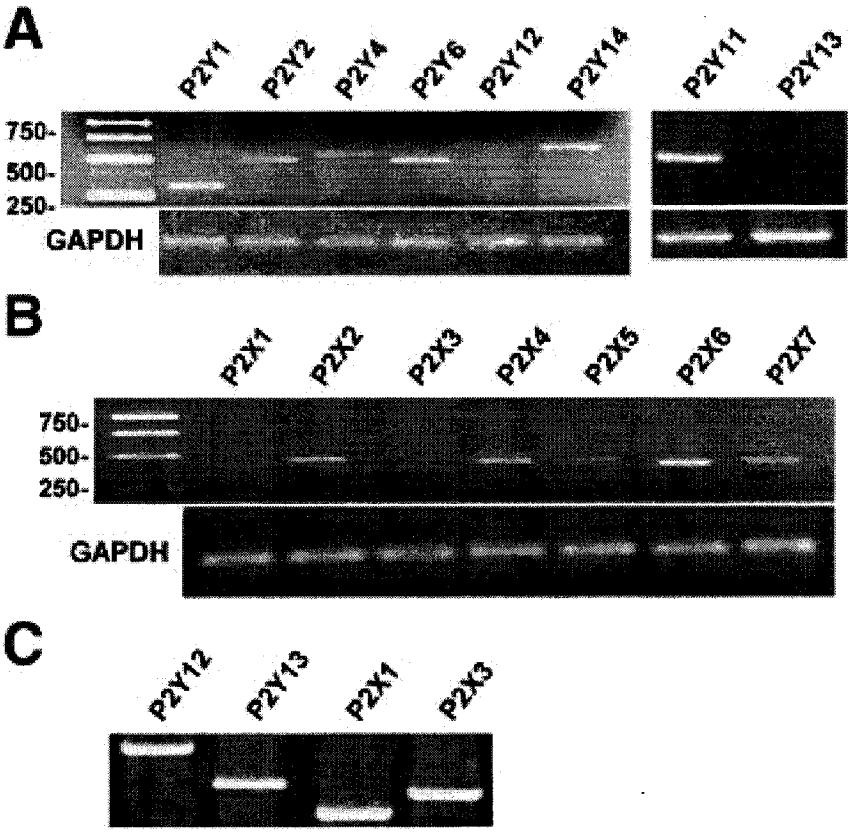

Figure 3. Primary cortical cultures express mRNA for a variety of purinergic receptors. (A) P2Y (metabotropic) receptors. mRNA for P2Y11 and P2Y13 was amplified in a separate trial. (B) P2X (ionotropic) receptors. (C) Positive controls for purinergic receptor mRNA not detected in primary cortical cultures.

The increase in *egr-1* expression following exposure to extracellular ATP was dose-dependent, with the most effective concentration being well below 100 μM (Fig. 4A). The concentration eliciting the greatest increase in *egr-1* expression was not toxic when applied to primary neuronal cultures for 15 min and the extent of cell survival assessed 24 h later (Fig. 4B). When primary cortical cell cultures were pretreated with pertussis toxin (200 ng/ml) for 14 to16 h prior to exposure to ATP for 15 min, the level of

egr-1 expression was significantly reduced (Fig. 4C). In cultures treated with the P2 receptor antagonist suramin (von Kugelgen and Wetter, 2000, 10 μM) for 1 h prior to treatment with ATP, the level of *egr-1* expression was also significantly reduced (Fig. 4D), indicating that P2 receptors are involved in mediating the response to ATP. Suramin by itself caused a significant increase ($P < 0.001$) in *egr-1* expression of 2.14-fold. Further observations indicate that the upregulation of *egr-1* by extracellular ATP is mediated primarily by P2Y receptors. The response was reduced by 68.6% when cultures were pretreated with the P2Y receptor antagonist Reactive Blue 2 (100 μM, 1 h) prior to exposure to ATP (Fig. 4F, Volonte et al., 1999). The response was reduced by 20.0% by pretreatment (100 μM, 1 h) with the P2X antagonist PPADS (Fig 4E, Zona et al., 2000) and 19.9% by pretreatment (100 nM, 1 h) with the adenosine A1 receptor antagonist DPCPX (Fig. 4G, Rebola et al., 2005).

The upregulation of Egr-1 at the protein level by exposure to extracellular ATP is not readily demonstrated by Western blotting because of the high levels of Egr-1 protein in untreated cultures. This was overcome by exposing cultures to the sodium channel blocker tetrodotoxin (TTX) prior to exposure to ATP (Fig. 5). This would be expected to cause a reduction in Egr-1 protein levels because primary cortical neurons began to generate spontaneous electrical activity after several days in culture (Opitz et al., 2002) and Egr-1 expression is thought to be determined in large part by ongoing synaptic activity (Knapska and Kaczmarek, 2004). Cultures were exposed to 1 μM TTX overnight, and then to 10 μM ATP for 15 min while still in the presence of TTX. Protein was then extracted immediately, or after 15 or 45 min in normal medium.

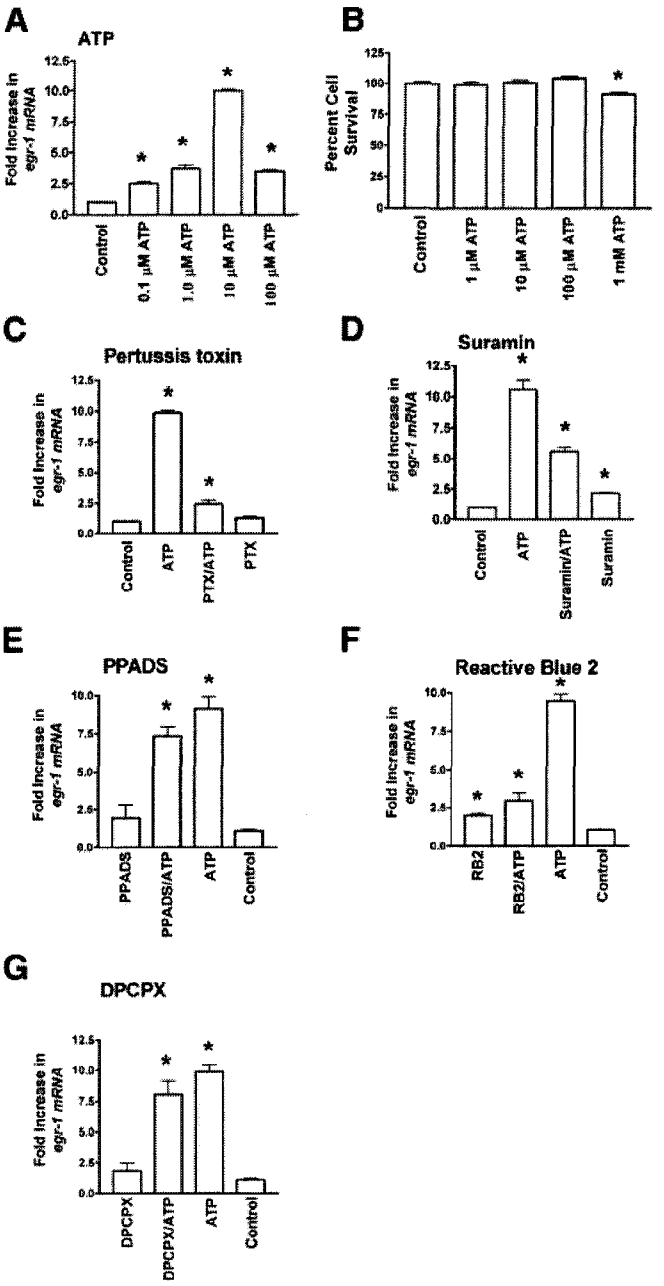

Figure 4. (A) Extracellular ATP upregulates egr-1 expression in a dose-dependent manner. (B) The concentration of ATP that elicits altered gene expression is not toxic to cultured neurons. Cultures were exposed to ATP for 15 min and cell viability assessed 24 h later. (C) Pertussis toxin (PTX, a G-protein receptor inhibitor) greatly reduces the upregulation of egr-1 by extracellular ATP. (D) The effect of the P2 receptor antagonist suramin (10 µM) on egr-1 expression following exposure to extracellular ATP. (E) The P2X receptor antagonist PPADS (100 µM) reduced the upregulation of egr-1 expression by about 20%. (F) The P2Y receptor antagonist reactive blue 2 (RB2, 100 µM) reduced the response to ATP by about 70%. (G) The adenosine A1 receptor antagonist DPCPX (100 nM) reduced the response to ATP by about 20%

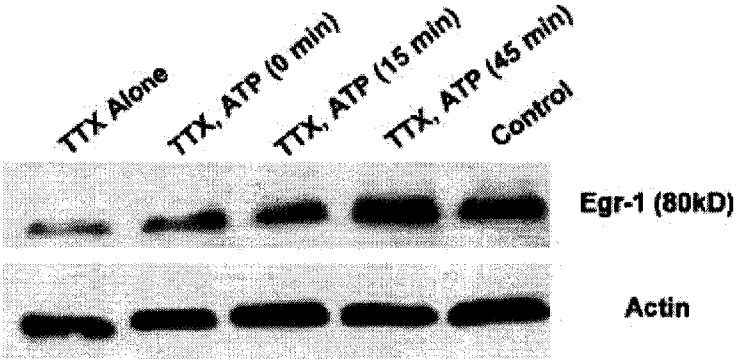

Figure 5. Extracellular ATP upregulates Egr-1 at the protein level. Primary cortical cultures were exposed to the sodium channel blocker tetrodotoxin (TTX, 1 μM) overnight and then to 10 μM ATP for 15 min, still in the presence of TTX. Cultures were then harvested immediately or returned to normal medium and harvested 15 or 45 min later.

2.4.3.Multiple ligands elevate the expression of *egr-1*

Exposure to extracellular ATP proved to be the most effective treatment of those used in the present study in elevating *egr-1* expression. Therefore, in a separate series of experiments, the induction of *egr-1* expression was assessed for a range of known nucleotide receptor agonists. For each agonist, cultures were exposed in triplicate to concentrations of 0.1, 1.0, 10 and 100 μM for 15 min and the level of *egr-1* mRNA assessed by real-time RT-PCR and compared to the mean value for 3 control cultures. When the greatest increase in *egr-1* expression induced by each agonist was ranked, the order of potency was ATP (9.97 ± 0.21-fold) > ADP (8.56 ± 0.29-fold) > α, β-methylene ATP (4.85 + 0.75-fold) > UTP (3.84 ± 0.63-fold) > cAMP (3.31 ± 0.22-fold) > UDP (2.83 ± 0.66-fold) > AMP (1.75 ± 0.35-fold) > adenosine (1.91 ± 0.38-fold). These results do not constitute a proper dose–response curve but do indicate that multiple ligands may be involved in mediating the response to extracellular ATP. The most effective concentration differed among agonists.

2.4.4. Signal transduction pathways mediating the response to extracellular ATP

In order to identify signal transduction pathways mediating the increase in expression of *egr-1* by primary cortical neurons in response to extracellular ATP, cultures were pretreated with inhibitors of pathways known to be activated by purinergic receptor activation in other cell types prior to treatment with ATP (Fig. 6). Cultures were exposed to either U0126 (10 μM), Rp-cAMPS (50 μM), LY294002 (50 μM), Bisindolymalimide IV (10 μM) or U73122 (10 μM) for 30 min and then to 10 μM ATP for 15 min before RNA extraction. Bisindoylmaleimide IV (inhibitor of Protein Kinase C), Rp-cAMPS (inhibitor of Protein Kinase A), LY294002 (inhibitor of PI3 kinase) and U73122 (inhibitor of phospholipase C) significantly reduced expression of *egr-1* compared to control cultures while U0126 (inhibitor of MEK 1/2) was without effect.

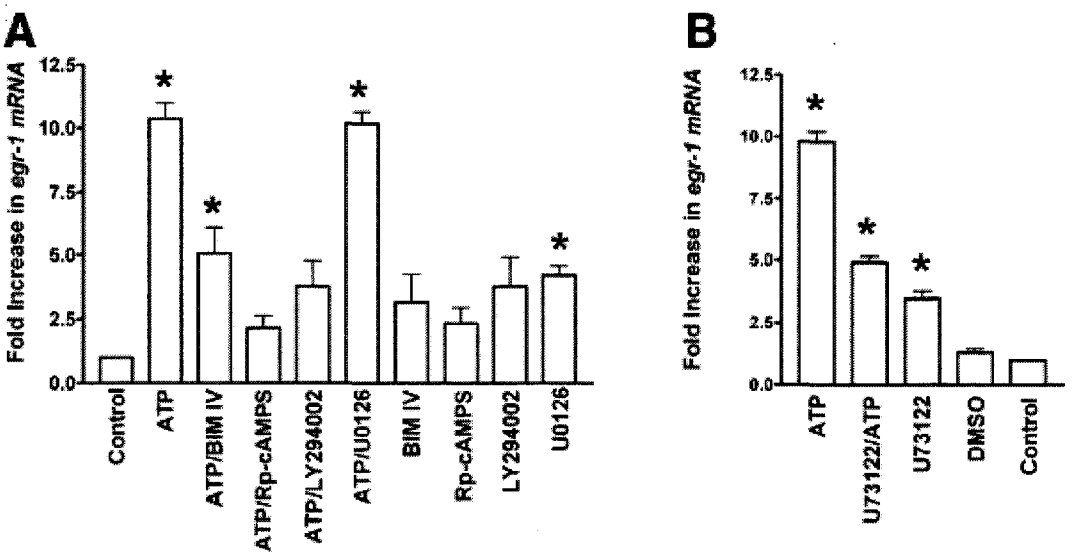

Figure 6. The effects of signal transduction pathway inhibitors on ATP-induced *egr-1* expression. (A) The protein kinase C inhibitor Bisindoylmaleimide IV (BIM IV), the protein kinase A inhibitor Rp-cAMPS and the PI3 kinase inhibitor LY294002 significantly reduced expression of *egr-1* while the MEK inhibitor U0126 was without effect. U0126 alone produced a statistically significant increase in *egr-1* mRNA. (B) The phospholipase C inhibitor U73122 significantly decreased *egr-1* expression.

While bisindoylmaleimide IV is a reasonably selective inhibitor of PKC relative to other Ser/Thr protein kinases and tyrosine kinases, similar K_m values have been reported for various isoforms of PKC (Toullec et al., 1991). Thus, it is not possible to identify which of the 11 different isoforms of PKC identified in rodent brain to date (Li et al., 2005) are mediating the response to ATP on the basis of the present results. When cultures were treated with these compounds in the absence of extracellular ATP, there was a trend toward increased *egr-1* expression in every case but only U0126 and U73122 produced a statistically significant increase in the level of *egr-1* mRNA.

2.4.5. Cytoplasmic calcium levels influence *egr-1* expression

Members of both P2X and P2Y families of purinergic receptors are known to alter levels of ionized calcium in the cytoplasm by allowing calcium from the extracellular medium to pass through ion channels in cell membranes or by activating phospholipase C and releasing calcium from intracellular stores, respectively. When cortical cell cultures were preincubated in calcium free medium for 60 min and then exposed to ATP in calcium-free medium for 15 min, there was a significant reduction in *egr-1* mRNA from 9.47 + 0.54 to 6.9 + 0.52-fold compared to control cultures ($0.001 < P < 0.01$, Fig. 7A). When cultures were incubated in medium containing thapsigargin (10 μM), which is known to deplete calcium stores in the endoplasmic reticulum (Thastrup et al., 1990), for 60 min prior to exposure to ATP, the increase in *egr-1* mRNA levels was completely eliminated (Fig. 7B). This indicates that release of calcium from the endoplasmic reticulum by P2Y receptor activation is a major factor in the induction of *egr-1* expression in primary cortical neurons by extracellular ATP. When cultures were

exposed to the calcium ionophore A23187, there was a substantial time-dependent increase in *egr-1* mRNA (Fig. 7C), indicating that the level of ionized cytoplasmic calcium itself is a major determinant of the ongoing rate of *egr-1* expression (Fig. 7).

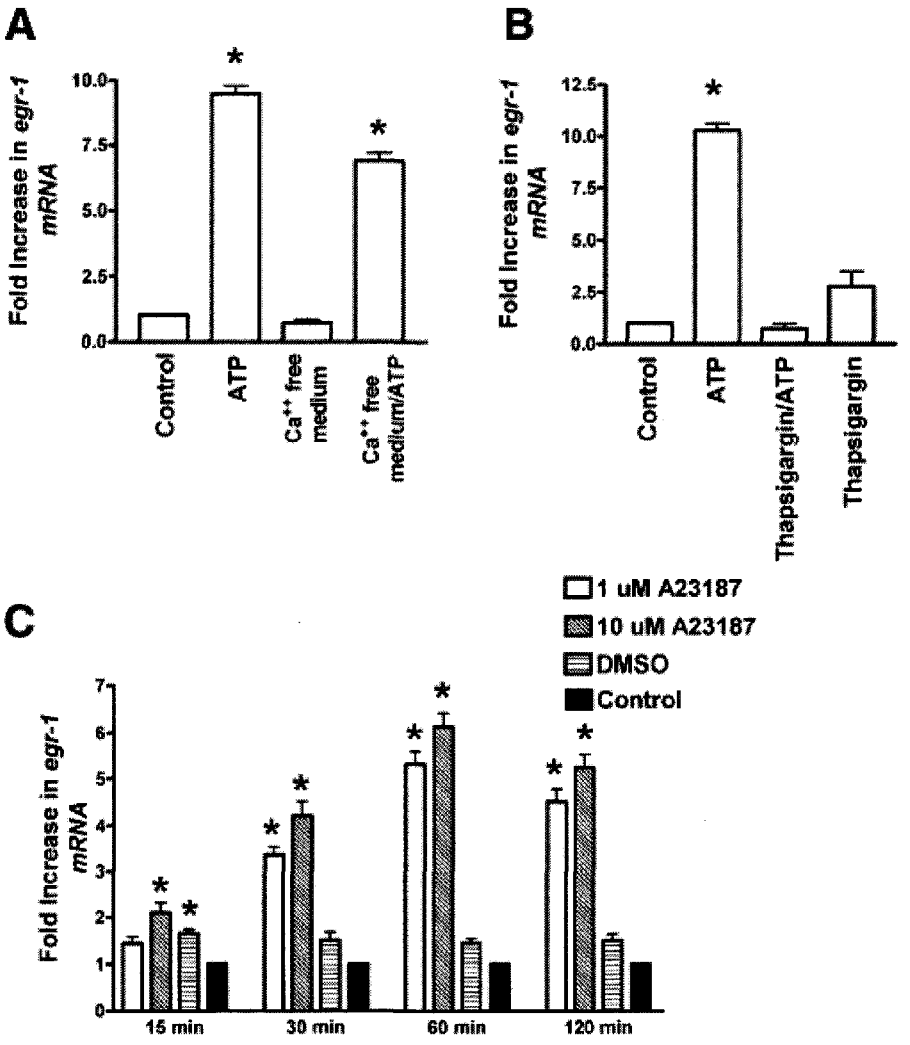

Figure 7. Cytoplasmic calcium ion levels affect *egr-1* expression. (A) When cortical cell cultures were exposed to ATP in calcium-free medium, the level of *egr-1* mRNA was significantly reduced after 15 min compared to cultures exposed to ATP in normal medium. (B) When cultures were pretreated with the endoplasmic reticulum calcium transporter inhibitor thapsigargin (10 μM) for 60 min prior to exposure to ATP, the response was completely eliminated. Exposure to thapsigargin alone for 60 min produced an increase in *egr-1* mRNA that was not highly significant ($P < 0.01$). (C) When cultures were exposed to the calcium ionophore A23187 (1 μM or 10 μM), there was a time-dependent increase in *egr-1* mRNA.

2.5 Discussion

Recent studies using DNA microarray technology have shown in several rodent models that there is an extensive genomic response to brain preconditioning (Carmel et al., 2004, D'Ambrosi et al., 2004, Kawahara et al., 2004 and Priller et al., 1995), and it is likely that these changes in gene expression contribute to the induction of ischemic tolerance. As immediate early gene transcription factors, early growth response family members may be involved in the regulation of the overall genomic response to preconditioning and the induction of ischemic tolerance.

In order to study the upregulation of *egr* genes in cultured primary cortical neurons, cultures were treated with conditions reported to occur in the brain during CSD and the level of expression of egr family members assessed by RT-PCR. Both exposure to extracellular glutamate and depolarization with high potassium medium caused an increase of *egr-1* expression of about 4-fold. Exposure to extracellular ATP proved to be much more potent in elevating *egr-1* expression. Extracellular ATP has previously been reported to induce the expression of a variety of genes including acetylcholine receptor and acetylcholinesterase subunits (Choi et al., 2003 and Tsim et al., 2003), interleukin 6 (Guthrie et al., 1999), heat shock proteins 70 and 90 (D'Ambrosi et al., 2001) and the immediate early genes c-*fos*, junB, c-*jun* and TIS11 (Okada et al., 2004).

Extracellular ATP has been reported to be toxic to cultured cerebellar granule neurons, dissociated striatal primary cells and hippocampal organotypic cultures with an EC_{50} in the range of 20–50 μM and cell death occurring within a few minutes (Amadio et al., 2002). The concentration of ATP and time of exposure used in the present study was far below the level required to cause detectable cell death and could thus be relevant to

the normal physiological function of neurons. It is likely that ATP is released into the extracellular space during CSD in vivo because ATP release from astrocytes has been shown to mediate the progression of glial calcium waves (Fumagalli et al., 2003) and glial calcium waves accompany spreading depression (Basarsky et al., 1998). ATP is also known to be released into the extracellular space in the brain following metabolic stress and ischemia (Salim et al., 2002).

Numerous studies have reported the expression of purinergic receptors by rodent neurons and glia (Bennett et al., 2003, Calvert et al., 2004, Fields and Stevens, 2000 and Lalo et al., 1998). In the present study, mRNAs encoding 11 of the 15 known purinergic receptors were found to be present in primary cortical cultures at 10 to 14 DIV. It is possible that some of these receptors are expressed only in the relatively small number of astrocytes present in the neuronal cultures used in this study, as rat cortical astrocytes have been shown to express all known P2X and P2Y receptors, with the exception of $P2X_6$ (Fields and Stevens, 2000). $P2X_6$ mRNA was detected in our cultures and is thus likely expressed by neurons. It is possible that more than one type of purinergic receptor is contributing to the response to extracellular ATP. Not only is mRNA encoding multiple purinergic receptor types present but G-protein-coupled receptors in general are known to form hetero-oligomeric complexes with altered receptor-ligand binding properties (Breitwieser, 2004 and Pignatelli et al., 2003) and purinergic receptors in particular may form hetero-oligomers with adenosine receptors (Li et al., 2005). It is likely as well that more than one ligand is contributing to the overall response to extracellular ATP. Extracellular ATP is rapidly degraded by a series of ecto-nucleotidases, both in vivo (Toullec et al., 1991) and in vitro (Joseph et al., 2004), so

shortly after the addition of ATP to cultured neurons ADP, AMP and adenosine may be generated. The observations that the non-hydrolyzable analogue α, β-methylene ATP does not elicit as great a response as ATP itself and that ADP elicits a greater response than α, β-methylene ATP suggest that ADP may make a significant contribution to the overall response.

One complication occurring in studies using the exogenous application of ATP is that responses mediated by adenosine receptors may occur (Cunha et al., 1998). In the present study, the exogenous application of adenosine elicited an increase in *egr-1* mRNA of less than 2-fold and the adenosine A1 receptor antagonist DPCPX reduced the response to extracellular ATP by about 20%. Thus, it is possible that adenosine receptor activation is contributing to the induction of *egr-1* expression by extracellular ATP. Pertussis toxin greatly reduced the induction of *egr-1* expression by extracellular ATP, indicating that the response is mediated primarily by G-protein-coupled receptors. The application of the P2 receptor antagonist suramin also reduces the response to ATP. Prior exposure to the P2Y receptor antagonist reactive blue 2 decreased the induction of egr-1 expression by ATP by about 70% while exposure to the P2X receptor antagonist PPADS reduced expression by about 20%. These observations indicate that the induction of *egr-1* expression by extracellular ATP is mediated primarily by one or more members of the P2Y family of purinergic receptors but P2X receptor activation may contribute to the response.

Observations presented here indicate that the level of expression of *egr-1* is controlled by multiple signal transduction mechanisms. P2Y receptors have been shown to couple to phospholipase C (PLC) in every case examined (Stenzel-Poore et al., 2003)

and the finding that the PLC inhibitor U73122 greatly reduces the response to ATP confirms that PLC activation is an important mediator. The finding that prior exposure to thapsigargin, an inhibitor of endoplasmic reticulum Ca^{++}-ATPase (Rebola et al., 2005), completely eliminates the response to ATP indicates that the generation of inositol triphosphate by PLC and the subsequent release of calcium from intracellular stores is the predominant signal transduction pathway mediating the response. However, other pathways may be involved in regulating *egr-1* expression. The inhibition of the response to ATP by various inhibitors indicates that protein kinase A, protein kinase C and PI3 kinase are all involved to a greater or lesser extent in mediating the response to ATP. The finding that inhibition of MEK and PLC with no further treatment significantly increases *egr-1* expression suggests that the moment to moment level of expression is subjected to complex control mechanisms involving active repression as well as stimulation. Stimulation of *egr-1* expression by ATP is greatly enhanced in the presence of cycloheximide, consistent with the idea that extracellular ATP also upregulates the expression of proteins that actively suppress *egr-1* transcription.

2.6 Experimental procedures

All surgical procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Immunohistochemistry - Cultured primary cortical neurons were rinsed twice in phosphate-buffered saline and fixed for 10 min in 4% PFA prior to staining. Primary

antibodies used were directed against Egr-1 (Santa Cruz Biotechnology), GFAP (Sigma, Inc.), MAP2 (Chemicon, Inc.) and NeuN (kindly provided by Michael W. McBurney). FITC and CY3-conjugated secondary antibodies were purchased from Chemicon, Inc. Nuclei were labeled by adding 10 μg/ml 4′6-diamidino-2-phenylindole (DAPI, Sigma) to the first rinse following exposure to the secondary antibody.

Primary cortical neuron culturing - Primary cortical neurons were cultured as described previously (Herdegen et al., 1993). Briefly, pregnant Sprague–Dawley rats (Charles River Canada, St. Constant, QC, Canada) at E-15/16 were anesthetized with halothane and sacrificed by cervical dislocation. Fetuses were decapitated and the cortical region dissected out and collected under sterile conditions. The tissue was then placed into 5 ml of Hank's Basic Salt Solution (HBSS) (Gibco-BRL). Upon completion of tissue collection, 100 μl of Trypsin-EDTA solution was added to dissociate tissue connection for 25 min at 37 °C. Subsequently, Trypsin inhibitor 125 μl (Sigma) and DNase I (10 μg/ml) (Sigma) were added and incubated for 5 min at 25 °C. The cells were spun down at 1000 rpm for 5 min, and the supernatant removed. The cells were resuspended by triturating 10 times using a 5 ml pipette with the same mixture as above. Tissue and cells were allowed to settle for 5 min and the supernatant containing the neurons was transferred to a new tube. These were then centrifuged at 1000 rpm for 5 min at room temperature. After removal of the supernatant, the cells were resuspended in serum-free Neurobasal Medium (Gibco-BRL) supplemented with B-27, N-2, glutamine (0.5 mM) and a combination of penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were plated on poly-d-lysine (100 μg/ml, Sigma) dishes and kept for 7 days in vitro (DIV) without replacing the culture medium in a humidified atmosphere of 95% air and

5% CO_2 at 37 °C. Cultures were grown for another 6 to 7 days, with one half of the medium being changed every 2 to 3 days, before being used in an experiment. For RNA isolation, cells were grown in 60 mm diameter culture dishes (2.5×10^6 cells/dish). In cultures stained with antibodies to GFAP (astrocyte marker) and MAP2 (neuronal marker) and DAPI (nuclear marker), 4.03 ± 1.9% of the cells were GFAP positive, 67.52 ± 7.13% were MAP2 and 28.45 ± 5.62% were unidentified ($N = 9$). At 12–14 DIV, all of the cells appeared healthy with no sign of crenated nuclei or axonal blebbing. ATP, suramin, thapsigargin, U0126, Bisindoylmaleimide IV, LY294002, pertussis toxin, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS) and tetrodotoxin were purchased from Sigma.

Total RNA Isolation - Primary cortical neurons were lysed in Trizol reagent (Invitrogen) and total RNA was extracted following the manufacturer's instructions. The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm, and the integrity was confirmed by RNA gel.

Reverse transcription and PCR - Total RNA (2 μg) from the primary cortical neurons was subjected to RT for 60 min at 42 °C using SuperScriptTM II (200 units) and First Strand Buffer (Invitrogen), in the presence of 0.5 μg/μl oligo dT, 10 mM DTT and 1 mM dNTP mix. Amplifications of cDNA (1 μl) obtained from RT were then performed in ThermoPol Buffer (NEB), containing 1 mM dNTP mix, in the presence of 10 μM of each specific primer and 2.5 units of Taq DNA polymerase (NEB). Control samples lacking reverse transcriptase were processed in parallel with the same experimental protocol. Amplifications were performed in an Eppendorf thermocycler (Brinkmann) and PCR

products obtained after 25–35 cycles (typically 94 °C for 1 min, 1 min at an annealing temperature ranging from 51–60 °C depending on the specific receptor subtype, 72 °C for 1 min), after an initial denaturation at 94 °C for 2 min. The sense and antisense primers used to detect P2 receptor mRNA in primary cortical neuronal cultures (Table 1) have been previously reported (Amadio et al., 2002 and Fumagalli et al., 2003). Each sample was run in triplicate. cDNA was also amplified with GAPDH primers (sense 5′-CAT GGC CTT CCG TGT TCC TAC CC-3′; antisense 5′-CCT CGG CCG CCT GCT TAC-3′) as a positive control. Five microliter aliquots of the PCR products were size-separated by electrophoresis on a 1–2% agarose gel.

Quantitative real-time PCR - Reverse transcription was performed as described above. Quantitative real-time PCR was performed using the SYBR Green I reagent. PCR reactions were carried out in a total volume of 25 µl containing 20.5 µl of Platinum PCR Supermix (Invitrogen), 1.25 µl of diluted first-strand cDNA (produced from 2 ng of total RNA), 1.25 µl of SYBR Green and 1 µl of forward and reverse primers each. The primers for Egr-1: sense 5′-AGC AGC GCC TTC AAT CCT CA-3′; antisense 5′-TCT CCA CCA TCG CCT TCT CA-3′ and the primer sequence for GAPDH: sense 5′-CAT GGC CTT CCG TGT TCC TAC CC-3′; antisense 5′-CCT CGG CCG CCT GCT TAC-3′ were used. All PCR reactions were performed in 0.1 ml tubes with caps (Corbett Research) in triplicates. Cycling conditions comprised an initial 3 min denaturing step at 95 °C, and cycling at 95 °C for 20 s, 57 °C for 20 s and 72 °C for 20 s. Forty cycles were run for amplification of templates. Data evaluation of real-time PCR experiments was performed by normalizing relative expression levels of target genes to the mean of relative

expression levels of the housekeeping gene GAPDH. For each primer pair, we performed a no template control which produced negligible signals.

Western blotting - Protein levels of Egr-1 were determined by Western blotting using a rabbit polyclonal antibody (Santa Cruz). Primary cortical neurons were grown on 60 mm plates coated with poly-d-Lysine (Sigma) and plated at a density of 2.5×10^6 cells/plate. To harvest the cells, plates were washed twice with cold PBS, and lysed in buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton-X, 1% NP-40, 0.5% Sodium-dideoxycholate, 0.1% SDS and 2 mM EDTA, pH 7.5), containing 10 mM NaF, 10 mM beta-glyc, 1 mM DTT, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin for 30 min on ice, vortexing every 5 min. Cell lysates were centrifuged at $10{,}000 \times g$ at 4 °C for 10 min. The protein concentration was determined by Bradford method (BioRad). Cell lysates containing 40 μg protein were added in equal volume of 2× reducing sample buffer (100 mM Tris–Cl pH 6.8, 200 mM dithiothretiol, 4% SDS, 20% glycerol and 0.2% Bromophenol blue) and heated at 100 °C for 5 min. The samples were electrophoresed on an 8% polyacrylamide gel under constant current (100 mV). Separated proteins were transferred onto a PDVF membrane (Perkin-Elmer). The blots were blocked with 5% milk in TBST (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C, washed three times with TBST and incubated (1–2 h) with horse radish peroxidase (HRP)-conjugated second antibody. The membranes were washed three times with TBST and the immunoblots were visualized on X-ray films after exposure to enhanced chemiluminescence reagent (ECL) (Amersham). Actin bands were monitored on the same blot to verify consistency of protein loading. Briefly, the immunoblots were

stripped with TBST containing Reblot Plus solution (Chemicon) for 20 min at room temperature. The blots were probed with anti-actin primary antibody and second antibody (anti-mouse) as described above. The molecular size of protein was determined by running prestained protein markers in an adjacent lane.

Statistics - Statistical significance was determined using the Student's *t* test of significance of difference of means. Error bars indicate standard deviation.

Chapter 3

Cortical spreading depression releases ATP into the extracellular space and purinergic receptor activation contributes to the induction of ischemic tolerance

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3.1 Author Contribution

Sarah C. Schock: Unless otherwise indicated all experiments were performed by the first author under the supervision of Dr. Hakim, Dr. Thompson and Dr. Sabourin. The manuscript was co-written by Dr. Thompson and with the first author.

Nzau Munyao: Finding for a portion of this stuy was provided by a grant issued to this author.

Yury Yakubchyk: All in vivo ATP recordings and CSD surgeries were performed by Yury Yakubchyk and Dr. Thompson.

Luc A. Sabourin: This author was the co-supervisor of the first author and contributed ideas and reagents to this paper.

Antoine M. Hakim: This author supervised the work on this paper, edited the manuscript and provided financial support.

Enrique C.G. Ventureyra: Provided funding for the ATP sensor used in this study.

Charlie S. Thompson: The manuscript was co-written by Dr. Charlie Thompson with the first author and this author performed all in vivo ATP recordings and CSD surgeries with Yury Yakubchyk.

3.2 Abstract

Cortical Spreading Depression (CSD) is a well-studied model of preconditioning that provides a high degree of tolerance to a subsequent ischemic event in the brain. The present study was undertaken in order to determine whether the release of ATP during CSD could contribute to the induction of ischemic tolerance. Direct measurement of ATP levels during CSD indicates that with each CSD wave ATP is released into the extracellular space at levels exceeding 100 μM. Cultures of rat primary cortical neurons exposed to low levels of extracellular ATP developed tolerance to subsequent oxygen-glucose deprivation (OGD) or metabolic hypoxia. The preconditioning effect requires new protein synthesis and develops with time, suggesting that a complex genomic response is required for the induction of tolerance. Multiple purinergic receptors are involved in mediating tolerance, with P2Y receptor activation having the greatest effect. Although extracellular adenosine or glutamate may make a small contribution, most of the tolerance was found to be induced independently of adenosine or glutamate receptor activation. Multiple signal transduction pathways mediate the response to extracellular ATP with the protein kinase A pathway and activation of phospholipase C contributing the most. The results are consistent with the proposal that CSD releases ATP into the extracellular space and the subsequent activation of P2Y receptors makes a major contribution to the induction of ischemic tolerance in the brain.

3.3 Introduction

Cortical spreading depression (CSD) was first described as a slowly propagating wave of suppressed electrical activity in rabbit cortex (Leao, 1944). Since then spreading depression (SD) has been characterized as a wave of depolarization of neurons and glia that may be elicited in virtually any grey matter in the nervous system (Sugaya et al., 1975 and Somjen 2001). Results of SD include a net influx of sodium, chloride and calcium, a large efflux of potassium and cell swelling. SD also imparts a substantial degree of tolerance to a subsequent ischemic insult to the brain. This was first shown in hippocampal CA1 neurons in rat brain by Kawahara et al. (1995) and other studies have confirmed this observation for cortical neurons (Kobayashi et al., 1995, Matsushima et al., 1996, Matsushima et al., 1998, Taga et al., 1997, Yanamoto et al., 1998, Yanamoto et al., 2000 and Otori et al., 2003). Ischemic tolerance develops with time (Kobayashi et al., 1995, Yanamoto et al., 1998 and Taga et al., 1997) and is transient (Yanamoto et al., 1998). In rat brain CSD may reduce cortical infarct volume following transient focal ischemia by up to 50% (Matsushima et al., 1996, Matsushima et al., 1998, Yanamoto et al., 1998 and Otori et al., 2003) and the protection is long lasting (Yanamoto et al., 2004).

ATP has been shown to act as extracellular signaling molecule in the brain (Zimmermann, 1994 and Fields and Stevens, 2000). It may be released from nerve terminals and act as a co-transmitter (Burnstock, 2004) or released from cells by way of ruptured cell membranes or pass through a variety of channels (Bao et al., 2004, Cortina et al., 1998, Okada et al., 2004 and Reigada and Mitchell, 2005). Two families of receptors for ATP and ADP have been identified. P2X receptors are ligand-gated cationic channels and P2Y receptors are G protein-coupled receptors, both families having a wide

distribution in the brain (Illes and Ribeiro, 2004). Extracellular ATP has been shown to mediate a range of effects including neurotransmission, modulation of the effects other neurotransmitters and growth factors, trophic actions and cytotoxicity (Di Virgilio, 2000, Amadio et al., 2002 and Volonte et al., 2003). Extracellular ATP has also been reported to induce the expression of a variety of genes (Tsim et al., 2003, Choi et al., 2003, Hanley et al., 2004, D'ambrosi et al., 2004, Priller et al., 1995 and McKee et al., 2006). Some of these genes are associated with enhanced cell survival and activation of P2Y receptors has been reported to suppress apoptosis and activate survival pathways in some tissues (Chrona et al., 2004, Tan et al., 2004 and Arthur et al., 2006).

These considerations led us to explore the potential role of extracellular ATP in mediating the increased resistance to ischemic damage imparted by CSD. Direct measurement of ATP levels during CSD shows that ATP is released during CSD waves. Exposure of cultured primary cortical neurons to low levels of extracellular ATP was found to induce a considerable tolerance to subsequent oxygen/glucose deprivation (OGD) and metabolic hypoxia that was not mediated by adenosine or glutamate receptor activation. These observations indicate that the release of ATP plays an important role in induction of ischemic tolerance in mammalian brain and may provide a therapeutic avenue aimed at neuroprotection.

3.4 Results

3.4.1 CSD releases ATP into the extracellular space

The response of the ATP sensor was linear in the range of 1 to 50 μM ATP (Fig. 1A). Simultaneous recording of extracellular ATP and the cortical DC potential in vivo

showed a spike in ATP concentration occurring at the same time as the negative extracellular potential of the CSD wave (Fig. 1B). This was observed in all rats tested ($N = 9$). The greatest concentrations of ATP recorded were in the range of 100 μM and the responses were up to 2 min in duration. When consecutive responses to CSD waves were recorded the second response was diminished in amplitude (Fig. 1C).

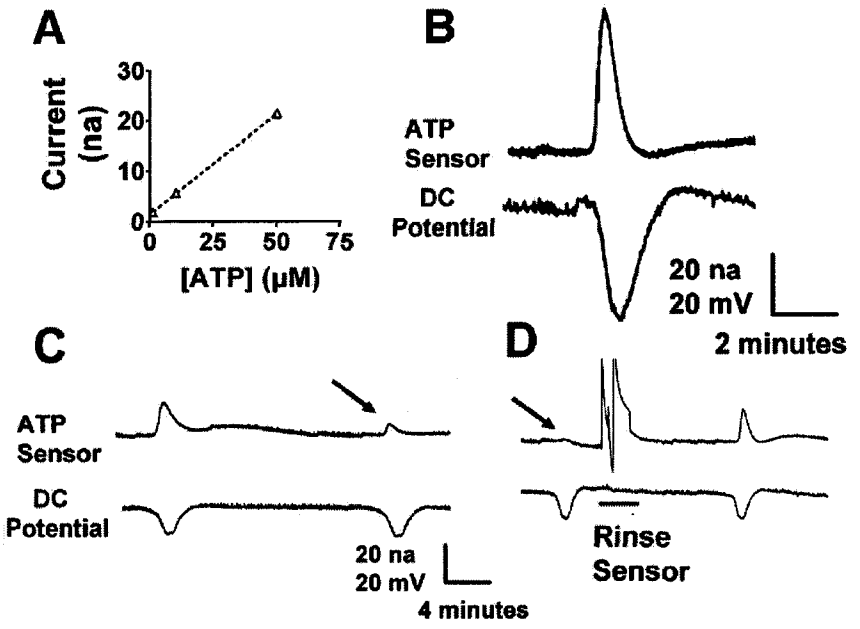

Figure 1. CSD waves release ATP into the extracellular space. (A) A standard curve relating the current response of the ATP sensor to ATP concentration made immediately before the recording shown in panels B. (B) A spike in extracellular ATP concentration, around 100 μMin amplitude and 99 s in duration, accompanies the negative extracellular DC potential. (C) When consecutive CSD waves are recorded the second wave is diminished in amplitude. (D) After several waves the ATP response is very small (arrow) but if the sensor is withdrawn and rinsed in buffer containing glycerol a larger response is recorded.

However, if the ATP sensor was withdrawn and rinsed in the layer of buffer containing glycerol the response increased in amplitude (Fig. 1D). To test the effect of cell damage caused by inserting the sensor into the cortex, a fresh ATP sensor was inserted into the cortex and responses of up to 1 mM ATP were recorded (not shown). This indicates that the sensor damages cells and releases ATP into the extracellular space.

The rise and fall in ATP concentration recorded during CSD is likely distorted by the relatively slow response time of the ATP sensor. When the sensor was dipped into an ATP solution, or removed and placed in buffer with no ATP, the time required to rise to 90% of the final current value or fall to 10% of the maximum current, respectively, was on the order of 20 s. Thus, the duration of the ATP response may be considerably shorter than that recorded.

3.4.2 Exposure of primary cortical neurons to extracellular ATP is neuroprotective

In order to assess the neuroprotective effects of exposure to extracellular ATP, cultures of rat primary cortical neurons were treated with 10 μM ATP for 15 min and then subjected to oxygen/glucose deprivation (OGD), camptothecin, potassium cyanide or hydrogen peroxide immediately, or up to 24 h later. Cultures were treated with ATP for 15 min and returned to normal medium for either 4 or 8 h. They were then exposed to OGD for 10 h and a significant protective effect was observed (Fig. 2B). For this experiment cell survival was assessed using a lactate dehydrogenase (LDH) assay, which measures LDH released from dead cells, rather than an MTT assay, which measures metabolism of the surviving cells, in order to demonstrate that the increase in cell survival was not due to cell proliferation. When cultures were exposed to the DNA damaging agent camptothecin (100 μM, 4 h) following ATP treatment there was also a significant protective effect 4 and 8 h after ATP treatment (Fig. 2C). ATP did not significantly protect against oxidative stress induced by H_2O_2 (Fig. 2D).

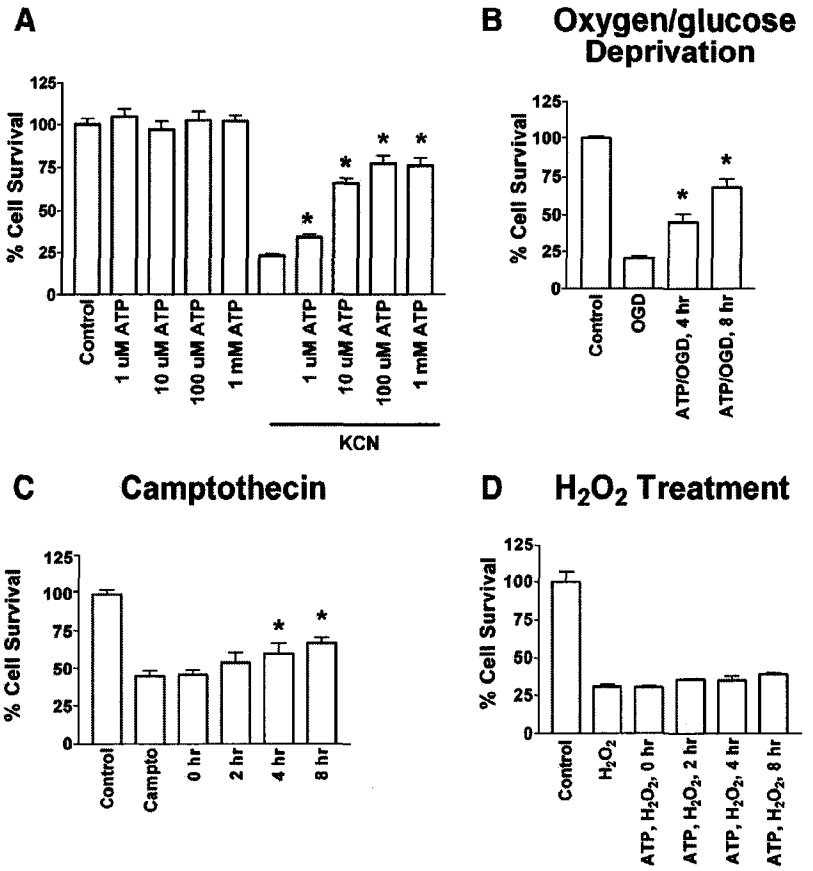

Figure 2. Exposure to extracellular ATP is neuroprotective in some models of cell death. (A) When cultures were exposed to extracellular ATP (1 μM to 1 mM for 15 min) and cell survival assessed 24 h later, there was no reduction in cell survival. When cultures were exposed to extracellular ATP for 15 min and returned to normal medium for 8 h and then exposed to KCN (1 mM for 4 h) there was a significant degree of protection for all concentrations tested when cell survival was assessed 24 h later. (B) ATP induces a considerable degree of tolerance to oxygen/glucose deprivation. Cultures were exposed to extracellular ATP (10 μM for 15 min) and returned to normal medium for 4 or 8 h and then exposed to OGD (6 h) there was a significant increase in cell survival, when determined 24 h after exposure to OGD. (C) ATP provides a somewhat less, but still significant, degree of tolerance to the topoisomerase inhibitor camptothesin. Cultures were exposed to extracellular ATP (10 μM for 15 min) and returned to normal medium for 8 h before exposure to camptothecin (100 μM, 4 h). Cell survival was determined 24 h after exposure to camptothecin. (D) In the same protocol as in panel C above ATP does not induce tolerance to free radical damage (H2O2, 10 μM, 30 min). N=4.

In order to further characterize the protective effect of extracellular ATP a model of metabolic hypoxia was chosen for reasons of convenience. Primary cortical cultures were exposed to 1 mM potassium cyanide (KCN) for 4 h and then returned to normal

medium. Cell death was then assessed 1, 3 or 7 days later. Exposure to extracellular ATP induced a degree of tolerance to metabolic hypoxia that was comparable to the tolerance induced to OGD. When cultures were treated with KCN immediately following exposure to ATP for 15 min, there was a significant protective effect. However, if cultures were returned to normal medium for a period of time before exposure to KCN the protective effect was maximal after 8 h (Fig. 3A). When cultures were pretreated with cycloheximide (10 μg/ml) for 1 h prior to and during ATP treatment the protective effect was lost, indicating that protein synthesis is required for the induction of tolerance (Fig. 3B). If cultures were treated with KCN and exposed to ATP (15 min) immediately afterwards, or 2 or 4 h later, there was a reduced but still significant protective effect (Fig. 3C). The protection from metabolic hypoxia was not permanent. When the degree of cell survival was assessed 3 days after exposure to ATP and then 8 h later to KCN there was reduced but still significant increase in cell survival. When cell survival was assessed 1 week later there was a statistically significant ($p = 0.0064$), but greatly diminished, difference between ATP treated and control cultures (Fig. 3D).

3.4.3 Purinergic receptors mediate the induction of tolerance

The activation of both glutamate receptors (Soriano et al., 2006) and adenosine receptors (Heurteaux et al., 1995) has been reported to induce cerebral preconditioning. In the present study ATP added to the extracellular medium could be degraded to adenosine by extracellular nucleotidases and the elevation of cytoplasmic calcium induced by exposure to extracellular ATP could cause the release of glutamate.

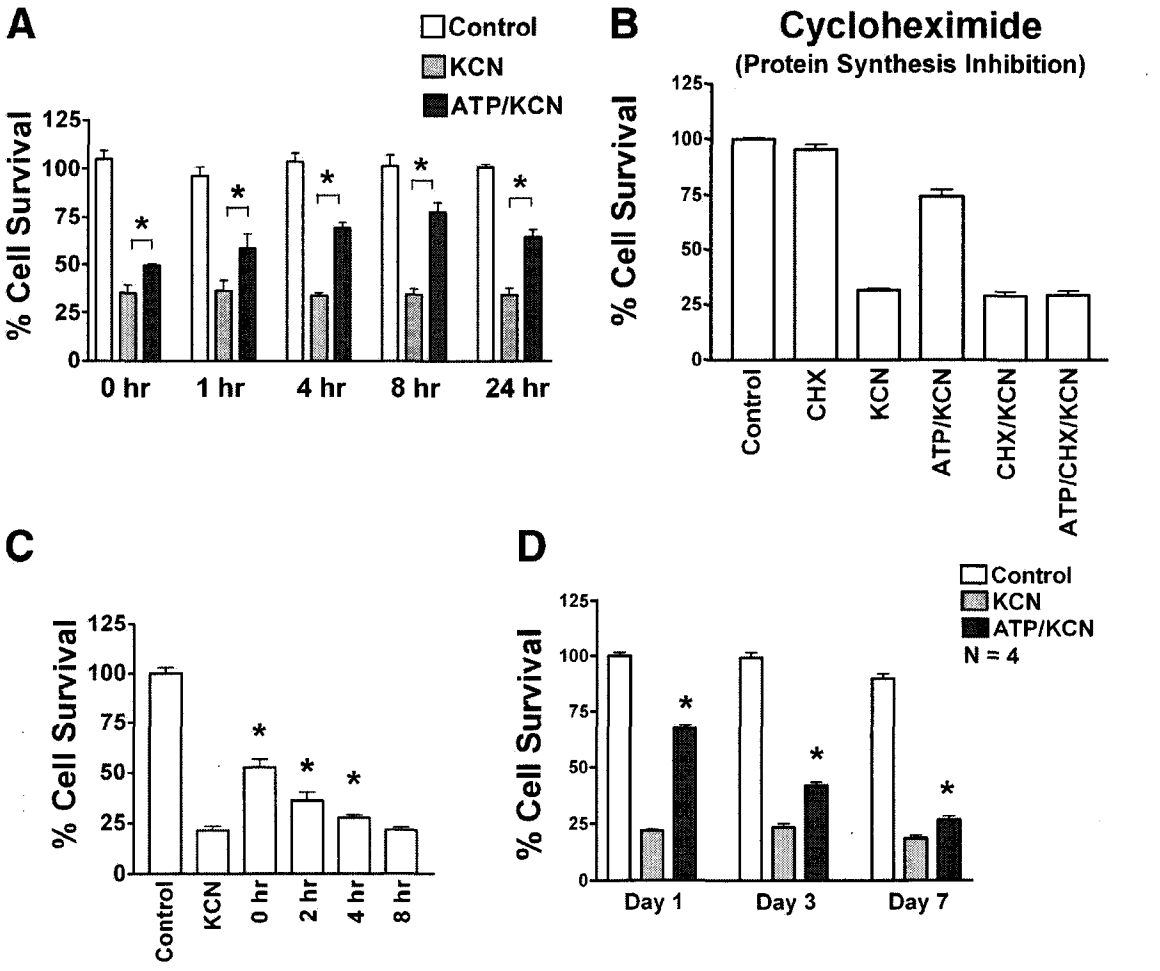

Figure 3. Characteristics of ATP induced neuroprotection. (A) Tolerance to metabolic hypoxia increases with time following exposure to ATP. Cultures were exposed to extracellular ATP (10 μMfor 15 min) and exposed to KCN (1mMfor 4 h) immediately or after 1 h, 4 h, 8 h or 24 h in normal medium. Cell death was assessed 24 h after exposure to KCN. (B) In the presence of cycloheximide the neuroprotective effect is lost. Cultures were pretreated with cycloheximide for 1 h and then exposed to ATP for 15 min in the presence of cycloheximide. Cultures were returned to normal medium for 8 h before exposure to KCN (1mM for 4 h) and cell survival assessed 24 h later. (C) Neuroprotection diminishes with time when cultures are treated with ATP following (immediately following or 2 h, 4 h or 8 h later) exposure to KCN (1mMfor 4 h). (D) The protection afforded by exposure to ATP is not permanent. Cultures were exposed to extracellular ATP (10 μMfor 15 min) and returned to normal medium for 8 h before exposure to KCN (1 mM for 4 h). Cell death was assessed 24 h, 3 days or 7 days after exposure to KCN. N=4.

In order to determine whether these processed could account for the observed degree of neuroprotection cultures were exposed to 10 μM, 100 μM or 1 mM glutamate for 15 min, and then, 8 h later, to KCN. A significant level of protection was observed for the 2 lower doses which was completely blocked by 100 μM MK-801 (Fig. 4A). When cultures were exposed to extracellular ATP in the presence of 100 μM MK-801 the percentage of cell survival was not significantly reduced (Fig. 4B). When cultures were exposed to 100 μM adenosine a small but significant protective effect did develop over the next few hours (Fig. 4C) but, even if all of the ATP added to the medium (10 μM) was immediately converted to adenosine, adenosine receptor activation could not account for the observed degree of neuroprotection.

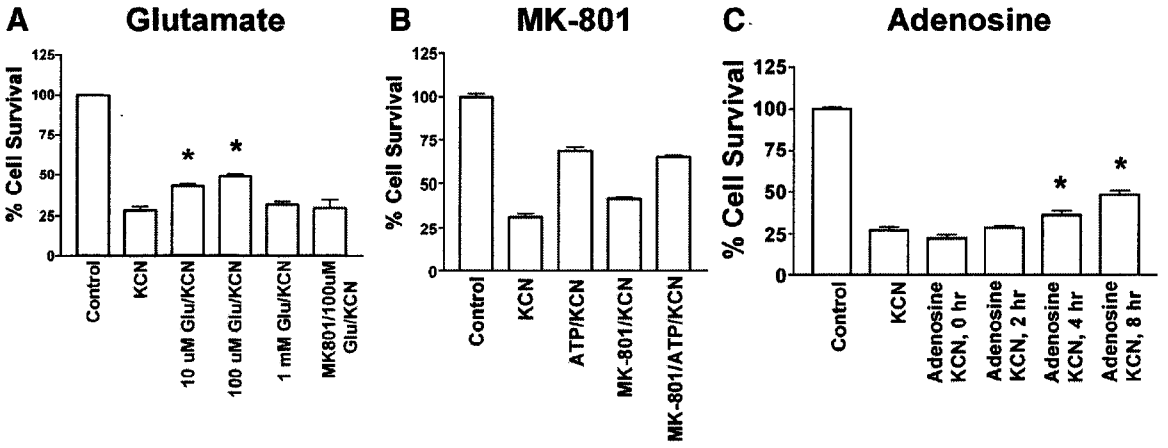

Figure 4. Glutamate or adenosine receptor activation does not account for the tolerance induced by exposure to extracellular ATP. (A) Exposure of cultured neurons to 10 μM or 100 μM glutamate for 15 min significantly increased cell survival when the cultures were exposed to KCN 8 h later. This effect was completely blocked by the NMDA receptor antagonist MK-801. (B) MK-801 does not block the increase in cell survival induced by exposure to extracellular ATP. Cultures were exposed to extracellular ATP (10 μM for 15 min) and returned to normal medium for 8 h before exposure to KCN (1 mM for 4 h). When ATP was applied in the presence of MK-801 (100 μM) there was not a significant decrease in cell survival, as determined 24 h later. (C) Exposure of cultured neurons to adenosine for induces a small but significant increase in cell survival. Cultures were exposed to extracellular adenosine (100 μM, 15 min) and returned to normal medium. Cultures were then exposed to KCN immediately or 2 h, 4 h or 8 h later. Cell survival was determined 24 h later. N=4.

In order to assess the contribution of purinergic receptor activation in the induction of tolerance to metabolic hypoxia, primary cortical cultures were treated with purinergic receptor antagonists for 1 h prior to and during exposure to extracellular ATP (10 μM) for 15 min. Eight hours later the cultures were exposed to 1 mM KCN for 4 h and the extent of cell survival was determined 24 h later. When cultures were treated with the general P2 receptor antagonist suramin (10 μM) there was reduction in cell survival from 71% to 46% (Fig. 5A). Suramin itself, however, provided a significant degree of protection in this model. When cultures were exposed to the P2X receptor antagonist PPADS there was a significant reduction in survival of about 11% (Fig. 5B), indicating that P2X receptor activation may be involved to some extent. When cultures were exposed to the P2Y receptor antagonist Reactive Blue 2 the protective effect of ATP was almost completely eliminated (Fig. 5C).

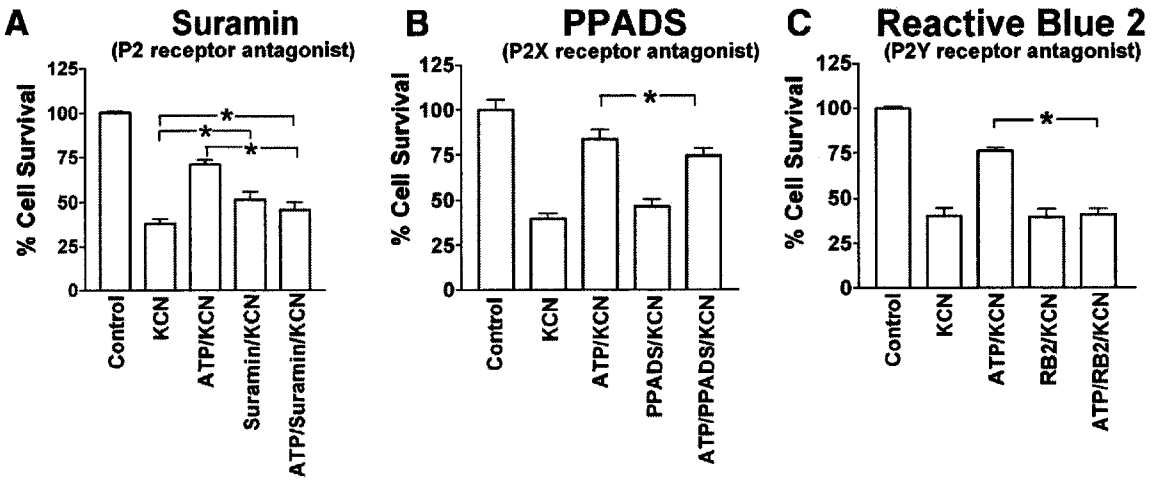

Figure 5. Induction of tolerance to metabolic hypoxia by exposure to extracellular ATP is mediated primarily by P2Y receptors. In panels A–C cultures were exposed to extracellular ATP (10 μM for 15 min) and returned to normal medium for 8 h before exposure to KCN (1 mM for 4 h). Cell survival was determined 24 h later. (A) Suramin blocks much of the protective effect of ATP. (B) PPADS causes a modest but significant ($p = 0.029$) reduction in the protective effect of ATP. (C) Reactive Blue 2 eliminates the protective effect of ATP. N=4. (D).

3.4.4 Signal transduction pathways

In order to identify signal transduction pathways that might be involved in the induction of tolerance to metabolic hypoxia, primary cortical cultures were treated with various kinase inhibitors for 1 h before and during exposure to extracellular ATP for 15 min. The cultures were then returned to normal medium for 8 h before exposure to 1 mM KCN for 4 h. The extent of cell survival was determined 24 h later.

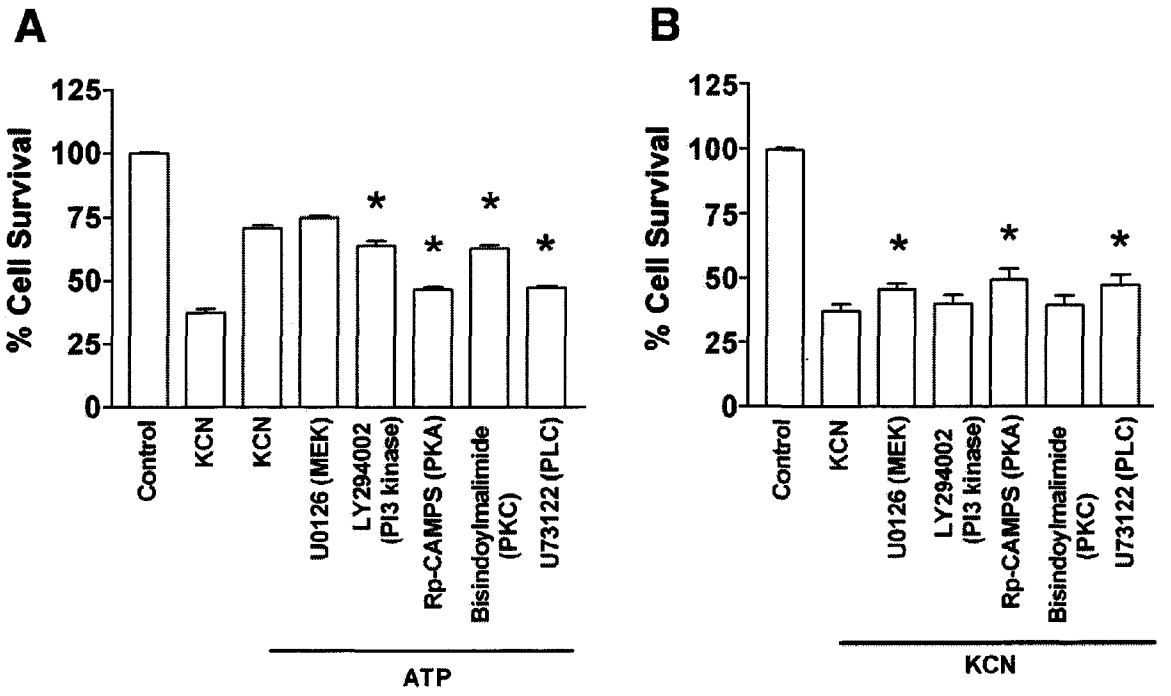

Figure 6. Signal transduction pathways mediating the induction of tolerance to metabolic hypoxia. (A) Cultures were exposed to extracellular ATP (10 μM for 15 min) alone, or in the presence of a signal transduction inhibitor, and returned to normal medium for 8 h before exposure to KCN (1 mM for 4 h). Cell survival was determined 24 h later. Inhibition of PI3 kinase or PKC significantly reduces tolerance induced by exposure to ATP while inhibition of PKA or PLC almost completely eliminates tolerance. A star indicates a statistically significant difference from ATP/KCN treatment alone. (B) Cultures were exposed to KCN (1 mM for 4 h) alone, or in the presence of an inhibitor. Inhibition of MEK, PKA or PLC during exposure to KCN with no ATP treatment provides a significant degree of protection. A star indicates a statistically significant difference from KCN treatment alone. $N = 4$.

Pretreatment of cultures with U0126 (10 μM, 30 min), an inhibitor of the phosphorylation of p44/42 mitogen activated protein kinases (ERK 1/2) by MEK, did not significantly affect cell survival (Fig. 6A). Inhibition of PI3 kinase or protein kinase C (PKC) moderately but significantly reduced cell survival while inhibition of protein kinase A (PKA) or phospholipase C (PLC) reduced cell survival from about 71% to about 47% (Fig. 6A). There was a moderate but significant increase in cell survival when cultures were treated with inhibitors of MEK, PKA and PLC before and during exposure to KCN alone (Fig. 6B).

3.5 Discussion

In the present study direct measurement of ATP concentration has demonstrated that ATP is released into the extracellular space during SD in rat cortex. It is likely that both glial cells and neurons release ATP during SD, as ATP has been shown to be released from astrocytes during the intercellular progression of cytoplasmic calcium waves ([Guthrie et al., 1999], [Stout et al., 2002] and [Coco et al., 2003]) and calcium waves accompany SD (Kunkler and Kraig, 1998). The exocytotic release of ATP from neurons during synaptic transmission is well established and ATP may also be released from dendrites and cell bodies in response to mechanical stress and other pathophysiological conditions (Fields and Burnstock, 2006). The method used to measure ATP concentration likely yields a lower value than is actually present and overestimates the duration of the ATP response. The ATP sensor requires glycerol to operate accurately and once inserted into the cortex glycerol is no longer available and gradually dissipates.

As the sensor is being inserted into the cortex it is exposed to high concentrations of ATP released from damaged cells, thus depleting glycerol from the enzyme matrix. Once released into the brain extracellular space ATP is rapidly degraded to adenosine by ecto-nucleotidases ([Zimmermann, 1996] and [Fields and Burnstock, 2006]). Indeed, the half-life for the conversion of ATP to adenosine in the extracellular space in the rat hippocampus is on the order of 200 msec (Dunwiddie et al., 1997). Thus, the time course of the pulse of ATP released by SD is likely much briefer than that indicated by the ATP sensor used here.

Extracellular adenosine has been shown to induce ischemic tolerance in a variety of in vivo and in vitro rodent model systems (Reshef et al., 2000). In mouse mixed primary cortical cell cultures adenosine protects against subsequent oxygen or glucose deprivation, but not exogenous glutamate, with an EC50 in the range of 100 μM (Goldberg et al., 1988). In this study exposure of cultures to 100 μM adenosine did induce a moderate but significant degree of tolerance to metabolic hypoxia, but 10 μM ATP produced a much greater effect. All of the experiments described here characterizing the induction of tolerance by ATP used a concentration of 10 μM. Thus, the contribution of adenosine receptor activation would be minimal in this model. Exposure of rat primary cortical cultures to 10 μM ATP provided a substantial degree of protection from subsequent OGD or metabolic hypoxia. A modest degree of tolerance to the topoisomerase inhibitor camptothesin was induced by this procedure but sensitivity to free radical damage (H_2O_2) was unaffected. The protective effect increases with time for at least 8 h following exposure to ATP and is blocked by cycloheximide. These observations indicate that protein synthesis is required and suggest that a complex

genomic response underlies the induction of the tolerant state. The effect of extracellular ATP is mediated primarily by P2Y receptors, with a small contribution from P2X and possibly P1 receptor activation. This is consistent with the results of other studies showing that P2 receptor activation protects from a variety of insults in several rodent in vitro models (Volonte et al., 1999, Chorna et al., 2004 and Arthur et al., 2006). We have previously reported that mRNAs encoding 6 of the 8 known P2Y receptors and 5 of the 7 known P2X receptors are present in the rat primary cortical cultures used in this study (McKee et al., 2006). Most of the known P2 receptors are expressed in rat nervous system in vivo as well (Norenberg and Illes, 2000 and Von Kugelen, 2006), so it is not possible to determine which P2 receptors are involved on the basis of the data presented here. Results of this study indicate that multiple signal transduction pathways are involved in determining the degree of tolerance to OGD and other stressors. In this study inhibition of the phosphorylation of ERK 1/2 by U0126 did not affect the induction of the tolerant state. This was unexpected in view of the demonstration that CSD causes an increase in the level of phosphorylated ERK 1/2 in rat cortical neurons and an increase in kinase activity (Chow et al., 2002). The activation of ERK 1/2 has been reported to be required for the inhibition of serum starvation-induced apoptosis by P2Y2 receptor activation in PC12 cells and dorsal root ganglion neurons in vitro (Arthur et al., 2006). This may reflect a difference between these cells and the primary cortical neurons used in this study. Jones and Bergeron (2004) found that hypoxic preconditioning (HI) of neonatal rat brain increased levels of phospho-ERK 1/2 in the brain and that inhibition by U0126 significantly reduced the neuroprotection afforded by HI. However, immunofluorescence staining showed that phospho-ERK 1/2 was found mainly in

microvessels and astrocytes in white matter tracts, neither of which are abundant in the cultures used in the present study. Also, Wick et al. (2002) have reported that HI increased levels of activated ERK 1 in cultures of cerebellar granule neurons and induced tolerance to subsequent potassium deprivation or exposure to glutamate or 3-nitroproprionic acid but blocking ERK activation with U0126 did not diminish the tolerance induced by HI. In this study the observation that U0126 significantly increases cell survival when cultures are exposed to KCN without prior exposure to ATP indicates that the state of activation of the ERK pathway does influence the degree of tolerance under resting conditions.

Inhibition of PI3 kinase or protein kinase C (PKC) prior to and during exposure to ATP moderately but significantly reduced cell survival following subsequent exposure to KCN. Several authors have reported that PI3 kinase is activated by ischemic preconditioning (IPC) in vivo and in turn activates protein kinase B (PKB, Akt). Inhibition of PI3 kinase by LY294002 or wortmannin during IPC reduces the activation of PBK and the protective effect of IPC (Yano et al., 2001, Hashiguchi et al., 2004 and Miao et al., 2005). Similar studies have implicated PKC in the induction of ischemic tolerance in vivo (Lange-Asschenfeldt et al., 2004) and in hippocampal organotypic cultures (Raval et al., 2003). Inhibition of PKA or of PLC during exposure of cultures to ATP almost completely eliminated the neuroprotective effects. Numerous studies have implicated the activation of PKA in the induction of ischemic tolerance in the heart (Sanada et al., 2004, Inserte et al., 2004 and Tong et al., 2005). It has been reported that preconditioning of rat primary cortical cultures by OGD activates PKA and that the PKA inhibitor H89 significantly reduces the neuroprotective effects of OGD preconditioning

(Meller et al., 2005). Inhibition of PLC during IPC or adenosine preconditioning in organotypic hippocampal slices has been reported to significantly attenuate the effects of preconditioning (Lange-Asschenfeldt et al., 2004). Activated PLC hydrolyzes the phosphatidylinositol PIP_2 into inositol triphosphate (IP_3) and diacylglycerol, an activator of PKC. Inhibition of PKC during ATP treatment moderately reduced the protective effect but inhibition of PLC resulted in a much larger reduction in cell survival, suggesting that IP3-mediated increases in cytoplasmic calcium ion levels is an important mediator of the induction of tolerance.

The results of this study support the idea that CSD causes the release of ATP into the extracellular space in the cortex and the subsequent activation of P2 purinergic receptors contributes to the induction of ischemic tolerance by CSD that is independent of adenosine receptor activation. Although P2Y receptor activation is the predominant mechanism in the in vitro model used here there is a small contribution from P2X and possibly adenosine receptor activation. When primary cortical cultures were treated with inhibitors of ERK 1/2 phosphorylation, PKA or PLC and then exposed to KCN without ATP treatment there was a significant increase in cell survival. This observation demonstrates that the degree of ischemic tolerance at any given time is dynamically determined by several signal transduction pathways. Extracellular ATP elicits a complex response in cortical neurons that requires new protein synthesis, develops with time and is mediated by multiple ligands, receptors and signal transduction pathways.

3.6 Experimental procedures

All surgical procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Induction and recording of CSD - Male Sprague–Dawley rats weighing between 175 and 200 grams were anesthetized with isoflurane (5% induction, 2% maintenance) in a 70:30 mixture of oxygen and nitrous oxide. The animals were mounted in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) and the skull exposed. A 2 mm diameter burr hole was drilled on the left side 3 mm anterior to and 3 mm laterally from bregma, without breaking the dura. A second 2 mm burr hole was drilled 4 mm posterior to and 4 mm laterally from bregma on the left side and a third 2 mm laterally from the second hole. CSD was elicited by placement of a cotton pledget soaked in 0.5 M KCl in the first hole directly in contact with the dura. A fresh pledget was reapplied every 15 min. In the second hole a chloridized silver wire recording electrode was inserted to a depth of 2 mm into the cortex and a reference electrode inserted through the skin at the back of the neck. CSD was recorded with a DC coupled amplifier and a chart recorder (Servogor 440, NGI Norma Goerz Instruments, Inc.). The ATP sensor, a null sensor and a chloridized silver wire reference electrode were inserted in the third hole to a depth of 2 mm.

Measurement of Extracellular ATP - Extracellular ATP was measured simultaneously with the electroencephalogram (EEG) in the cortex using an ATP sensor (*sarissa*probe-ATP, Sarissa Biomedical Ltd., Coventry, England) and a potentiometer (MicroC Carbon Fiber Potentiostat, World Precision Instruments, Inc., Sarasota, FL, USA) coupled to a chart recorder. The ATP sensor consists of a platinum wire, 50 μm in diameter, coated

with a matrix containing the enzymes glycerol kinase and glycerol-3-phosphate oxidase. Glycerol is phosphorylated and then oxidized to yield glycerone phosphate and H_2O_2. The concentration of ATP is then proportional to the electrical current generated when platinum catalyzes the decomposition of H_2O_2. A standard curve was made by immersing the sensor and reference electrode in a buffer solution containing different concentrations of ATP. The solution consisted of 2 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$ and 2 mM glycerol. When detecting extracellular ATP *in vivo* the surface of the cortex where the ATP sensor was inserted was covered with buffer solution.

Primary cortical neuron cultures - Pregnant Sprague–Dawley rats (Charles River Canada, St. Constant, QC, Canada) at E-15/16 were anesthetized with halothane and sacrificed by cervical dislocation. Fetuses were decapitated and the cortical region dissected out and collected under sterile conditions. The tissue was then placed into 5 ml of Hank's Basic Salt Solution (HBSS) (Gibco-BRL). Upon completion of tissue collection, 100 µl of Trypsin-EDTA solution was added to dissociate tissue connection for 25 min at 37°C. Subsequently, Trypsin inhibitor 125 µl (Sigma) and DNase I (10 µg/ml) (Sigma) were added and incubated for 5 min at 25°C. The cells were spun down at 1000 rpm for 5 min, and the supernatant removed. The cells were resuspended by triturating 10 times using a 5 ml pipette with the same mixture as above. Tissue and cells were allowed to settle for 5 min and the supernatant containing the neurons was transferred to a new tube. These were then centrifuged at 1000 rpm for 5 min at room temperature. After removal of the supernatant the cells were resuspended in serum-free Neurobasal Medium (Gibco-BRL) supplemented with B-27, N-2, glutamine (0.5 mM), a

combination of penicillin (Invitrogen, 100 IU/ml) and streptomycin (Invitrogen, 100 μg/ml) and the mitotic inhibitors 5-fluoro-2′-deoxyuridine (10 μM) and uridine (10 μM). The cells were plated on poly-d-lysine (100 μg/ml, Sigma) dishes and kept for 7 days *in vitro* (DIV) without replacing the culture medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were grown for another 6 to 7 days, with one half of the medium being changed every 2 to 3 days, before being used in an experiment. When cultures prepared in this manner were stained with antibodies to MAP2 (neuronal marker), GFAP (astrocytes marker) and DAPI (nuclear stain), 67.5 ± 7.1% of the cells were MAP2 positive, 4.0 ± 1.9% were GFAP positive and 28.5 ± 5.6% were unidentified ($N = 9$). ATP, glutamate and adenosine were purchased from Sigma.

Treatment of cultures - Four commonly used models of cell death were used to assess the effect of exposure to extracellular ATP on cell survival. Cultures of rat primary cortical neurons were treated with 10 μM ATP for 15 min and then returned to normal medium for up to 8 h. They were then exposed to H_2O_2 (10 μM, 30 min), oxygen glucose deprivation (OGD, 10 h), potassium cyanide (KCN, 1 mM, 4 h) or camptothecin (Sigma, 100 μM, 4 h) and returned to normal medium. Cell survival was determined 24 h later by MTT or LDH assay. OGD was carried out in a hypoxic glove box (Coy Laboratory Instruments, Inc., Grass Lake, Michigan). Metabolic hypoxia (KCN) was chosen as a model to further characterize the protective effects of extracellular ATP and identify receptors and signal transduction pathways involved. Unless otherwise stated, cultures were pretreated with the compound being tested for 1 h and then exposed to ATP (10 μM) in the presence of the compound for 15 min. Cultures were then returned to normal medium for 8 h before exposure to KCN (1 mM) for 1 h. Cultures were returned

to normal medium and the extent of cell survival determined 24 h later by MTT assay (Cell Proliferation Kit I (MTT), Roche Applied Science, Mannheim, Germany). The assay was performed using manufacturer's instructions. Briefly, 10 µl of MTT solution per 100 µl of media was added to the cells and incubated for 4 h at 37°C with 5%C02. Following this incubation, 100 µl of solubilization solution was added to each well. The plate was allowed to stand overnight in the incubator at 37°C with 5%C02. The following day plates were checked for complete solubilization of the purple formazan crystals and absorbance was measured using a spectraMax 340 microplate ELISA reader (Molecular Devices). The wavelength used to measure the absorbance of the product was 550 nm and the reference wavelength used was 690 nm. Cell injury was also quantitatively assessed in some cases by measurement of lactate dehydrogenase (LDH) activity released in the media 24 h after exposure to KCN. The LDH assay was performed using the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's protocol. Cytotoxicity was determined by measuring wavelength absorbance at 490 nm. Ischemia-induced LDH release was expressed as a percentage of experimental LDH release/maximal LDH release.

Cycloheximide, Reactive Blue 2, PPADS, suramin, U0126, Bisindoylmaleimide IV, LY294002, U73122 and Rp-adenosine 3′, 5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS) were purchased from Sigma.

Egr-1 is required for the induction of ischemic tolerance by extracellular ATP application

Using siRNA we knocked down the expression of the transcription factor Egr-1 in PC12 cells to determine if this gene was required for the full onset of protection by extracellular ATP application. RT-PCR experiments were performed to confirm the knockdown in Egr-1 expression (Appendix A, Figure 1). Cells were then exposed to 10 pMol Egr-1 siRNA for 24 hours prior to application of various concentrations of ATP. Cells were returned to normal media for 8 hours prior to treatment with 1mM KCN for 4 hours. Results show that cell survival induced by extracellular ATP is reduced in Egr-1 siRNA treated cells (Appendix A, Figure 2). This implicates a role for the induction of Egr-1 by extracellular ATP in the onset of ischemic tolerance.

Chapter 4

ATP release by way of connexin 36 hemichannels mediates ischemic tolerance in vitro

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4.1 Author Contribution

Sarah C. Schock: Unless otherwise indicated all experiments, were performed by the first author under the supervision of Dr. Antoine Hakim and Dr. Charlie Thompson. The manuscript was co-written by Dr. Charlie Thompson with the first author.

Danielle LeBlanc: This author produced all primary cortical cultures used for these studies.

Antoine M. Hakim: This author supervised the work on this paper, edited the manuscript and provided financial support.

Charlie S. Thompson: Work was done under the supervision of this author and the manuscript was co-written by Dr. Thompson with the first author.

4.2 Abstract

Spreading depression (SD) is a self-propagating wave of neuronal and glial depolarization that may occur in virtually any grey matter region in the brain. One consequence of SD is an increased tolerance to ischemia. It has been shown that during cortical SD ATP is released into the extracellular space and activation of purinergic receptors leads to the induction of ischemic tolerance. In the present study we show that depolarization of cultured neurons induces ischemic tolerance which is mediated by purinergic receptor activation. Depolarization causes the release of ATP into the extracellular medium, which may be prevented by treatment with the connexin hemichannel blockers flufenamic acid and quinine, but not the pannexin hemichannel blocker carbenoxolone. Knockdown of connexin 36 expression by siRNA greatly reduces the amount of ATP released during depolarization and the subsequent degree of ischemic tolerance. We conclude that during depolarization neurons release ATP by way of connexin 36 hemichannels.

4.3 Introduction

The resistance of the brain to ischemic injury may be transiently increased by exposure to a non-injurious preconditioning stimulus. It has been shown that ischemic tolerance reprograms the response to ischemia, leading to a protected state [1]. Cortical spreading depression (CSD) is a well studied phenomenon in the brain that induces a substantial degree of ischemic tolerance [2], [3], [4]. CSD is described as a slowly propagating wave of depolarized neurons and glia that causes depressed synaptic activity [5], [6], [7]. CSD results in transmembrane redistributions of K^+, glutamate, Ca^{2+}, Na^+ and Cl^-, which further lead to cell swelling and a reduction in the extracellular space. The induction of CSD has been shown to occur *in vivo* in the setting of epileptic seizures [8], hypoxia [9], traumatic brain injury [10], focal ischemia [11] and migraine [12]. Experimental induction of CSD is accomplished with the use of mechanical trauma, electrical stimulation, or the application of high potassium or a variety of other chemical agents [13].

In the brain ATP is known to act as a signaling molecule where it may influence cellular proliferation and differentiation [14], [15]. ATP also modulates the excitability of neurons [16] and is known to alter gene expression [17]. Two families of purinergic receptors, ligand gated cation channels (P2X) and G-protein coupled receptors (P2Y), mediate the effects of extracellular ATP. There are a variety of ways that ATP may be released in the brain including ruptured cell membranes, vesicular release from nerve terminals and passage through an assortment of channels including voltage-dependent anion channels [18], the cystic fibrosis transmembrane conductance regulator, CFTR

[19], P2X7 receptor-channels [20], connexin 43 hemichannels [21], [22], [23], connexin 32 hemichannels [24] and pannexin I hemichannels [25].

We have previously reported that during CSD ATP is released into the extracellular space and the activation of purinergic receptors contributes to the induction of ischemic tolerance [26]. As well, in cultured rat primary cortical neurons, exposure to extracellular ATP provides protection against potassium cyanide, a chemical form of hypoxia, and oxygen glucose deprivation (OGD).

Previous studies have implicated a role for connexins in various models of preconditioning and ischemic injury. Connexin 43 hemichannels have been implicated in the induction of ischemic tolerance in the heart [27] and Naus et al., [28] found that blocking gap junctions during exposure to glutamate in co-cultures of astrocytes and neurons resulted in increased neuronal injury. These authors also report that connexin 43 heterozygous null mice show a significantly larger infarct volume following focal ischemia in the brain. Connexin 36 forms a neuron specific channel in the brain. When these channels are blocked with various pharmacological agents such as quinine, quinidine and mefloquine the progression of CSD waves is inhibited [29]. In the present study, we show that potassium chloride (KCl) induced depolarization results in the release of ATP from cultured cells by way of connexin 36 hemichannels and leads to protection against ischemia *in vitro*. We also show that activation of the P2Y purinergic receptors as well as induction of the PKA and PLC signal transduction pathways are required for the induction of ischemic tolerance.

4.4 Materials and Methods

All surgical procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Primary cortical neuron cultures

Pregnant Sprague–Dawley rats (Charles River Canada, St. Constant, QC, Canada) at E-15/16 were anesthetized with halothane and sacrificed by cervical dislocation. Fetuses were decapitated and the cortical region dissected out and collected under sterile conditions. Primary cortical neurons were cultured as described previously [26]. The cells were plated on poly-d-lysine (100 μg/ml, Sigma) dishes in neurobasal medium (Invitrogen) containing N2 and B27 supplements (Invitrogen) and kept for 7 days *in vitro* (DIV) without replacing the culture medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were grown for another 6 to 7 days, with one half of the medium being changed every 2 to 3 days, before being used in an experiment.

Cerebellar granule neuron cultures

Cerebellar granule neurons (CGN) were isolated from 7- to 9-day-old mice, plated and cultured as described [30] on poly-d-lysine (20 μg/ml; PDL)-coated surfaces in neurobasal medium (Invitrogen) containing N2 and B27 supplements (Invitrogen). Cells were plated on poly-D-lysine coated plates at the same densities as stated above.

Treatment of cultures

Cultures of rat primary cortical neurons were treated with 100 mM KCl for 30 min and returned to normal medium for up to 8 h. They were then exposed to oxygen glucose deprivation (OGD, 8 h) or potassium cyanide (KCN, 1 mM, 4 h) and returned to normal medium. Cell survival was determined 24 h by LDH assay. OGD was carried out in a hypoxic glove box (Coy Laboratory Instruments, Inc., Grass Lake, Michigan). Metabolic hypoxia (KCN) was chosen as a model to further characterize the protective effects of KCl and identify the signal transduction pathways involved. Unless otherwise stated, cultures were pretreated with the compound being tested for 1 h and then exposed to KCl (100 mM) in the presence of the compound for 30 min. Cultures were then returned to normal medium for 8 h before exposure to KCN (1 mM) for 4 h. Cultures were returned to normal medium and the extent of cell survival determined 24 h later by measurement of lactate dehydrogenase (LDH) activity released in the media. The LDH assay was performed using the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's protocol. Cytotoxicity was determined by measuring wavelength absorbance at 490 nm. Ischemia-induced LDH release was expressed as a percentage of experimental LDH release/maximal LDH release.

ATP measurements

ATP release from cells was measured using the Adenosine 5'-triphosphate Bioluminescent Assay Kit (Sigma Aldrich) according to the manufacturer's instructions. Samples were read using the SpectraMax luminometer (Molecular Devices). A standard curve was performed prior to all experiments.

siRNA transfections

Connexin 36 siRNA was purchased from Santa Cruz Biotechnology containing 3 target specific sequences. 10 pmol of siRNAs were transfected into CGN cultures using Lipofectamine 2000 according to the manufacturer's instructions. Downregulation and quantitation of connexin 36 mRNA was evaluated 24 h post-transfection using RT-PCR as described previously [17] with the following primers: Cx36 sense (5'-TGTCAATGGGGTGCTCCAGA-3') and Cx36 antisense (5'-TCTGCCTTGGGGCTA CTTGC-3'). The primers for GAPDH were: GAPDH sense (5'- CATGGCCTTCCGTG TTCCTACCC-3') and GAPDH antisense (5'-CTTCGGCCGCCTGCTTCA-3').

Statistics

Statistical significance was determined using the Student's *t* test of significance of difference of means. Error bars indicate standard deviation.

4.5 Results and Discussion

Potassium chloride protects against in vitro models of ischemia

To determine the protective effects of depolarization in primary cortical neurons, cultures were exposed to various concentrations of potassium chloride (KCl) in normal medium. At the concentrations used KCl alone did not induce any cell death or proliferation (Fig. 1A). To precondition the cells, cultures were exposed to elevated KCl for 30 minutes and returned to normal medium for 8 hours [26]. Following this the cells were exposed to potassium cyanide (KCN) for 4 hours. All concentrations tested

provided protection against KCN, however 100 mM provided the greatest degree of protection and was the concentration used throughout the duration of this study (Fig. 1A).

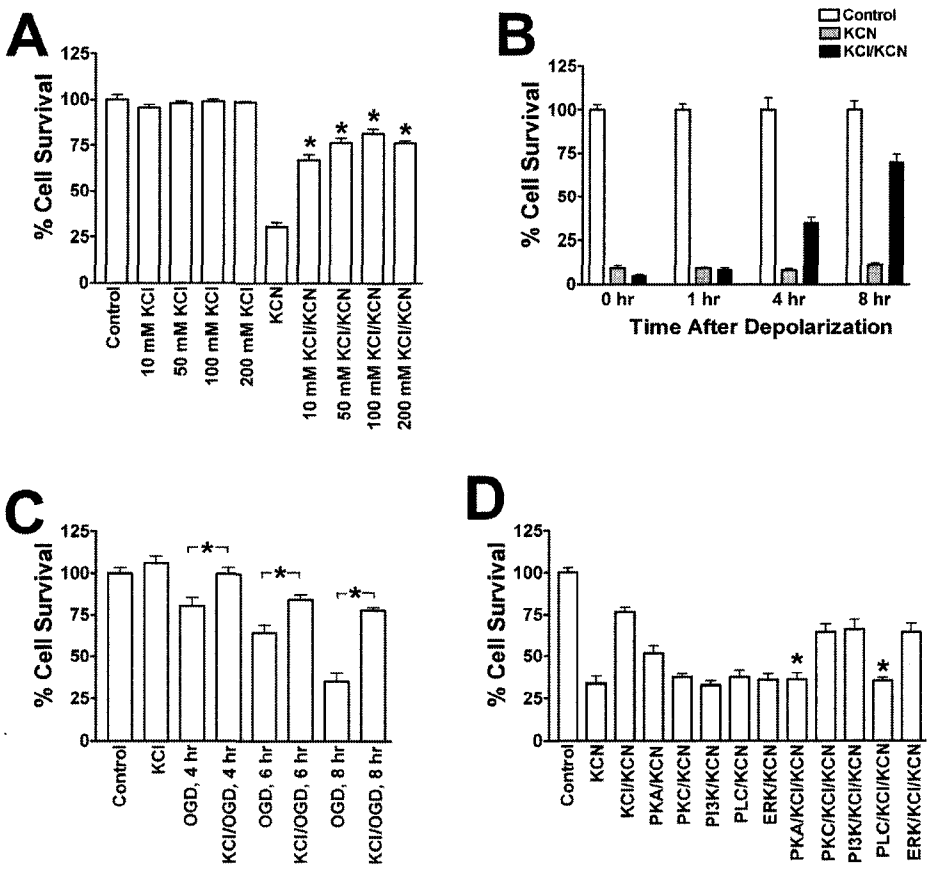

Figure 1. Induction of tolerance to *in vitro* forms of hypoxia by depolarization. A. Primary cortical cultures were exposed to elevated KCl for 30 min at the concentrations indicated. After 8 hours the cells were exposed to 1mM KCN for 4 h. Cell survival was determined 24 hours later. All concentrations of KCl provided significant protection against KCN with 100 mM being the most significant. B. Induction of tolerance requires time to develop, no protection was seen at 0 and 1 hr following pretreatment, however maximal protection was seen 8 hours following exposure to KCl. C. Exposure to 100mM KCl also provided protection against oxygen glucose deprivation. D. Signal transduction pathways mediating the induction of tolerance to metabolic hypoxia. Cultures were exposed to KCl (100 mM for 30 min) alone, or in the presence of a signal transduction inhibitor, and returned to normal medium for 8 h before exposure to KCN (1 mM for 4 h). Cell survival was determined 24 h later. Inhibition of PKA or PLC almost completely eliminates tolerance, whereas inhibition of the PI3 kinase, ERK or PKA pathways did not show a significant inhibition of protection. A star indicates a statistically significant difference from KCl/KCN treatment alone. Cultures were also exposed to KCN (1 mM for 4 h) alone, or in the presence of an inhibitor. N=4.

When cultures were treated with KCN immediately following exposure to KCl for 30 min, there was no significant protective effect. However, if cultures were returned to normal medium for a period of time before exposure to KCN the protective effect was maximal after 8 h (Fig. 1B). Cultures were also exposed to oxygen glucose deprivation for various times to further characterize the protection by KCl induced depolarization. Cells were pretreated with 100mM KCl for 30 min and returned to normal media for 8 h. Cells were then exposed to OGD for either 4, 6 or 8 h. A significant increase in cell survival was also observed in this model of ischemia for all OGD conditions with the greatest significance occurring at the 8 h OGD exposure (Fig. 1C). These results are comparable to that of KCN induced cell death and in subsequent experiments KCN was used to induce ischemic cell death for reasons of convenience.

The PLC and PKA signal transduction pathways play a role in the induction of ischemic tolerance by depolarization.

To identify signal transduction pathways that may be involved in the induction of tolerance by KCl induced depolarization, primary cortical neurons were treated with various kinase inhibitors for 1 h prior to and during exposure to 100 mM KCl for 30 min. The cultures were then returned to normal medium for 8 h before exposure to 1 mM KCN for 4 h. Cell survival was determined 24 h later. Inhibition of MEK 1/2, PKC and PI3K signal transduction pathways did not significantly affect cell survival (Fig. 1D). Inhibition of the PKA pathway with Rp-cAMP reduced cell survival from about 76% to about 35%. As well, inhibition of the PLC pathway with U73122 also caused a decrease in cell survival of 39% (Fig. 1D). These results implicate a role for both the PLC and

PKA pathways in the induction of tolerance by KCl induced depolarization. In previous experiments the PLC and PKA signal transduction pathways were also shown to be involved in the onset of protection against KCN induced cell death by application of extracellular ATP [26].

ATP is involved in the induction of ischemic tolerance by KCl depolarization through activation of P2Y purinergic receptors

To test the hypothesis that purinergic receptor activation is mediating the induction of tolerance by KCl depolarization, primary cortical cultures were treated with either suramin, a general P2 receptor antagonist, PPADS, a P2X receptor antagonist, or reactive blue 2 (RB2), a P2Y receptor antagonist, for 1 h prior to and during exposure to KCl. Cells were returned to normal media for 8 h and then exposed to 1mM KCN for 4 h and the extent of cell survival was assessed 24 h later. Suramin (10 uM) reduced cell survival from 80% to 39% (Fig. 2A). When cultures were exposed to PPADS, there was a significant reduction in survival of about 20%, indicating that P2X receptor activation is involved in KCl induced protection. RB2 caused the protective effect of KCl to be reduced 36% also indicating activation of these receptors during the induction of tolerance (Fig. 2A).

In order to determine the involvement of ATP in depolarization-induced tolerance, primary cortical neurons were pretreated with apyrase at various concentrations prior to and during application of 100mM KCl for 30 min. A dose dependent decrease in protection induced by exposure to KCl was observed with the most significant loss in protection corresponding to the highest concentration of apyrase

applied (Fig. 2B). Direct measurement of ATP release during KCl induced depolarization revealed levels of extracellular ATP approximately 7 fold higher than in control cultures. According to the standard curve performed, extracellular ATP levels in control cultures were in the range of 1-10 nM whereas KCl treated cultures had an extracellular ATP concentration in the range of 100 nM – 1 uM (Fig 3C), leading to an approximate release of 225 fmol per cell in a 24 well plate containing 200 uL of medium.

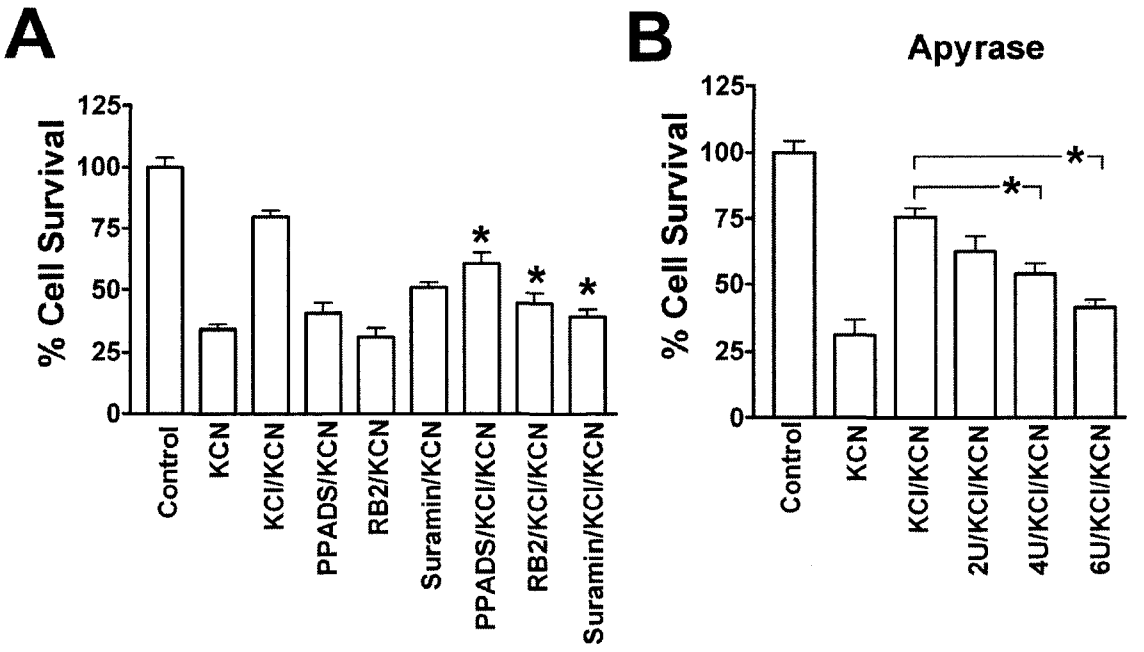

Figure 2. Protection afforded by KCl is in part due to the release of ATP and the activation of purinergic receptors. A. Pretreatment with purinergic receptor antagonists reduce the protection elicited by KCl. Suramin, a general purinergic receptor antagonist blocks the protection induced by KCl. The P2X antagonist, PPADS, slightly but significantly blocks protection whereas the P2Y receptor inhibitor, RB2, almost completely blocks protection. B. Application of apyrase, an enzyme that hydrolyzes ATP to AMP, 30 min before treatment with KCl reduced protection in a dose dependant manner. N=4.

Connexin channels release ATP during KCl induced depolarization

To determine how ATP is being released during KCl induced depolarization, primary cortical neurons were treated with the pannexin channel blocker, carbenoxolone (CBX), or the connexin channel blocker, flufluemic acid (FFA), 1 h prior to and during treatment with 100 mM KCl for 30 min. Cultures were returned to normal medium for 8 h before the addition of KCN for 4 h. Cell survival was assessed 24 h later. Exposure to CBX prior to and during depolarization results in a slight but significant reduction in cell survival. However exposure to FFA almost completely eliminates the protection elicited by KCl, from about 79% survival to 30% (Fig. 3A). As well, CBX exposure causes a slight but significant reduction in the release of ATP from KCl treated neurons, while FFA causes a reduction to control levels in the treated cultures (Fig. 3B). To determine the involvement of connexin hemichannels in ATP release cultured neurons were exposed to the connexin channel blocker quinine (Quin), which has been shown to specifically block Cx36 channels [31]. Exposure to Quin caused a 25% reduction in protection elicited by depolarization (Fig. 3C), as well as completely reducing the release of ATP to that of control levels (Fig 3B). A standard curve was performed in the presence of Quin and compared to the normal standard curve. Since Quin is also know to inhibit chloride channels, the chloride channel blocker 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS) was used to assess the role of these channels in the release of ATP by KCl induced depolarization. DIDS caused a slight but significant reduction in ATP release in KCl treated cultures (Fig. 3D). These results indicate that during depolarization ATP is being released into the extracellular space mainly via connexin

hemichannels. They also indicate that some ATP may pass through pannexin hemichannels and chloride channels.

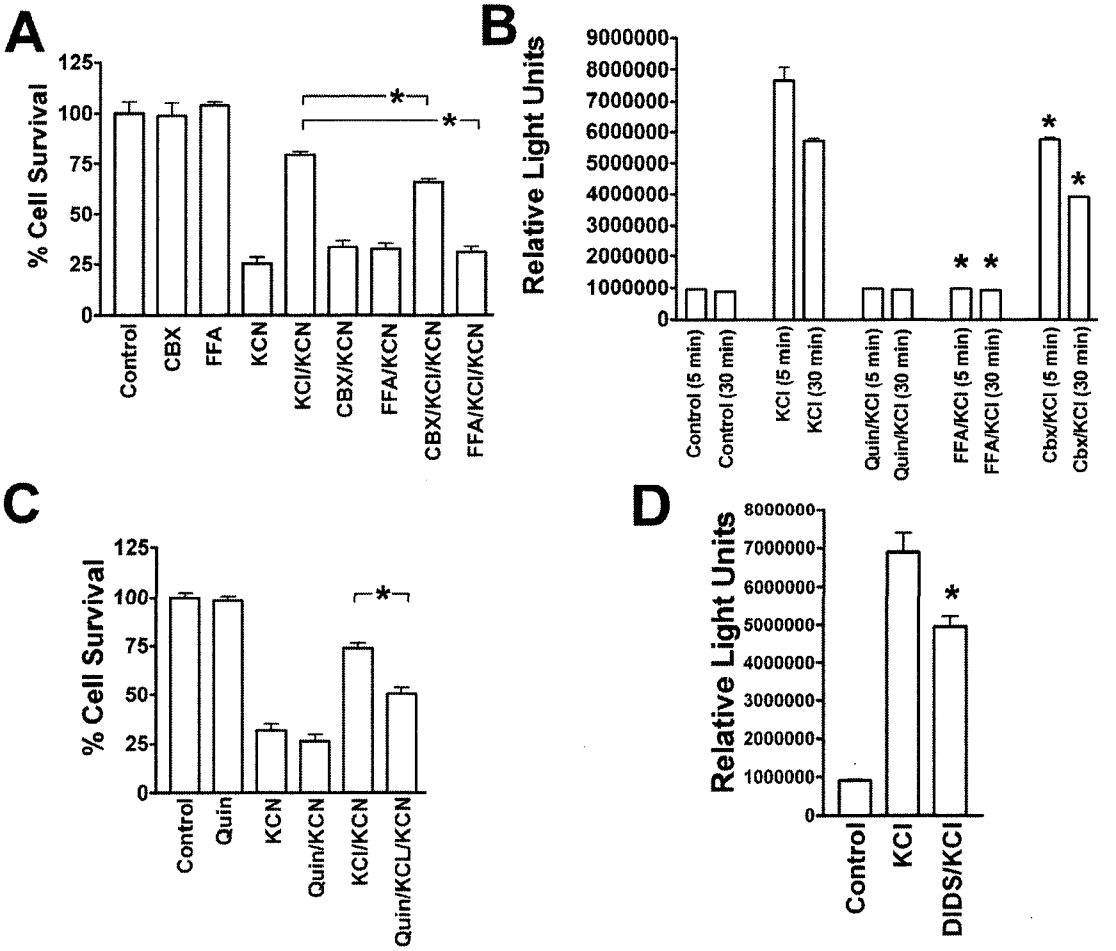

Figure 3. Exposure to KCl causes the release of ATP though connexin hemichannels. A. Primary cortical cultures were exposed to either CBX (50uM), an inhibitor of pannexin channels, or FFA (50uM), and inhibitor of connexin channels for 30 min prior to exposure to 100 mM KCl for 30 min. Cells were returned to normal media for 8 h before exposure to 1 mM KCN for 4 h. Results show that both FFA and CBX significantly block the protection elicited by KCl pretreatment, although FFA is much more effective. B. Direct measurement of extracellular ATP released by cortical neuron cultures in response to KCl induced depolarization. Results show that there is a 7 fold increase in relative light units (RLU) in KCl treated cultures compared to controls. This increase is blocked by FFA and quin and to a slight degree Cbx. C. Cells were pretreated with quinine (quin) (50 uM), an inhibitor of connexin 36 channels, and then treated as above. Results show that quinine significantly blocks the protection elicited by KCl. D. DIDS, a chloride channel inhibitor, also slightly but significantly reduced the RLU compared to KCl treated cultures alone. N=4.

Connexin 36 is the main channel releasing ATP during depolarization

To further determine a role for Cx36 in the release of ATP during depolarization we made use of Cx36 specific siRNA in CGN cultures. Expression of Cx36 mRNA was almost completely abolished following siRNA transfection, whereas no effect was observed in siRNA control treated cultures (Fig. 4A). In addition, a 25% reduction in survival was observed in the absence of Cx36 (Fig. 4B), suggesting a role for Cx36 in KCl-mediated neuroprotection in cultured cells. As well, in Cx36 reduced cells, there was a significant reduction in the release of ATP in response to KCl compared to control siRNA treated cultures (Fig. 4C). However, there is not a complete reduction in protection or in ATP release from the Cx36 siRNA transfected cells. This may be due to the fact that Cx36 expression was not completely eliminated by the siRNA method. Also, ATP may be able to pass through some other type of channel such as those formed by other connexins or pannexin I or a chloride channel.

Taken together our study suggests that depolarization of neurons induces a degree of tolerance against KCN insult or OGD *in vitro*. This tolerance state is due mainly to the release of ATP through Cx36 hemichannels and activation of purinergic receptors in an autocrine or paracrine manner. P2Y purinergic receptors are known to activate the PLC signal transduction pathway that produces IP_3 and causes the release of calcium from intracellular stores [32], and it was shown in this study that PLC as well as PKA pathway activation is required for the onset of protection induced by depolarization.

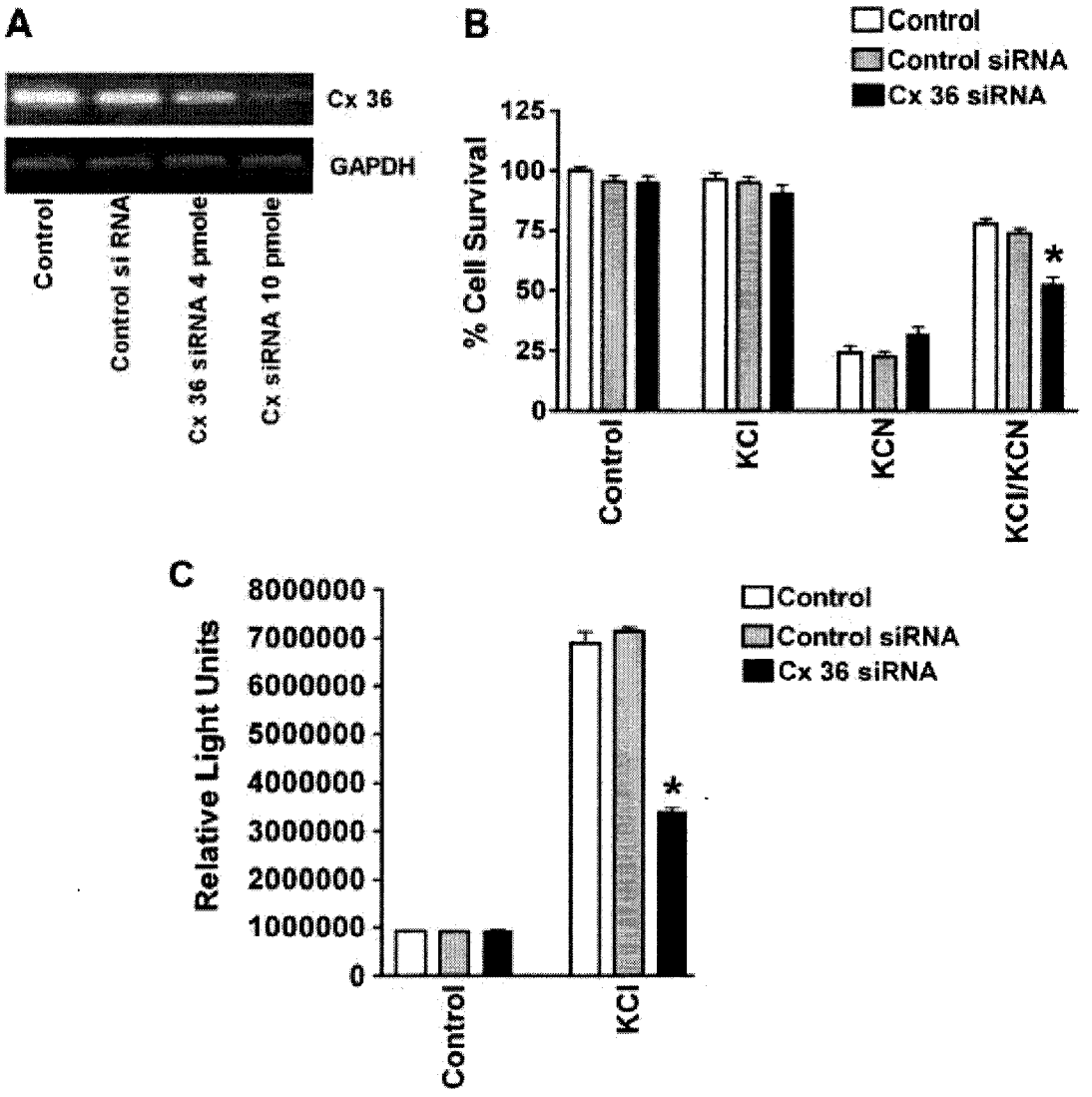

Figure 4. ATP is released through connexin 36 hemichannels in response to depolarization. A. RT-PCR showing the reduction in Cx36 mRNA in siRNA transfected CGN cultures. GAPDH was used as a loading control. B. Cell survival assay showing the reduction in protection in Cx36 siRNA transfected cells compared to control siRNA transfected cells. C. Luciferase measurement of extracellular ATP also showed that transfection of CGN cultures with Cx36 siRNA reduced ATP release in response to depolarization.

Chapter 5: General Discussion

In this series of individual studies we have examined the roles of extracellular ATP and KCl in the induction of protection through the activation of purinergic receptors, signal transduction pathways and the transcription factor Egr-1. Taken together, our results indicate that during depolarization neuronal cells release ATP into the extracellular space in part by way of connexin hemichannels and this ATP acts on P2Y purinergic receptors to activate signal transduction pathways such as the PLC and PKA pathways. These pathways are involved in upregulating the expression of Egr-1 mRNA which is dependent on the release of calcium from intracellular stores and is required for the onset of protection elicited by application of extracellular ATP. Egr-1 may serve to regulate the overall genomic response to preconditioning. Results of these studies have furthered our understanding of the molecular events that underlie the protective mechanisms of cortical spreading depression.

For many years the protective effects of CSD have been explored in order to gain a better understanding of the molecular mechanisms of preconditioning. In vitro studies have demonstrated the protective effects of depolarization both in a beta-amyloid model of neurotoxicity (Pike et al., 1996) as well as in sympathetic neurons (Crowder and Freeman, 1999). In the present study, it was revealed that KCl preconditioning also induces tolerance in rat primary cortical neuronal cultures exposed to either KCN or OGD. Previous studies have shown that the induction of protection requires both new RNA and protein synthesis (Marini and Paul, 1992; Bruno et al., 1997). The protection induced by KCl develops over time and is maximal eight hours following treatment with

KCl prior to the application of the ischemic insult and also requires new protein synthesis.

During CSD various ligands, such as glutamate and acetylcholine, are known to be released into the extracellular space (Basarsky et al., 1999; Reuter et al., 1998). In our study, it was shown that ATP is also released into the extracellular space both in vivo and in vitro in response to depolarization (Figure 1). Direct measurement of extracellular ATP levels was recorded both in vivo, with the use of an ATP sensor, and in vitro, with the use of a luciferase assay. Results for both suggest that during depolarization extracellular ATP concentrations significantly increase over controls. However, these recordings may actually be artificially lower due to the ectonucleotidases that are also present in the extracellular space and cause the breakdown of ATP (Wang and Guidotti, 1996; Zimmerman and Braun, 1999). Experiments, taking advantage of the enzyme apyrase to catalyze the hydrolysis of ATP to AMP, implicated a clear role for extracellular ATP in the onset of tolerance induced by KCl against various forms of ischemia in vitro. ATP has previously been shown to induce an increase in hyperoxia-induced oxidative stress (Ahmad et al., 2004) as well as play a role in preventing the collapse of mitochondrial membrane potential, and thus apoptotic cell death, against nitric oxide (NO) (Beltrán et al., 2000). However, since ATP may be rapidly degraded, some of this protection observed may be due to one of the breakdown products of ATP. Indeed, previous studies have implicated a role for both ADP and adenosine in the prevention of apoptosis in rat CGN cultures (Vitolo et al., 1998). As well, studies have also implicated cAMP as a critical element in neuroprotective signalling pathways (Silveira and Linden, 2006). Our studies also show that adenosine provides a degree of

protection for primary cortical neurons against KCN induced cell death; however it is to a much lesser degree than that of ATP. In addition, ADP causes an increase in Egr-1 8 fold above controls, comparable to that of ATP which causes an increase of 10 fold above controls. Glutamate, which is also released into the extracellular space during depolarization, also induced protection, but again to a smaller degree than that of ATP. Other studies have implicated a role for extracellular ATP in the onset of apoptosis; however this is at a higher concentration than used in our studies (Bulanova et al., 2005). Taken together these results indicate that during depolarization extracellular ATP as well as its breakdown products and other neurotransmitters contribute to the induction of ischemic tolerance.

Since cells may release ATP through a variety of channels we sought to determine which channel was the major conduit for ATP in response to depolarization. Results implicate a role for connexin hemichannels in the release of ATP, corresponding with other studies that have previously shown ATP is released by these channels, and more specifically Cx43 and Cx32 (De Vuyst et al., 2006; Pearson et al., 2005; Gomes et al., 2005; Cotrina et al., 1998; Eltzschig et al., 2006). As well, previous studies have suggested that there is a need for the release of ATP through connexin hemichannels in the propagation of calcium waves that accompany SD (Stout et al., 2002). However, our studies also show an involvement of chloride channels as well as the pannexin channels in the release of ATP in response to depolarization. In the Cx36 siRNA knock down experiments there is a reduction in the release of ATP from CGN cultures in response to depolarization as well as a significant reduction in the protection elicited by KCl against a KCN insult, suggesting that other connexins may also be involved in the release of ATP.

It is known that there are three major connexin proteins expressed in neurons, Cx36, Cx45 and Cx57 with Cx36 being the most abundant (Condorelli et al., 20003; Sohl et al., 2005). These results implicate the Cx36 hemichannels in the release of ATP and the onset of neuroprotection but do not rule out the involvement of other connexin hemichannels, pannexin hemichannels or even chloride channels.

Once ATP is released into the extracellular space it can activate the P2Y or P2X family of purinergic receptors, many of which are present both in cortical neurons as well as in the nervous system (McKee et al., 2006; Norenberg and Illes, 2000; Von Kugelgen, 2006). In our experiments both extracellular ATP and KCl induced protection required the activation of the P2 purinergic receptors, mainly the P2Y purinergic receptors and to a lesser degree the P2X purinergic receptors (Figure 1). This was similar to the upregulation of Egr-1 by extracellular ATP, which also required the activation of mainly the P2Y purinergic receptors. This result was confirmed in human 293 cells which showed upregulation of Egr-1 biosynthesis in cells overexpressing $P2X_1$ or $P2X_7$ as well as in cells endogenously expressing many of the P2Y receptors, including $P2Y_1$, $P2Y_2$, and $P2Y_4$ (Stefano et al., 2007). Previous studies have shown that the P2Y receptors and more specifically the $P2Y_2$ receptors activate neuroprotective mechanisms (Chorna et al., 2004) and inhibit apoptosis (Arthur et al., 2006), whereas the P2X receptors have been implicated in the onset of apoptosis (Solini et al., 2007; Kong et al., 2005). It has also been shown that connexin channels are involved in coordinating the propagation of intracellular calcium waves that are generated by P2Y receptor activation (Suadicani et al., 2004); these calcium waves are seen to accompany CSD.

Following activation of the P2Y purinergic receptors, signal transduction pathways are induced. With the use of various chemical inhibitors we uncovered a role for the induction of the PLC and PKA signal transduction pathways in the onset of tolerance by both extracellular ATP and depolarization (Figure 1). These pathways were also required for the increase in expression of Egr-1 by extracellular ATP. However, the upregulation of Egr-1 also required the activation of the PI3K and the PKC pathways although to a lesser degree than that of the PLC and PKA pathways. In all three cases the ERK1/2 pathway did not play a role in either the onset of protection or the upregulation of Egr-1 expression. Although ERK1/2 was shown to be phosphorylated during CSD (Chow et al., 2002), activation of this pathway has been implicated in the onset of reactive oxygen species (ROS)-induced cell death (Dong et al., 2004) and inhibition of this pathway causes a reduction in apoptosis caused by transient focal ischemia (Noshita et al., 2002). The PLC pathway has previously shown to be required for the onset of neuroprotection against hypoxia (Donohoe et al., 2001; Rogel et al., 2006) and the phosphorylation of PKA has also been shown to induce protection in CGN cultures (Wang et al., 2005) and reduce cell death in response to nitric oxide insult (Maiese et al., 2005). The PLC pathway has previously been shown as a downstream target of the P2Y purinergic receptors (Exton 1996) and produces inositol triphosphate (IP_3) which is involved in the release of calcium from intracellular stores, a requirement for the upregulation of Egr-1 expression induced by extracellular ATP. However the roles of the other signal transduction pathways studied in our experiments can not be ruled out since most of these pathways have also been previously implicated in the induction of ischemic tolerance; such as the PI3K, and the PKC signal transduction pathways and even other

studies which report the protective effects of the ERK1/2 pathway (Cavanaugh et al., 2006; Hetman et al., 2002; Zhang et al., 2007; Yu et al., 2004; D'Astous et al., 2006; Weinreb et al., 2004; Fujiki et al., 2006). One reason that these pathways may not have shown involvement is that only one concentration was used for each pathway inhibitor rather than a range. Therefore a higher dose may have been required to eliminate the function of these pathways. As well, only a limited number of signal transduction pathways were investigated and therefore activation of other pathways may also been required for the full induction of protection by extracellular ATP and KCl. Taken together our results implicate a role for the PKA and PLC signal transduction pathways in the induction of tolerance by extracellular ATP and KCl as well as for the increase in Egr-1 mRNA expression in response to extracellular ATP application.

CSD has also been shown to induce the expression of many genes that may play roles in coordinating the overall response to preconditioning (Choudhuri et al., 2002; Jander et al., 2001; Stenzel-Poore et al., 2007). One of the genes induced by CSD and other preconditioning stimuli is Egr-1 (Hanley et al., 2004; Jacobs et al., 1994; Carmel et al., Kawahara et al., 2004). Our experiments also showed that extracellular ATP increased the expression of Egr-1 and that Egr-1 is required for the onset of tolerance elicited by ATP application. The induction of Egr-1 by extracellular ATP was also previously shown in a HOBIT cell line through activation of the PKC signal transduction pathway (Romanello et al., 2003). However, in the Egr-1 siRNA knock down experiments, a complete reduction in protection was not seen, this may be due to other Egr family members compensating for the loss of Egr-1, since they were also seen to be upregulated by extracellular ATP. Another option is that extracellular ATP is

upregulating other transcription factors that also play roles in the induction of tolerance. This is known to be true for at least two transcription factors; NXF and LMO4 are both upregulated by extracellular ATP and are required for the full onset of protection elicited by pre-treatment with extracellular ATP (Chen et al., 2007; Hester et al., 2007). It has been previously suggested that Egr-1 may play a role in the coordinating the upregulation of various genes that underlie ischemic stress (Yan et al., 2000). In our studies Egr-1 may also be playing a similar role in upregulating protective genes and downregulating detrimental genes to induce the protection elicited by extracellular ATP or KCl. This was confirmed with the protein synthesis inhibitor cycloheximide, showing that in the absence of protein synthesis there is also an absence of protection. Inhibition of protein synthesis also caused an increase in Egr-1 mRNA levels in response to extracellular ATP application suggesting that ATP is also upregulating proteins which actively inhibit Egr-1 mRNA expression. Two such genes which are known to repress Egr-1 are NAB1 and NAB2; these genes have been shown to be induced by proliferative and differentiative stimuli (Svaren et al., 1996). Various target genes of Egr-1 have been studied, however, a limited number of genes have been identified to be upregulated by Egr-1 in models of preconditioning. Egr-1 is known to induce the expression of various anti-inflammatory- and anti-apoptotic genes as well as numerous growth factors. For example the anti-apoptotic genes Bcl2 and p35 are downstream targets of Egr-1 and may play a role in protecting the cells during an ischemic event. Egr-1 also upregulates serpine1, interleukin-1 and TNF-α (Mishra et al., 2006) all of which have been implicated in the onset of neuroprotection (Gustavsson et al., 2007; Carlson et al., 1999; Marchetti et al., 2004). As well, Egr-1 is known to induce the expression of BDNF which is involved in

the protection of cells against glutamate cytotoxicity. Future studies exploring other downstream target genes of Egr-1 in a preconditioning model would be beneficial, in hopes to further discover genes involved in the onset of neuroprotection.

All of these results taken together seem to suggest a pathway of activation for the induction of tolerance (Figure 1), implicating the release of ATP through connexin hemichannels in response to depolarization. This ATP acts on the P2Y purinergic receptors to activate the PLC and PKA signal transduction pathways. The PLC pathway is involved in the release of calcium from intracellular stores and the activation of both pathways is required for the induction of Egr-1 expression. Egr-1 may then cause the upregulation of protective proteins required for the onset of tolerance.

The signalling mechanisms leading to preconditioning are as of yet poorly understood but these experiments have given insight into the potential pathways and genes involved in the onset of protection. Parts of this pathway along with downstream targets of Egr-1 may have the potential for providing important pharmaceutical targets for the treatment of patients at risk for ischemic injury.

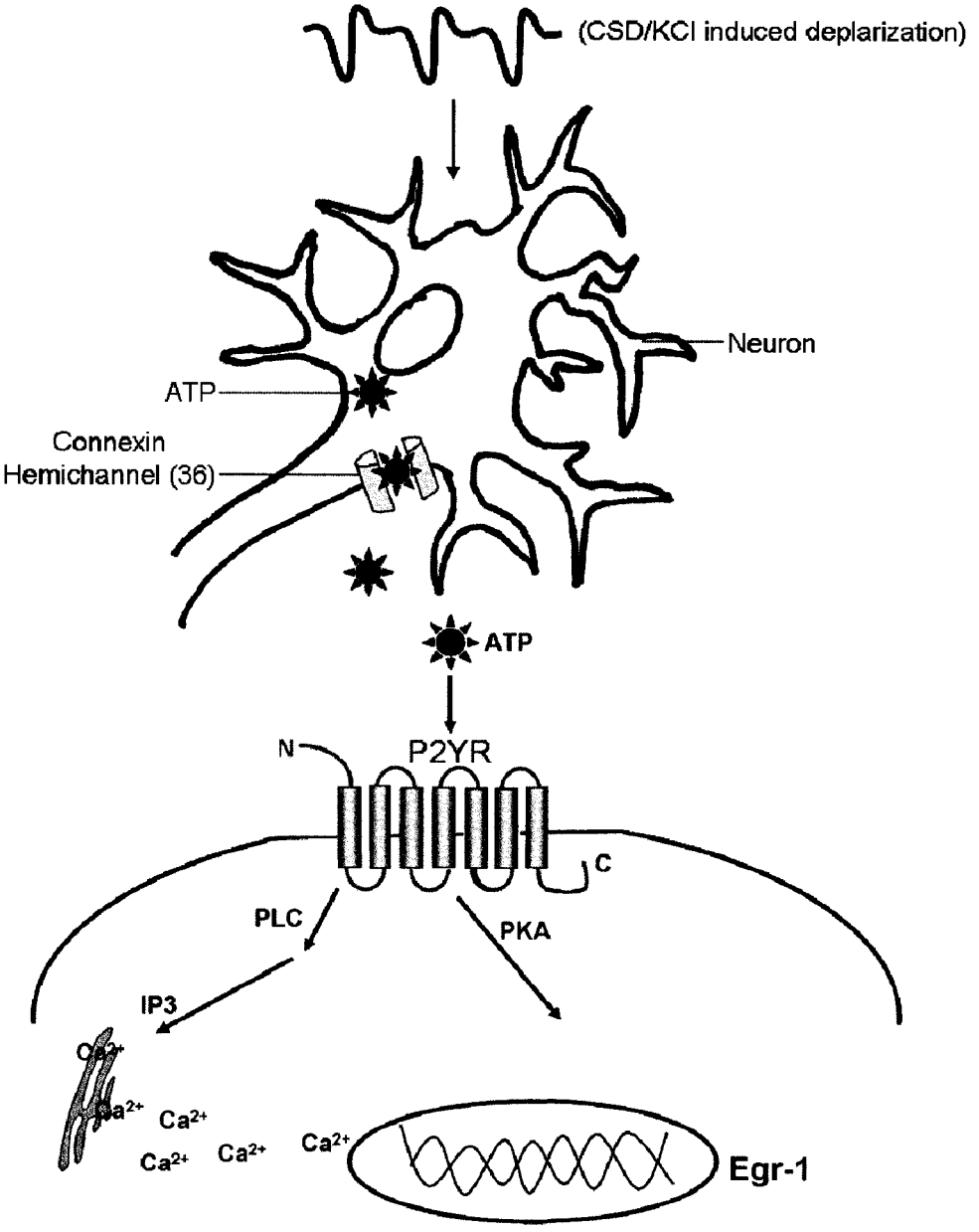

Figure 1. Schematic diagram of the pathway induced by depolarization caused by KCl application or CSD. Depolarization causes the release of ATP from neuronal cells by way of connexin hemichannels, specifically the connexin 36 channels. This ATP is acting on P2Y purinergic receptors to activate the PLC and PKA signal transduction pathways. The PLC pathway is involved in the production of IP_3 which induces the release of calcium from intracellular stores. The release of calcium as well as the activation of the PKA and PLC pathways is required for the upregulation of the transcription factor Egr-1.

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Appendix A: Additional figures from Chapter 3

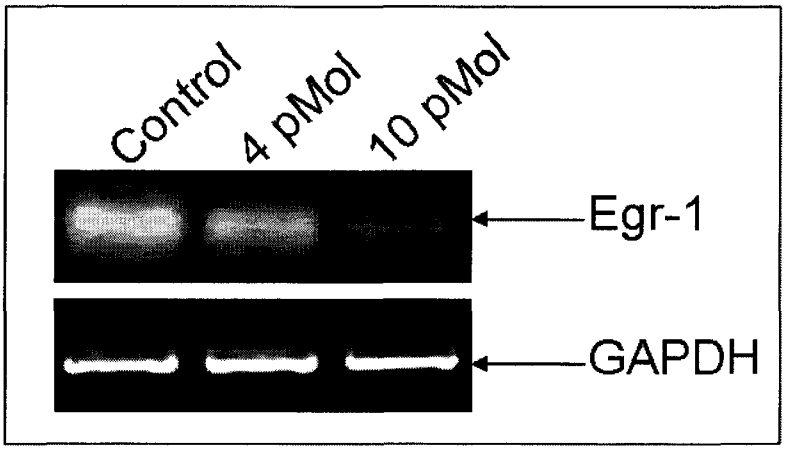

Figure 1. RT-PCR showing the reduction in Egr-1 mRNA with siRNA transfection. PC12 cells were transfected with either 4 or 10 pMol of siRNA and harvested 24 hours later. RNA was extracted and subjected to RT-PCR. GAPDH was used as a loading control.

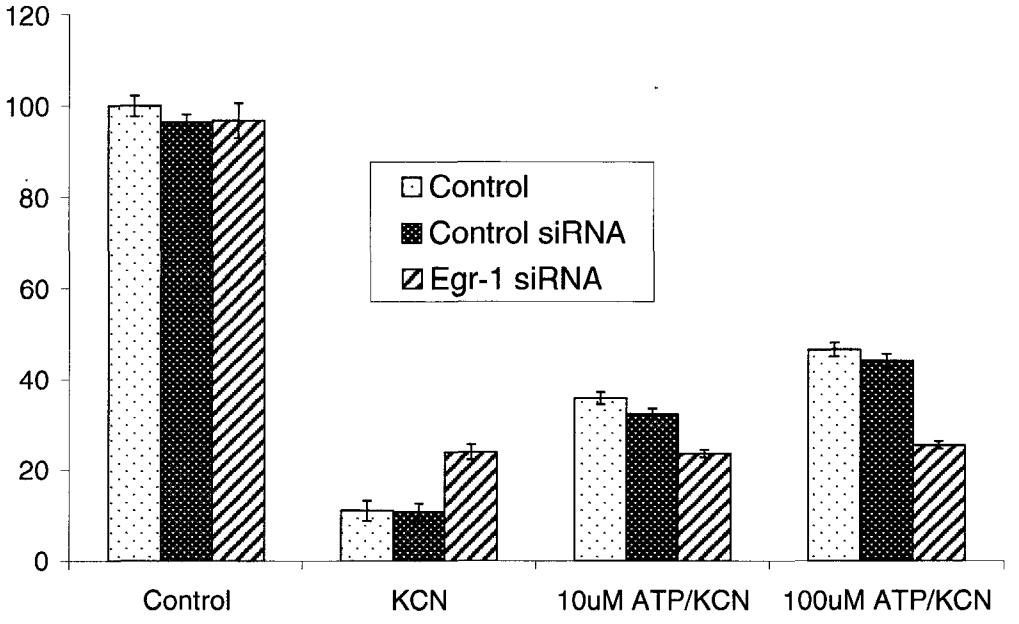

Figure 2. Cell survival assay showing the reduction in protection in Egr-1 siRNA knock-down cells exposed to extracellular ATP. PC12 cells were exposed to 10 pMol Egr-1 siRNA for 24 hours prior to treatment with ATP. Cells were returned to normal media for 8 hours prior to exposure to 1mM KCN for 4 hours. Cell survival was determines 24 hours later by LDH assay. N=4.

Appendix B: Publications

1. Chen, H. H., Schock, S. C., Xu, J., Safarpour, F., Thompson, C. S., and Stewart, A. F. (2007). Extracellular ATP-dependent upregulation of the transcription cofactor LMO4 promotes neuron survival from hypoxia. Exp Cell Res *313*, 3106-3116.

2. McKee, S., Hester, I., Pelletier, P., Thompson, C., Storbeck, C., Mears, A., Schulz, J. B., Hakim, A. A., and Sabourin, L. A. (2007). Transient expression of Nxf, a bHLH-PAS transactivator induced by neuronal preconditioning, confers neuroprotection in cultured cells. Brain Res *1135*, 1-11.

3. McKee, S. C., Thompson, C. S., Sabourin, L. A., and Hakim, A. M. (2006). Regulation of expression of early growth response transcription factors in rat primary cortical neurons by extracellular ATP. Brain Res *1088*, 1-11.

4. Schock, S. C., Munyao, N., Yakubchyk, Y., Sabourin, L. A., Hakim, A. M., Ventureyra, E. C., and Thompson, C. S. (2007). Cortical spreading depression releases ATP into the extracellular space and purinergic receptor activation contributes to the induction of ischemic tolerance. Brain Res *1168*, 129-138.

5. Schock, S.C., LeBlanc, D., Hakim, A.M., and Thompson, C.S. (2008). ATP release by way of connexin 36 hemichannels mediates ischemic tolerance in vitro. BBRC Vol. 328(1): 138-44.