CYCLOPENTYL-ADENOSINE DECREASES CASPASE-3 ACTIVITY AND LDH RELEASE FOLLOWING SIMULATED ISCHEMIA IN CEREBELLAR GRANULE NEURONS.

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ABSTRACT

Activation of adenosine A1 receptors *in vivo* or *in vitro,* prior to simulated ischemic insults have been demonstrated to be neuroprotective. Preconditioning with A1 receptor agonists, both *in vivo* and *in vitro,* has been shown to induce neuroprotective effects against necrosis even when administered 24-72 hours before ischemic insult. This thesis examined the neuroprotective effects of the selective A1 receptor agonist, N^6-cyclopentyl-adenosine (CPA), against both apoptotic and necrotic cell death following simulated ischemia in cultured rat cerebellar granule neurons. Using antibodies directed towards neuronal nuclear protein and glial fibrillary acid protein, the purity of cerebellar granule cell cultures was found to be 98.6 ± 0.4%. This indicates that the results from these experiments were likely mediated by effects of the drugs and simulated ischemia on neurons and not astrocytes. CPA (1μM) administered during incubation of cells with media deprived of oxygen and glucose (OGD) or for 1, 3 or 6 hours prior to OGD, resulted in significant reductions in caspase-3 activity and LDH release. A 3 hour incubation of CPA immediately prior to treating cells with OGD medium resulted in the greatest reduction in caspase-3 activity and LDH release (98.1 ± 1.5% and 80.2 ± 1.7% respectively). Additionally, administration of 1μM CPA decreased both caspase-3 activity and LDH release when administered for 3 hours and removed 0, 1, 3, 6 or 24 hours before treating cells with a simulated ischemic medium. Treatment with 1μM dipropyl-cyclopentyl-xanthine (DPCPX), a selective adenosine A1 receptor antagonist caused complete antagonism of CPA mediated neuroprotection. DPCPX alone increased both caspase-3 activity and LDH release at all times of pretreatment in cells exposed to control Krebs' solution. To determine if the neuroprotection induced by CPA treatment was mediated by activation of A1 receptors, a concentration response curve for DPCPX was generated in the presence and absence of 1μM CPA. Results indicated that DPCPX inhibited CPA mediated neuroprotection in a concentration-dependent manner. These data demonstrate that in cultured cerebellar granule neurons, CPA produces A1 receptor-mediated protection against increased caspase-3 activity and increased LDH release when administered 24 hours prior to simulated ischemia.

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NOTATION USED:

A1 - adenosine receptor subtype 1

A2 - adenosine receptor subtype 2

A3 - adenosine receptor subtype 3

ADA - adenosine deaminase

AMPA - α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid

ANOVA - analysis of variance

ATP - adenosine triphosphate

cAMP - cyclic-adenosine monophosphate

CAT - catalase

CGNs - cerebellar granule neurons

CHA - cyclohexyl-adenosine

COX2 - cyclo-oxygenase 2

CNS - central nervous system

CPA - N6-cyclopentyl-adenosine

DABCO - antioxidant anti-fading agent

DAG - diacylglycerol

DEVD - aspartate-glutamate-valine-aspartate

DNA - deoxyribonucleic acid

DPCPX - 8-dipropyl-1,3-cyclopentylxanthine

EC_{40} - concentration producing 40% of maximal effect

ER - endoplasmic reticulum

GABA - gamma-aminobutyric acid

GFAP - glial fibrillary acidic protein

GSHR - glutathione reductase

GSH-Px - reduced glutathione peroxidase

H_2O_2 - hydrogen peroxide

HS - horse serum

ICAM - intracellular cell adhesion molecules

IFκB - inhibitory factor kappa B

IL - interleukin

IP_3 - inositol-1,4,5- trisphosphate

K_{ATP} - ATP-dependent potassium channel

LDH - lactate dehydrogenase

MEM - minimal essential media

mGluR - metabotropic glutamate receptor

μM - micromolar

mM - millimolar

MnSOD - manganese superoxide dismutase

mRNA - messenger ribonucleic acid

NADH - reduced nicotidamine adenine dinucleotide

Na^+/ K^+ ATPase - ATP dependent sodium potassium pump

NeuN - neuronal nuclear protein

NFκB - nuclear factor kappa B

NMDA - N-methyl-D-aspartate

NO - nitric oxide

O_2° - superoxide anion

OGD - oxygen and glucose deprivation

OH° - hydroxy radical

$ONOO^{\circ}$ - peroxinitrite

PBS - phosphate buffered saline

PBS-HS-T - phosphate buffered saline containing 2% horse serum and 0.3% triton-X

PKC - protein kinase C

ρNA - ρ-nitroanilide

ROS - reactive oxygen species

R-PIA - N^6-(R)-phenylisopropyladenosine

SOD - superoxide dismutase

TNF-α - tumor necrosis factor-alpha

TNFR-1 - tumor necrosis factor receptor -1

VOCC - voltage operated calcium channel

VOSC - voltage operated sodium channel

1.0. General introduction

In Canada, the leading cause of hospitalization in women (other than childbirth) and in men is cardiovascular disease, including acute myocardial infarction, ischemic heart disease, congestive heart failure and stroke (Statistics Canada, 2000). Every year an average of 40 out of every 100,000 people suffer a stroke (Statistics Canada, 2000). As a result of stroke damage, 30% of patients die or have varying degrees of paralysis, loss of vision and disabilities in speech (Ter Horst and Postigo, 1997; Dirnagl *et al.*, 1999). Although mortality associated with stroke has decreased by 50% over the past 20 years, rates of stroke occurrence have not changed significantly in the last decade, suggesting that this trend has reached its plateau (Statistics Canada, 2000).

Stroke not only decreases quality of life for patients, it also places financial strain on the health care system. In Canada, average annual costs of the morbidity associated with stroke are estimated to be $4.9 billion for hospitalization, $1.6 billion for pharmaceuticals, $900 million for physicians and $60 million for research (Statistics Canada, 2000). In addition, the government or insurance companies must compensate patients and their families due to loss of employment and/or possible long-term home care. One approach to lowering both the emotional toll and financial cost of stroke to society is to develop pharmacological agents that effectively decrease morbidity and mortality associated with stroke. Development of these agents is dependent on further research into the biochemical and cellular mechanisms involved in stroke damage. This chapter reviews the literature on stroke-induced cell death,

and the role of adenosine in stroke.

1.1. Definition and mechanisms of stroke-induced cell death

A stroke, or cerebrovascular accident, occurs when there is a sudden decrease in blood flow to the brain. There are two types of strokes, namely, embolytic and hemorrhagic. Strokes are caused by the blockage of a cerebral artery (embolytic)(80%) or leakage of blood from these arteries (hemorrhagic)(20%)(Ter Horst and Postigo, 1997; Statistics Canada, 2000). The maintenance of cerebral blood flow is essential because the blood supplies neurons and glial cells with glucose and oxygen. The loss of these nutrients results in inhibition of oxidative phosphorylation, decreased pH due to loss of ion homeostasis, increased free radical production and an increase the number of depolarizations of neurons (Hossman, 1994b; Sweeney *et al.*, 1995; Choi, 1996; Juurlink and Sweeney, 1997; Martin *et al.*, 1998; Dirnagl *et al.*, 1999; Lipton, 1999).

The area of the brain most severely damaged by a stroke is termed the "core". Here, blood flow decreases to <15% of normal (Nedergaard *et al.*,1986; Duverger and MacKenzie, 1988). During ischemia, the core of the damage (infarct) undergoes anoxic depolarization which is defined as a sudden efflux of K^+ from a neuron. This efflux results in an increase in the concentration of extracellular K^+ and causes the depolarization of post-synaptic neurons (Kristian and Siesjo, 1997). Anoxic depolarization is responsible for the spreading of ischemic damage from the core to the penumbra, which is the area surrounding the core. The

spreading depression or damage is the result of action potentials in adjacent post-synaptic neurons which increase glutamate release causing further action potentials and consequent increases in intracellular Ca^{2+} (Chen *et al.*, 1993; Back *et al.*, 1996).

Ischemia in the core also affects the penumbra where a decrease in blood flow of up to <40% of normal has been observed (Hossmann, 1994a; Back *et al.*, 1996). Cell death in the penumbra occurs over a delayed period ranging from hours to weeks (Kirino *et al.*, 1984; Du *et al.*, 1996). The amount of delayed cell death and the size of the penumbra are determined by the severity of the ischemia and the length of time before reperfusion (Zhoa *et al.*, 1997). Injury also results from reperfusion due to the increased production of reactive oxygen species (ROS) and the decrease in mitochondrial function (Lipton, 1999). ROS are highly reactive molecules that contain an unpaired electron in the outer orbit. ROS will be discussed in further detail in section 1.1.2.

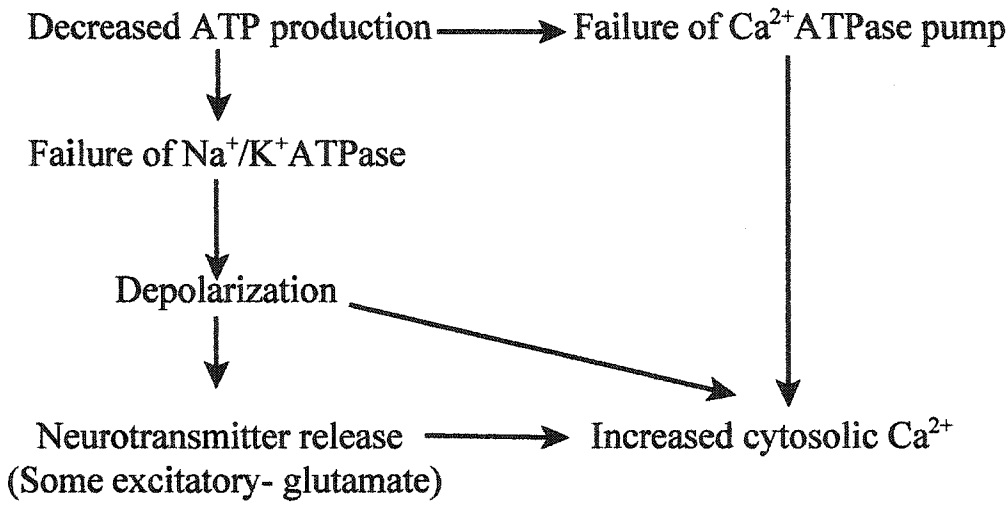
1.1.1. Hypoglycemia and hypoxia-induced decrease in ATP production

In the brain, cellular energy from adenosine triphosphate (ATP) is dependent on a constant supply of both glucose and O_2. Glucose is a metabolic precursor for pyruvate which is used in the formation of ATP, and O_2 is required as an electron acceptor for oxidative phosphorylation, therefore the loss of either O_2 or glucose results in a decrease in ATP production. The mechanism of ATP production is also disrupted in ischemia by ROS. Oxidants in the mitochondria cause the disruption of inner membranes, oxidation of proteins

involved in electron transport, and H^+ extrusion. Loss of the proton gradient, which is essential for oxidative phosphorylation, results in decreased ATP production (Picklo *et al.*, 1999). Intracellular concentrations of ATP in neurons drop to 25% of basal levels in the core of an infarct (Welsh *et al.*, 1991; Folbergova *et al.*, 1992), and to 50-70% of basal levels in the penumbra (Nedergaard *et al.*, 1986; Back *et al.*, 1996). Astrocytes are not as sensitive to ischemia as they have glycogen stores and can partially maintain ATP concentrations through anaerobic metabolism (Lipton, 1999). Other sources of ATP include the metabolism of amino acids, proteins and lipids.

The significant decrease in ATP concentration following ischemia causes decreased activity of many ATP-dependent processes in cells. Na^+/K^+ ATPase and Ca^{2+}ATPase which are responsible for maintaining ionic gradients in neurons following action potentials are examples of enzymes which are inhibited by a decrease in ATP concentration (Figure 1). Under physiological conditions, the Na^+/K^+ ATPase pumps 3 Na^+ molecules out of the neuron in exchange for the influx of 2 K^+ molecules. The pump is dependent on ATP and loss of ATP during ischemia inhibits the ability of the neurons to compensate for both the loss of K^+ through leak channels and the increased intracellular Na^+ concentrations that result from depolarization. The decreased functioning of the Na^+/K^+ ATPase contributes to the loss of membrane potential detected after ischemic injury (Figure 1). This is demonstrated by the increase in extracellular K^+ from 5mM to 60mM, and a decrease in extracellular Na^+ from 145mM to 50mM during the first 5 minutes following the onset of an ischemic insult (Lauritzen and Hansen, 1992; Kral *et al.*, 1993). The decrease in

Figure 1: Flow chart demonstrating mechanisms of hypoglycemia-induced increases in intracellular Ca^{2+}concentration in neurons (adapted from Lipton, 1999).



extracellular Na^+ concentrations leads to a doubling of intracellular concentrations following anoxia in rat hippocampal slices (Fried *et al.*, 1995). If the cell does not return to resting membrane potential, voltage operated Ca^{2+} channels (VOCC) and voltage operated Na^+ channels (VOSC) on neuronal membranes remain open. This allows further influx of Ca^{2+} and Na^+ and results in depolarization of the cell (Ter Horst and Postigo, 1997). Cl^- and Ca^{2+} ions also show decreased extracellular concentrations from 110mM and 1.2mM respectively to 0.1 mM (Lauritzen and Hansen, 1992; Kral *et al.*, 1993).

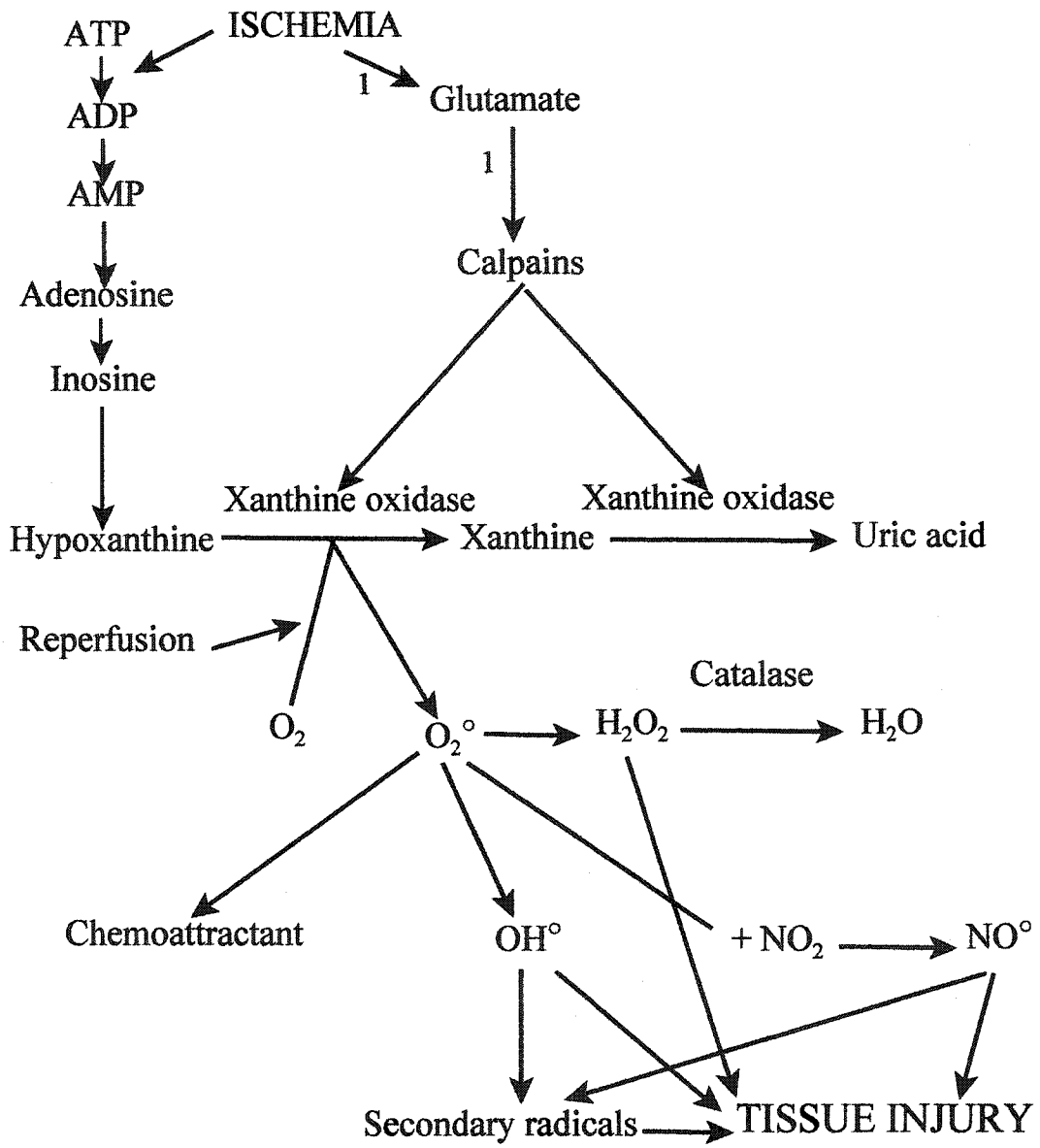
The concentration of intracellular Ca^{2+} is regulated by Ca^{2+}ATPase (Figure 1), a Ca^{2+} pump which requires phosphorylation by ATP to transport Ca^{2+} across the neuronal membrane. This mechanism would also be less active during ischemia, due to the decrease in ATP supply, and was shown to cause a sustained increase in cytosolic Ca^{2+} concentrations of up to 24μM in cortical neurons from mice exposed to either 50mM K^+ or 300μM glutamate (Hyrc *et al.*, 1997). In addition, the excess glutamate in the extracellular fluid would depolarize the neuron, open VOCCs and cause further Ca^{2+} influx. Under physiological conditions the Na^+/Ca^{2+} exchanger pumps 2 Ca^{2+} molecules out of the cells in exchange for the influx of 3 Na^+ molecules. However, under ischemic conditions, as cytosolic Na^+ concentrations increase, the exchanger is reversed, bringing in 2 Ca^{2+} ions and pumping out 3 Na^+ ions (Kristian and Siesjo, 1997)(Figure 1). The reversal of this mechanism increases the resting membrane potential and causes further depolarization. Further discussion of Ca^{2+}-induced cellular damage is described in section 1.1.3.

1.1.2 Hypoxia-induced increase in reactive oxygen species (ROS)

ROS are produced in cells during oxidative phosphorylation in the mitochondria and normally do little damage due to the presence of endogenous antioxidant pathways. The human body has many mechanisms to scavenge oxidative radicals, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px)(Mark *et al.*, 1998), thioredoxin peroxidase (Chae *et al.*, 1999) and phase 2 enzymes such as glutathione reductase (GSHR)(Thornalley, 1998) and glutathione S-transferases (Goon *et al.*, 1993). Oxidants cause increased permeability of the mitochondrial membrane (Marchetti *et al.*, 1996; Kroemer *et al.*, 1998), which allows Ca^{2+} release into the cytosol (Turrens *et al.*, 1991). This would add to the increase in cytosolic Ca^{2+} caused by a decrease in ATP production as mentioned above. ROS also cause damage to tissues by a number of other mechanisms including lipid oxidation and covalent binding in proteins. ROS-mediated tissue damage causes disruption of cellular and mitochondrial membranes and results in increased membrane rigidity, disruption of normal functioning and increased membrane permeability (Martin *et al.*, 1998; reviewed in Chakraborti *et al.*, 1999).

Under ischemic conditions, production of ROS is increased and scavenging mechanisms are unable to maintain equilibrium (Juurlink, 1999). An example of these are superoxide anions ($O_2^{-\circ}$), which are formed during oxidative phosphorylation (Matsuyama, 1997). During ischemia, superoxide anions can be converted to hydroxy radicals (OH°) or hydrogen peroxide (H_2O_2)(Figure 2). Ischemic neurons also produce cyclooxygenase 2 (COX2), which

Figure 2: Possible mechanisms of increased production of reactive oxygen species after ischemic injury. SOD - superoxide dismutase, NOS - nitric oxide synthase. (adapted from Sussman *et al.*, 1989; [1] Velazquez *et al.*, 1997).



facilitates the production of superoxides (Nogawa *et al.*, 1997). In addition, increased intracellular Ca^{2+}, associated with ischemia increases the activity of nitric oxide synthase which catalyzes the formation of nitric oxide (NO)(Palmer *et al.*, 1988)(Figure 2). Superoxide anions also interact with NO in mitochondria resulting in formation of peroxynitrite ($ONOO^{-}$)(Lipton, 1999).

It has been demonstrated that the concentration of ROS generated in the core of an ischemic infarct is significantly less than in the penumbra. Solenski *et al.* (1997) found a 50% higher concentration of ROS in the penumbra compared to the core. Two hours after 3-vessel occlusion in the rat, the concentration of ROS in the core was not different from sham but ROS in the penumbra was significantly higher (Solenski *et al.*, 1997).

During reperfusion, in the presence of O_2, the ATP metabolite hypoxanthine is converted to xanthine by the enzyme xanthine oxidase (Figure 2)(Sussman *et al.*, 1989). The reaction byproduct is superoxide. Superoxide can play many roles in reperfusion injury, including direct roles as both a chemoattractant for immune cells and a cause of tissue damage (Sussman *et al.*, 1989). In the presence of NO and nitric oxide synthase, superoxide can be converted to peroxynitrite. Superoxide can also be metabolized into two hydroxy radicals (Steinbeck *et al.*, 1989) or hydrogen peroxide by SOD (Friedovich, 1978; Deby and Goutier, 1990). Hydrogen peroxide can then be inactivated by CAT or GSH-Px to form H_2O (Matsuyama, 1997)(Figure 2).

Ischemic injury leads to a sustained increase in ROS in affected tissue, which can cause damage to neurons through at least five pathways including: immune cell-mediated, increased intracellular Ca^{2+} concentration, deoxyribonucleic acid (DNA) damage, protein covalent binding and mitochondrial damage. All of these pathways can lead to either apoptosis or necrosis (Tan *et al.*, 1998). An increase in ROS also causes an upregulation of pro-inflammatory genes such as: tumor necrosis factor-α (TNF-α)(Wallach, 1997), interleukin-1 (IL-1)(Wallach, 1997), intracellular cell adhesion molecule-1 (ICAM-1)(Kato *et al.*, 1996), P-selectins and E-selectins (Haring *et al.*, 1996). By increasing ICAM, the permeability of the blood-brain barrier is increased and allows the influx of immune cells which can cause further damage (Clark and Zivin, 1997).

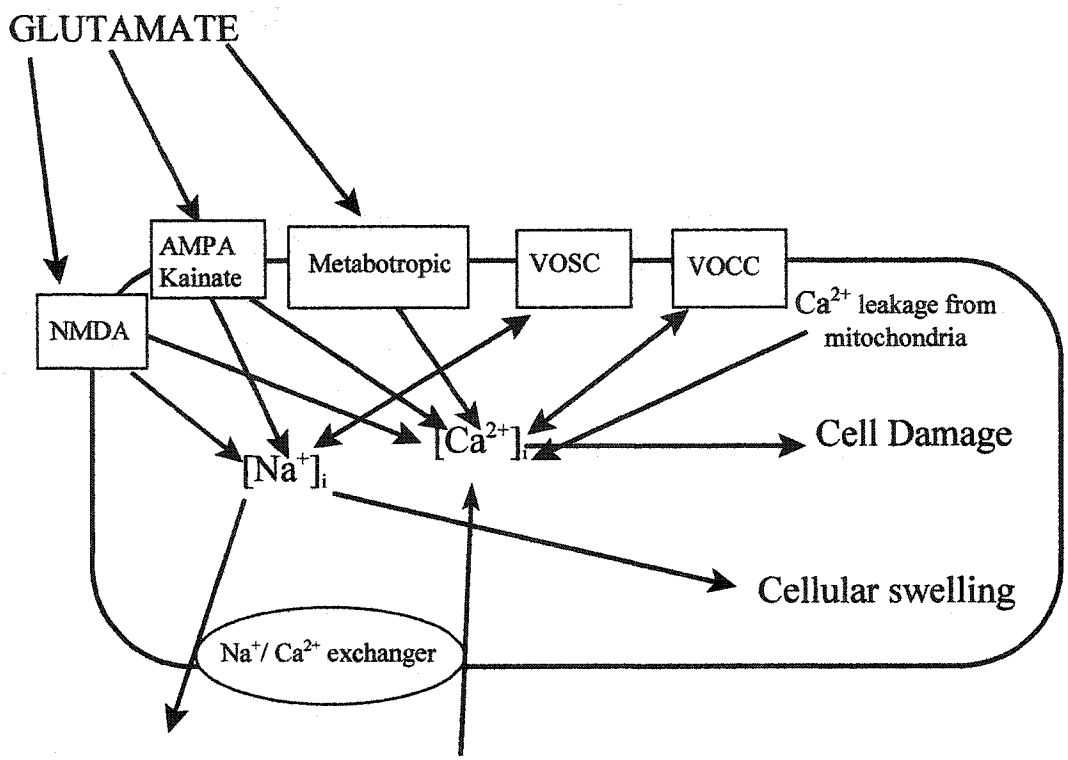
1.1.3. Contribution of Ca^{2+} and glutamate to excitotoxicity

There are certain areas of the brain (*eg.* hippocampus) which are more susceptible to ischemia than others (Kirino *et al.*, 1984). This phenomenon is thought to be a result of varying concentrations of glutamate receptors between regions of the brain. Glutamate is an excitatory neurotransmitter and the key mediator of excitotoxic cell death (Gill and Lodge, 1997). Extraneuronal glutamate concentrations, normally 1-3μM, can rise to 50-90μM in the core and 30-50μM in the penumbra during ischemia in rats (Wahl *et al.*, 1994; Baker *et al.*, 1995). *In vivo*, extracellular hippocampal concentrations of glutamate have been shown to return to basal levels 5-20 minutes following focal ischemia in the gerbil (Miyashita *et al.*, 1992).

Activation of ionotropic glutamate receptors such as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) or kainate cause Na^+ and/or Ca^{2+} influx (Cooper *et al.*, 1977)(Figure 3). An influx of cations results in changes to the resting membrane potential of the neuron and opens voltage operated ion channels, which allows further cation influx and depolarization of the cell. Ca^{2+} influx via ionotropic glutamate receptors has been shown to cause in an increase in ROS production (Carriedo *et al.*, 1998). This is thought to be due to increased activity of inducible nitric oxide synthase, phospholipases and xanthine oxidase (Carriedo *et al.*, 1998). As a result of the massive increase in cation concentration in the cytosol, the neuron becomes hypertonic relative to the extracellular fluid and water enters the cell. Water influx and cellular swelling may lead to cell membrane breakage (Lipton, 1999). Cell lysis and increased ion concentrations in the extracellular fluid cause an influx of water and immune cells to the infarct which can further inhibit perfusion of tissues (Dirnagl *et al.*, 1999).

Activation of metabotropic glutamate receptors (mGluRs) can produce either excitation or inhibition depending on which of the 8 receptors (mGluR1-8) is stimulated. Activation of group I mGlu receptors leads to stimulation of phosphoinositide-phospholipase C that catalyzes the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (Dingledine and McBain, 1999). DAG stimulates the activation of protein kinase C (PKC) which has a wide variety of effects (Dingledine and McBain, 1999). IP_3 stimulates receptors on the endoplasmic reticulum (ER) resulting in release of bound Ca^{2+} (Berridge and Irvine, 1989). This substantial increase in free Ca^{2+}

Figure 3: Simplified general mechanisms for glutamate mediated loss of ion homeostasis after ischemic damage (adapted from Gill and Lodge, 1997). VOSC - voltage operated sodium channel; VOCC - voltage operated calcium channel.



stimulates release of neurotransmitters from neurons via exocytosis (Leist and Nicotera, 1999). If the neurons are excitatory, as in the case of cerebellar granule neurons (CGNs), an excitatory neurotransmitter (*e.g.* glutamate) is released and can act at post-synaptic neurons to cause depolarization (Gallo *et al.*, 1982; Schousboe *et al.*, 1985; Kingsbury *et al.*, 1988; Schousboe *et al.*, 1989; Simmons and Dutton, 1992).

The role of mGluRs in neuroprotection has been studied. Activation of mGluR groups I and II has been shown to be neuroprotective against both colchicine-induced apoptosis and necrosis induced by oxygen-glucose deprivation (OGD) in rat CGN cultures (Kalda and Zharkovsky, 1999). Group II receptors have also demonstrated neuroprotective properties against excitotoxic cell death induced by NMDA in mouse mixed cortical cell cultures (Battaglia *et al.*, 1998). Activation of mGluR groups I and III receptors was found to be neuroprotective against NO-induced apoptosis in rat primary hippocampal cultures (Vincent and Maiese, 2000). Activation of mGluR groups II and III inhibits adenylyl cyclase activity, decreasing the production of cyclic adenosine monophosphate (cAMP) and therefore inhibiting the excitation of the neuron (Dingledine and McBain, 1999).

Depolarization of the ischemic core is associated with ionotropic glutamate receptors and is thought to originate from glutamate released at the core of an infarct (Back *et al.*, 1996; Chen *et al.*, 1996). Mechanisms which normally regulate extracellular glutamate concentrations such as re-uptake of excitatory amino acids by astrocytes and neurons are inhibited in ischemia (Swanson *et al.*, 1992; Volterra *et al.*, 1994). Na^+-dependent glutamate influx is

reversed due to increased intracellular Na^+ concentrations, which adds to the sustained increases in extracellular glutamate (Lyden, 1997; Dirnagl *et al.*, 1999). The ATP-dependent intracellular metabolism of glutamate to glutamine, by the glutaminase enzyme, is inhibited due to lack of ATP (Lyden, 1997).

In addition to glutamate-induced damage, the lack of ATP production compounds the problem of ionic homeostasis (Lipton, 1999). Ca^{2+} concentrations in the cell are normally maintained at 0.1μM due to poor membrane permeability of Ca^{2+} (Siesjö and Bengtsson, 1989) and mechanisms which pump Ca^{2+} out of the cell or store Ca^{2+} in mitochondria or ER (Leist and Nicotera, 1999). During ischemia, the lack of ATP renders these mechanisms inadequate to compensate for the accumulation of Ca^{2+} in the cytosol (Lipton, 1999). VOCCs and VOSCs are opened during ischemia due to the depolarization of the cell caused by glutamate. The lack of ATP inhibits Ca^{2+}ATPase activity and prevents the return to basal Ca^{2+} levels. Thus, these channels remain open allowing further influx of cations. The Na^+/Ca^{2+} exchanger can also reverse as the concentration of intracellular Na^+ increases causing Na^+ efflux and resulting in influx of Ca^{2+} (Ter Horst and Postigo, 1997).

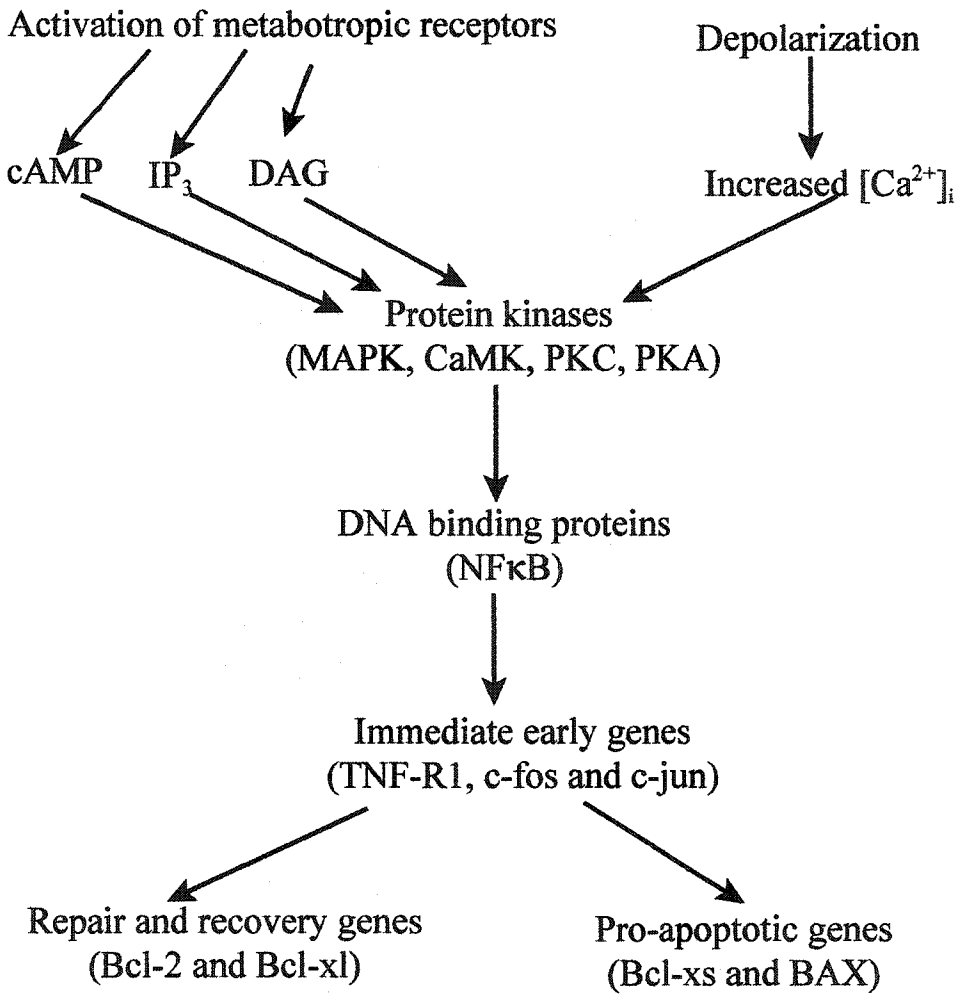
When intracellular concentrations of Ca^{2+} are increased and maintained, Ca^{2+}-dependent enzymes are activated. These enzymes include lipases, endonucleases, proteases and phospholipases, all of which can lead to apoptotic or necrotic cell death (Leist and Nicotera, 1999). Phospholipases lead to release of arachidonic acid which can cause the formation of free radicals through the activation of lipoxygenase (Yamamoto, 1992). In addition, Ca^{2+} can

activate calpains causing cell damage by activating xanthine oxidase and increasing free radical production (Gill and Lodge, 1997)(Figure 2). Ca^{2+} chelators have been demonstrated to decrease glutamate-induced excitotoxicity in mouse spinal explant cultures and ischemic cell death in a rat focal ischemic model (Tymianski and Wallace, 1993). These data support a role for Ca^{2+} as a mediator in ischemic cell damage.

Changes in Ca^{2+} content in cells also cause changes in gene expression patterns. Ca^{2+}-induced changes in expression can be mediated by transcription factors such as c-fos (Coulon *et al.*, 1999), calmodulin-dependent protein kinase (Shaywitz and Greenberg, 1999) and mitogen activated protein kinase (Dudek and Fields, 2001). Another mechanism of Ca^{2+} modulated gene expression is through an increase in ROS concentrations. Increased ROS leads to the activation of the transcription factor, nuclear factor kappa B (NFκB)(Christman *et al.*, 2000). Transcription factors can be activated by a number of other stimuli which are increased during ischemia, including cAMP, IP_3 and DAG (Bading *et al.*, 1993; Akins *et al.*, 1996; Bading *et al.*, 1997; Bito *et al.*, 1997; Papadopoulos *et al.*, 2000)(Figure 4).

Transcription factors, by definition, mediate expression of genes. Although they have been studied for many years, direct interactions between individual genes and transcription factors are difficult to demonstrate. This is mostly because the apparent mechanisms involved are stimulus and cell type dependent (Corcoran and Means, 2001). However, NFkB has been linked to transcription of pro-inflammatory, pro-apoptotic (Camandola and Mattson, 2000), anti-apoptotic (Mattson *et al.*, 1997; Tamatani *et al.*, 1999) and receptor proteins such as the

Figure 4: Generalized mechanisms for the induction of gene expression by ischemia (adapted from Akins *et al.*, 1996). cAMP - cyclic adenosine monophosphate; IP_3 - inositol trisphosphate; DAG - diacylglycerol; MAPK - mitogen activated protein kinase; CaMK - calmodulin activated kinase; PKC - protein kinase C; PKA - protein kinase A; NFκB - nuclear factor kappaB; TNF-R1 - tumour necrosis factor receptor 1.



adenosine A1 receptor (Nei *et al.*, 1998).

1.1.4. Immune-mediated cell damage

The immune response that follows ischemia results from the release of cellular contents into the extracellular space from necrotic cells and subsequent increased expression of pro-inflammatory genes (Lipton, 1999). Some of the pro-inflammatory factors involved in the immune response to ischemia are NFκB, hypoxia inducible factor 1, interferon regulatory factor I, IL-1β and TNF-α (Wallach, 1997). TNF-α and IL-1β upregulate ICAM (Rothlein, 1997), E-selectins (Haring *et al.*, 1996) and P-selectins (Rothlein *et al.*, 1988), all of which facilitate influx of immune cells into the brain.

Activated neutrophils produce inducible nitric oxide synthase which increases production of NO (Iadecola *et al.*, 1996). Activated microglia produce NO and TNF-α and IL-1β (Sharkey *et al.*, 1997). An immune response also results in increased TNF-α, which stimulates apoptosis (Sastry and Rao, 2000). The presence of activated immune cells also causes demyelination, resulting in further tissue damage (Becker *et al.*, 1997). One strategy shown to decrease ischemic damage in infarct regions has been to inhibit immune-mediated damage using immunosuppressants (Arvin *et al.*, 1996; Hartl *et al.*, 1996; Clark and Zivin, 1997).

1.2. Apoptosis

For many years it was believed that necrosis was the only type of cell death resulting from a pathological event such as ischemia (Dessi *et al.*, 1993; Dirnagl *et al.*, 1999). This type of cell death is characterized by cellular swelling, cell membrane lysis and an inflammatory response in the tissue (Majno and Joris, 1995; Martin *et al.*, 1998; Dirnagl *et al.*, 1999). Necrosis is the dominant form of cell death in the core of an infarct (Dirnagl *et al.*, 1999).

Only in the past 8-9 years have researchers found evidence of another type of cell death associated with ischemic damage: apoptosis (Heron *et al.*, 1993; Rink *et al.*, 1995; Choi, 1996; Barinaga, 1998; Kalda *et al.*, 1998; Martin *et al.*, 1998; Namura *et al.*, 1998). Apoptosis or programmed cell death occurs primarily in areas of the brain that experience mild to moderate ischemic damage (Hossmann, 1994a; Back *et al.*, 1996; Richter *et al.*, 1996; Dirnagl *et al.*, 1999). This type of cell death is characterized by cellular shrinkage, cytosolic condensation, cell compartmentalization into apoptotic particles and phagocytosis of these particles by local macrophages (Majno and Joris, 1995; Dirnagl *et al.*, 1999). Apoptosis is an optimal target for decreasing ischemic damage because of the increased length of time between initiation of the mechanism and commitment to death. The characteristics of apoptosis are very different from those of necrosis, as necrosis may occur in a matter of minutes, and with few possible mechanisms of intervention. In contrast, apoptosis involves highly complicated and tightly regulated pathways that provide researchers with many targets for intervention. The prevention of apoptosis is now a major

focal point for stroke research (Schulz *et al.*, 1999b).

1.2.1. Biological significance

Apoptosis is a physiological mechanism for removal of excess or damaged cells. Examples of this include deletion of lymphocytes after an immune response and the removal of 50% of sensory neurons during development (Silos-Santigo *et al.*, 1995; Hale *et al.*, 1996; Sastry and Rao, 2000). Cellular crowding results in insufficient supplies of essential factors such as glucose, oxygen and trophic factors. The depletion of any of these factors can induce apoptosis, supporting the role of this type of cell death as a regulatory mechanism (Rubin *et al.*, 1994; Aloyz *et al.*, 1998; Martin *et al.*, 1998). The importance of apoptosis is exemplified in situations where intracellular mechanisms do not function properly, the most significant example of this being cancer. Normally, overabundant cells are destroyed by apoptosis; however, a dysfunction in the apoptotic mechanism may allow cells to avoid death and continue multiplying (Martin *et al.*, 1998).

Apoptotic cell death is the least harmful to surrounding tissue as it does not result in lysis of the cell membrane, and therefore does not stimulate an immune response. Unlike necrosis, apoptotic cells are compartmentalized and then taken up by local macrophages, avoiding an overt immune response which can increase the amount of damage to tissue. This type of cell death was long thought to be involved only in physiological, not pathological cell death (Dessi *et al.*, 1993; Dirnagl *et al.*, 1999). Apoptosis has now been demonstrated to play a

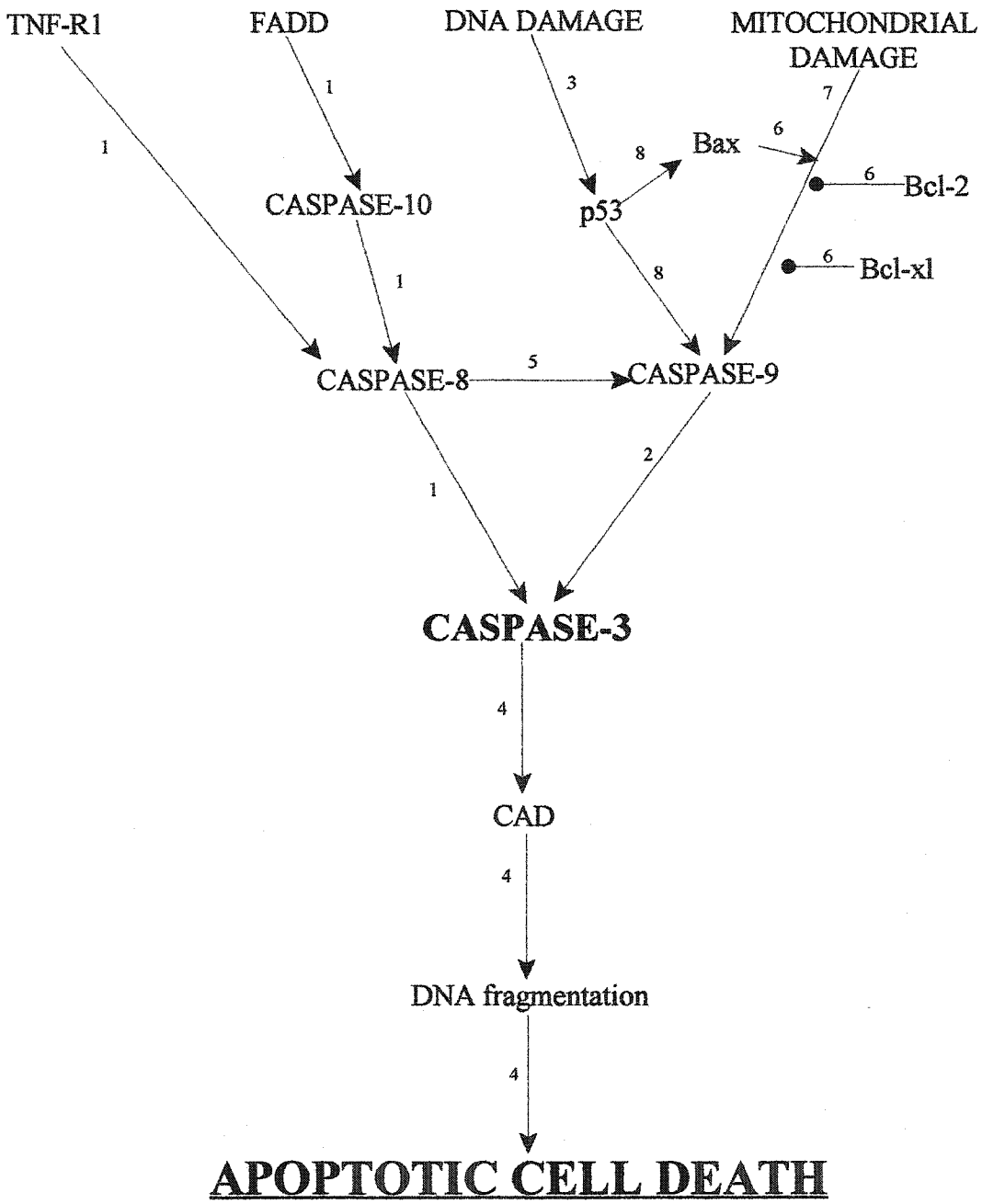
significant role in many diseases such as Alzheimer's disease, Parkinson's disease, amylotrophic lateral sclerosis and stroke (Thompson, 1995).

1.2.2. Induction of apoptotic pathways

Apoptosis can be initiated by a wide variety of stimuli. Events such as withdrawal of neurotrophic factors including brain derived neurotrophic factor (BDNF) (Sastry and Roa, 2000), increased or decreased intracellular Ca^{2+} (Zhong *et al.*, 1993), decreased ATP production (Richter *et al.*, 1996) and hypoxia (Chen *et al.*, 1997) result in apoptosis. Receptors that are associated with apoptosis include fas associated death domain and tumor necrosis factor receptor-1 (TNFR-1), both of which potentiate the apoptotic cascade (Budihardjo *et al.*, 1999; Denecker *et al.*, 2001)(Figure 5). DNA damage can also cause the activation of the pro-apoptotic tumor suppressor p53 gene (Cregan *et al.*, 1999)(Figure 5) which can activate the pro-apoptotic genes Bax, Bcl-Xs or the enzyme caspase-9, and result in the release of cytochrome C from the mitochondria (Shen and White, 2001).

Many enzymes are involved in the apoptotic cascade, but the most significant appear to be a large family of cysteine proteases, collectively called caspases (Sastry and Rao, 2000). Caspase enzymes are expressed as inactive pro-caspases which are activated through cleavage by identical or related enzymes (Cohen, 1997). Caspases are responsible for cleaving 30 different proteins during apoptosis including the DNA repair enzyme poly ADP-ribose polymerase (Ueda *et al.*, 1995) and cytoskeletal proteins such as gelsolin (Sastry and

Figure 5: Generalized mechanisms of apoptotic cell death. Methods of stimulation and inhibition of various steps in apoptosis. ([3]Fraser *et al.*, 1996; [6]Reed, 1997; [7]Hengartner, 1998; [2]Budihardjo *et al.*, 1999; [5]Earnshaw *et al.*, 1999; [1]Rosen and Casciola-Rosen, 1999; [4]Zheng and Flavell, 2000; [8]Shen and White, 2001). TNF-R1 - tumor necrosis factor receptor 1; FADD - fas activated death domain; CAD - caspase activated DNase; → Stimulate; —● Inhibit.



Rao, 2000) and spectrin (Nath *et al.*, 1998).

There are many mechanisms involved in apoptosis which can either inhibit or potentiate cell death. Additionally, the length of time between initiation of apoptosis and commitment to cell death is much longer than that of necrotic cell death (Lipton, 1999). This provides researchers with a longer "window of opportunity" to inhibit the neurodegenerative process (Lipton, 1999), however this "window" has not yet been defined and is dependent on the apoptotic stimulus. The many stimuli which can lead to apoptosis may begin the process that leads to cell death by activating initiator caspases (caspases 8, 9 or 10). These enzymes are closely coupled to pro-apoptotic signals and activate downstream processes which result in cell death (Cohen, 1997; Budihardjo *et al.*, 1999). The complexity of the apoptotic process increases, due to the many regulatory steps which occur prior to cell death. The activation of caspase-9 is regulated by four genes, pro-apoptotic Bax and p53, and anti-apoptotic Bcl-2 and Bcl-Xl (Reed, 1997), as well as the caspase-8 enzyme (Figure 5). Both Bcl-2 and Bcl-Xl proteins can inhibit p53-mediated cell damage (Shen and White, 2001). Bcl-2 is also believed to be associated with a Ca^{2+} channel on the mitochondrial membrane which has not yet been defined, but is believed to aid in the regulation of mitochondrial Ca^{2+} (Zhu *et al.*, 1999).

Activation of caspase-3 is one of the few events in the cascade which is common to all pathways (Cohen, 1997; Eldadah *et al.*, 2000) and has been demonstrated to become activated only in apoptotic cell death (Armstrong *et al.*, 1997; Gottron *et al.*, 1997).

Additionally, in CGNs, caspase-3 activity has been shown to be regulated by Ca^{2+} and cAMP (Moran *et al.*, 1999). There appear to be forms of apoptosis that rely completely on the constitutively expressed proteins in the body and are not dependent on time or ATP for new protein synthesis (Ter Horst and Postigo, 1997). Therefore, apoptosis can be activated immediately and the loss of ATP resulting from ischemia may not affect activity of caspase-3. This may be especially true in cells such as CGNs in which pro-caspase-3 is constitutively expressed (Ni *et al.*, 1998). However, caspase-3 mRNA is upregulated in hippocampal CA1 neurons between 8 and 24 hours following global ischemia in the rat with the appearance of protein after 8 hours (Ni *et al.*, 1998; Schulz *et al.*, 1999a). This finding would suggest that a combination of constitutively expressed pro-caspase-3 protein and pro-caspase-3 expressed during or after ischemia are responsible for ischemia-induced caspase-3 activity. After ischemic insult there is an increase in the ratio of pro-apoptotic genes (Bax, Bcl-Xs) to anti-apoptotic genes (Bcl-2, Bcl-xl) that results in an increased incidence of apoptosis (Ferrer *et al.*, 1997; Reed, 1997).

Caspase-3 induces caspase-activated DNAase (Figure 5), an endonuclease responsible for internucleosomal DNA fragmentation (Inohara *et al.*, 1999; Zheng and Flavell, 2000). DNA is also believed to be cleaved by Ca^{2+}/Mg^{2+} dependent endonucleases which also produce the nucleosomal (180-200 base pair) fragments (Clarke, 1999). It was previously believed that this type of DNA fragmentation (seen as a "DNA ladder" using gel electrophoresis) only occurred in apoptotic cell death. Intranucleosomal cleavage (seen as a DNA smear) was characteristic of necrotic cell death. Recently, it has been demonstrated that internucleosomal

and intranucleosomal DNA fragmentation are also associated with necrotic cell death (Rink *et al.*, 1995; Ueda *et al.*, 1995; Kaasik *et al.*, 1999) including necrosis-induced by ischemic cell damage (Clarke, 1990; Martin *et al.*, 1998). As a result of this finding, many methods previously used for quantification of apoptosis, such as gel electrophoresis and terminal deoxynucleotidyl transferase-mediated d-UTP biotin nick end labeling staining are used less frequently. It is for this reason many researchers now focus on other indicators such as caspase activity, cytochrome C release or exclusion of phosphatidylserine, to quantify apoptosis.

1.2.3. Cellular morphology

In the past, apoptotic cell death was identified by chromatin condensation into crescent formations along the nuclear membrane (Li *et al.*, 1995). Condensing of the cytosol, cellular shrinkage and blebbing of intact cell membranes are also characteristics of this type of cell death (Lipton, 1999). In the later stages of apoptosis, cells are compartmentalized into membrane-encased packages containing cellular organelles which are believed to remain intact and functional (Li *et al.*, 1995). One of the last stages of apoptosis is the transfer of phosphotidylserine to the outer membrane signaling local macrophages to begin phagocytosis (Walton *et al.*, 1997).

1.2.4. Significance of apoptotic cell death in ischemic injury

Apoptotic cell death involving caspase-3 activity is prominent in the ischemic penumbra (Chen *et al.*, 1998; Schulz *et al.*, 1999b) possibly due to the chronic low level of ATP (Richter *et al.*, 1996). However, ATP-dependent activity of caspase-3 has been demonstrated to result from constitutive expression of the mRNA (Moran *et al.*, 1999). The exact contribution of this type of cell death is still unclear. However, immediate cell death following ischemia is thought to be necrotic, while delayed cell death is probably apoptotic (Northington *et al.*, 2001). Stroke patients can accumulate tissue damage for up to a year before the total amount of ischemic damage can be accurately quantified (Martin *et al.*, 1998; Dirnagl *et al.*, 1999). As delayed cell death has been shown to be apoptotic, and there are many mechanisms by which to inhibit apoptosis, this is now an area in which stroke researchers are exploring.

1.3. Necrosis

1.3.1. Biological significance

Necrosis is the most common type of cell death involved in pathological events (Martin *et al.*, 1998). This mechanism of cell death occurs over a relatively short amount of time (seconds to minutes) when compared to that of apoptosis which can take hours to days depending on the intensity of the stimulus (Ankarcrona *et al.*, 1995). Unlike apoptosis,

necrosis does not consist of complex regulatory mechanisms. The short time span for necrotic cell death and the lack of inherent inhibitory mechanisms makes protection from necrosis difficult.

1.3.2. Induction of necrotic cell death

Necrotic cell death is associated with a number of pathological states including trauma, excitotoxicity and toxicity. Necrosis can be initiated by disruptions of cell volume homeostasis, mitochondrial function or oxidative phosphorylation (Dirnagl *et al.*, 1999). Damage to cell membranes or transport proteins alters membrane permeability allowing uncontrolled ion influx and results in further loss of ion homeostasis (Martin *et al.*, 1998). Mitochondrial damage from ROS or increased mitochondrial Ca^{2+} cycling results in increased ROS production and leads to cellular swelling and eventually lysis (Chalmers-Redman *et al.*, 1997).

1.3.3. Cellular morphology

Using light microscopy, necrotic cells can be characterized by cellular swelling and the presence of swollen mitochondria with disrupted cristae (Martin *et al.*, 1998). There is also dissolution of other organelles including the Golgi apparatus, ER and polysomes, which can only be seen as fragments (Lipton, 1999). Ribosomes are dispersed throughout the cytosol making it appear dense and granular (Martin *et al.*, 1998) and microtubules and other

filamentous structures are absent. The chromatin is also found in irregular shapes in the nucleus. Endonucleases cause intra- and internucleosomal DNA fragmentation, which is apparent when DNA is examined using gel electrophoresis (Ueda *et al.*, 1995; Kaasik *et al.*, 1999).

The final stage of necrosis involves lysis of cell membranes allowing release of antigenically active intracellular components (Martin *et al.*, 1998). As organelles are destroyed, enzymes are released including glutaminase which catalyzes the formation of glutamate from glutamine (Wahl *et al.*, 1994). Intracellular glutamate is released and therefore post-synaptic glutamate receptors may be activated (Lipton, 1999), which can compound damage by causing excitotoxicity in post-synaptic neurons (Dingledine and McBain, 1999) as described in Section 1.1.3.

1.3.4. Significance of necrosis in ischemic injury

Necrotic cell death as a result of ischemia is concentrated in the core of the infarct. It is here that excitotoxicity and subsequent spreading of damage begin. The characteristic lysis of cells causes the release of glutamate, K^+ and Ca^{2+} into extracellular spaces where they then affect surrounding neurons. Physiological indicators of necrotic cell death include measurement of lactate dehydrogenase (LDH) activity. LDH is an intracellular enzyme only released when cell membranes are disrupted. As the morphological features of apoptosis do not include membrane disruption, LDH in the extracellular media can be attributed to

necrotic cell death (Juurlink and Hertz, 1993).

1.4. Adenosine

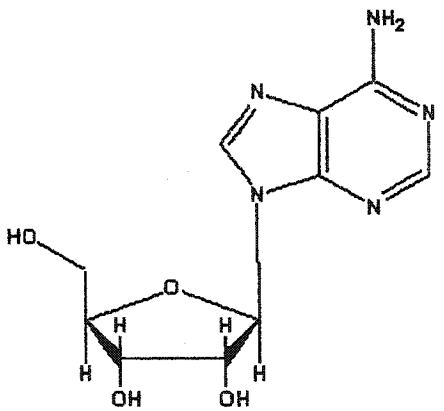
1.4.1. Overview

The neurotransmitter adenosine is comprised of a purine base attached to a ribose sugar (Figure 6a). Basal extra-neuronal concentrations of adenosine vary widely (25-250nM) throughout the central nervous system (Ballarin *et al.*, 1991; Dunwiddie and Diao, 1994). Adenosine has a wide variety of functions in nerve, muscle, adipose and vascular tissue (Dunwiddie and Masino, 2001). However, for the purpose of this thesis, only studies describing the properties of adenosine in nervous tissue, where it has been characterized as an inhibitory neurotransmitter (Fredholm, 1997; Sweeney, 1997; Dunwiddie and Masino, 2001) are discussed.

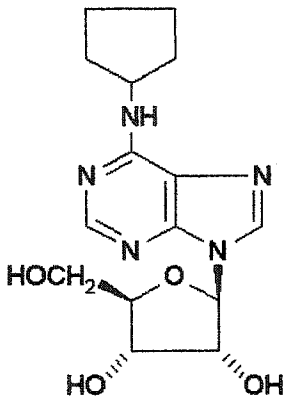
1.4.2. Synthesis and release

The major mechanism of adenosine synthesis is dephosphorylation of ATP to adenine diphosphate (ADP) by ATPases. ADP or cAMP are then converted to 5'-AMP by adenylate kinase or phosphodiesterases respectively. 5'-AMP is then converted to adenosine by 5'-nucleotidase (Dunwiddie and Masino, 2001)(Figure 7). The 5'-nucleotidase enzyme has been found in many tissues both in the cytosol and in the extracellular space indicating that

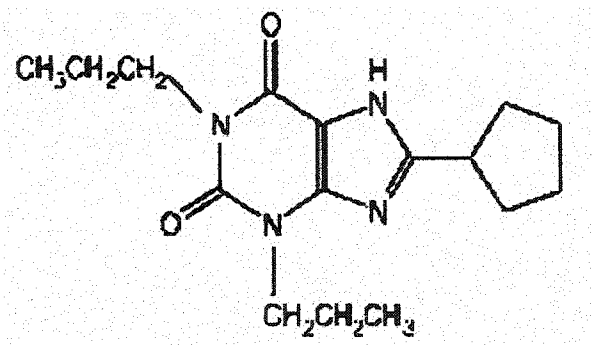
Figure 6: Chemical structures of A) adenosine, B) cyclopentyl-adenosine (CPA) and C) dipropylcyclopentyl-xanthine (DPCPX). (A and B from Linden, 2001; C from Illek *et al.*, 1998).



A) Adenosine

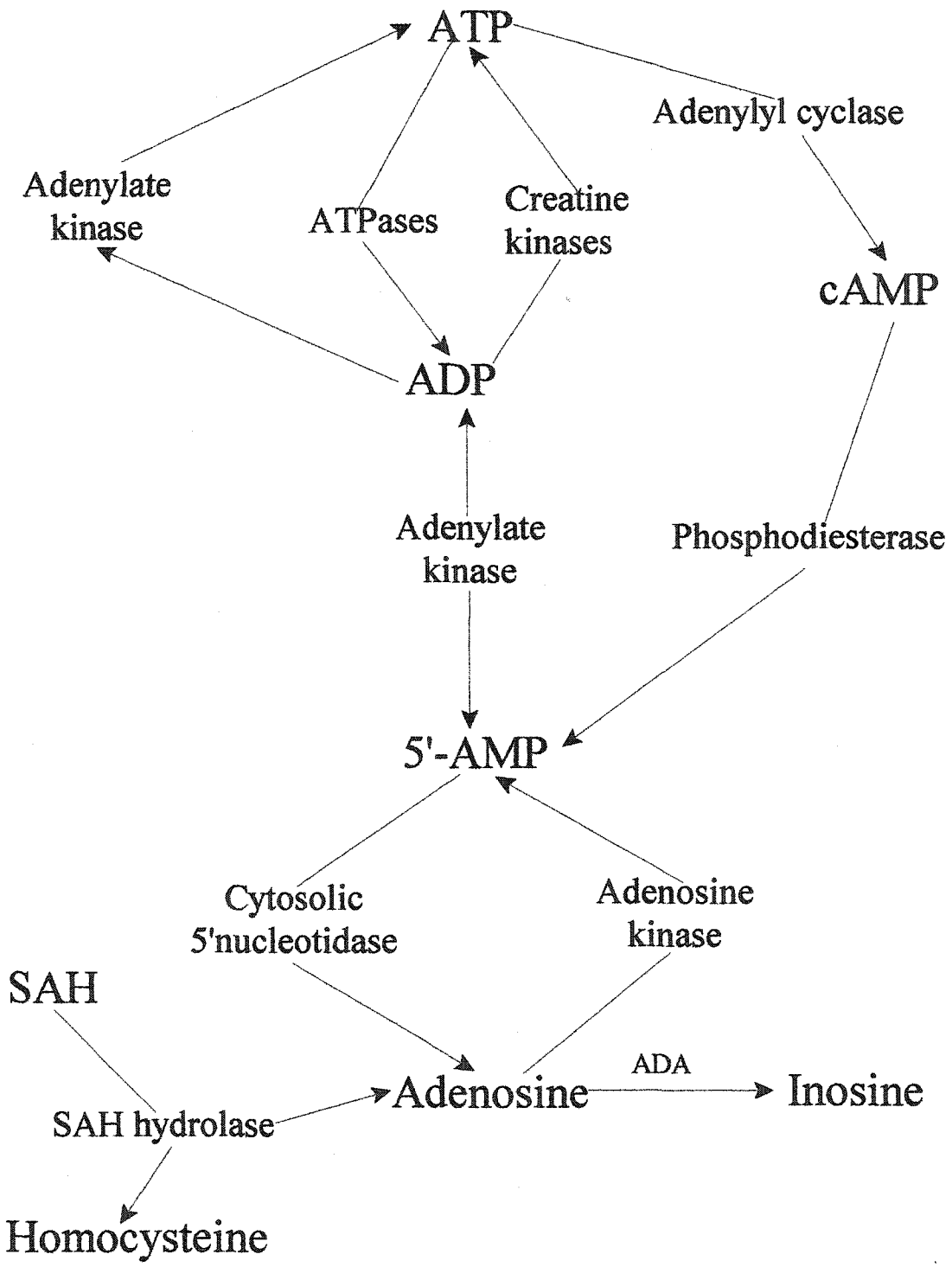


B) CPA



C) DPCPX

Figure 7: Synthesis and metabolism of adenosine (adapted from Dunwiddie and Masino, 2001). ADA - adenosine deaminase, SAH - S-adenosylhomocystein hydrolase.



adenosine can be synthesized in both places (Fredholm, 1997). Intracellular ATP concentrations are very high (approximately 3mM) such that if only 1% of endogenous ATP were converted to adenosine there would be a 100-fold increase in the concentration of adenosine in the cell (Fredholm, 1997).

There is evidence to suggest that phosphodiesterase and 5'-nucleotidase are in close proximity to the adenosine A1 receptors, which would result in a constant supply of adenosine for receptor activation (Dunwiddie and Masino, 2001). During ischemia, there can be a 300-fold increase in the extracellular concentration of adenosine from 100nM to 30μM in hippocampal slices cultures (Latini *et al.*, 1999).

Extracellular concentrations of adenosine can also be increased by adenosine release from surrounding cells. Release by neurons is induced via a variety of stimuli including hypoxia, anoxia, ischemia, hypoglycemia, seizures, increased temperature, free radicals, neurotransmission, ion shifts and drugs (Dunwiddie and Masino, 2001). It is also thought that adenine nucleotides such as ATP are co-released with a number of neurotransmitters including gamma-aminobutyric acid (GABA) and glutamate. Evidence suggests that these adenine nucleotides are then metabolized into adenosine in less than a second (Dunwiddie *et al.*, 1997). Adenosine has been shown to be released by both Ca^{2+} dependent and independent mechanisms (Fredholm, 1997). Although adenosine release is a main mechanism used to increase extracellular concentrations, adenosine uptake is also important. Pharmacological blockers of transport proteins have been shown to both increase

extracellular adenosine concentrations (Jonzon and Fredholm, 1985), by blocking adenosine uptake (Schubert *et al.*, 1994), and to decrease extracellular concentrations (Phillis *et al.*, 1994) by inhibiting adenosine release (Schubert *et al.*, 1994).

The two main types of adenosine transporters are "passive" and "active". There are 6 known types of active transporters, designated N1 through N6, which are dependent on the concentration of Na^+ in the cell, as adenosine influx is coupled to Na^+ influx (Geiger and Fyda, 1991). This mechanism may be reversed in conditions of hypoxia or ischemia due to loss of Na^+ gradient, causing the release of adenosine (Lipton, 1999). The two passive transporters (E1 and E2) do not depend on ATP or ionic gradients and equilibrate the concentrations of adenosine on either side of the cell membrane (Geiger and Fyda, 1991; Brundege and Dunwiddie, 1996). These transporters have been identified in both neurons and glial cells (Anderson *et al.*, 1999a,b).

Under normal conditions, the intracellular adenosine concentration is kept low by phosphorylation of adenosine by adenosine kinase (Fredholm, 1997)(Figure 7) to allow adenosine influx down its gradient through the E1 and E2 transporters. Release of adenosine can be caused by the reversal of the passive adenosine transport. This can be caused by a decrease in activity of adenosine kinase or an increase in production of intracellular adenosine (Brundege and Dunwiddie, 1996).

1.4.3. Metabolism

Adenosine is metabolized by two enzymes, adenosine kinase (intracellular) and adenosine deaminase (ADA)(intra- and extracellular)(Figure 7). ADA removes an amino group from adenosine resulting in the formation of inosine (Dunwiddie and Masino, 2001) (Figure 7). In hypoxic conditions, the activity of ADA dramatically decreases to 6% of control resulting in adenosine concentrations remaining high in the cell (Dunwiddie and Masino, 2001). Hypoxia does not affect the enzyme, 5'-nucleotidase, which suggests that the increase in adenosine concentration after ischemia or hypoxia is not due to an increase in adenosine synthesis but rather a decrease in metabolism (Dunwiddie and Masino, 2001).

The key mechanism for the removal of extracellular adenosine appears to be reuptake, as it has been demonstrated that under physiological conditions degradation of adenosine is only a "minor mechanism as the inhibition of ADA has little effect on the concentration of adenosine in the cell" (Pak *et al.*, 1994; Dunwiddie *et al.*, 1997). During ischemic conditions, the conversion of adenosine to inosine by ADA becomes the main mechanism for adenosine regulation in rat hippocampal slices (Lloyd and Fredholm, 1995).

1.4.4. Physiological functions of adenosine

The effects of adenosine are mediated by 4 adenosine receptors which have distinct and opposing effects on neuronal physiology. Adenosine has many functions in normal

physiology including promoting sleep, regulating neuronal excitability and regulating cerebral blood flow (Dunwiddie and Masino, 2001). Adenosine can hyperpolarize or depolarize neurons by respectively decreasing or increasing the formation of cAMP which is a second messenger. cAMP activates protein kinase A and increases the activity of phospholipase C which catalyzes the formation of DAG and IP_3. Both DAG and IP_3 activate receptors on the mitochondria and ER causing Ca^{2+} release into the cytosol (Dunwiddie and Masino, 2001). In the central nervous system, adenosine has also been linked to the inhibition of every classical neurotransmitter except GABA (Fredholm, 1997). Adenosine has been demonstrated to have the ability to halt glutamatergic transmission completely (Dunwiddie and Hoffer, 1980).

Peripherally, adenosine has been shown to induce vasodilation of cerebral blood vessels which is believed to be a mechanism of autoregulation. As the concentration of ATP decreases, the subsequent increase in adenosine acts on adenosine A1 receptors to cause vasodilation, increasing blood flow and therefore glucose and oxygen supply to the brain (Wahl and Schilling, 1993).

Stimulation of one cell can cause the release of adenosine which will act selectively to inhibit excitatory post-synaptic potentials acting both as an anterograde and retrograde messenger (Fredholm, 1997). Adenosine acts on pre-synaptic adenosine A1 receptors to inhibit depolarization but has not been shown to inhibit GABA-mediated inhibitory post-synaptic potentials in cultured rat hippocampal neurons (Dunwiddie and Hoffer, 1980).

1.4.5. Adenosine receptors

Adenosine interacts with 4 guanosine (G)-protein linked metabotropic receptors (A1, A2a, A2b and A3) (Fredholm and Dunwiddie, 1988; Fredholm, 1997; Sweeney, 1997; Olah and Stiles, 2000; Dunwiddie and Masino, 2001) in order to execute its functions. Some properties of adenosine receptors are summarized in Table 1.

1.4.5.1. A1 receptor

The most abundant adenosine receptor is the A1 receptor, which is concentrated in the cerebellum, hippocampus and cerebral cortex and has the highest affinity (70nM) for adenosine (Dunwiddie and Masino, 2001). Due to the high affinity for adenosine, the A1 receptor becomes activated during physiological events because basal extra-neuronal concentrations of adenosine range from 25-250nM (Ballarin *et al.*, 1991). This suggests that the A1 receptor is partially responsible for basic purinergic tone in the body (Dunwiddie and Masino, 2001).

The A1 receptor is a pertussis toxin-sensitive receptor linked to G_{i1}, G_{i2}, G_{i3} and G_o (Fredholm, 1997). Activation of this receptor is coupled to G-protein dependent inward rectifying K^+ efflux channels which are opened without the involvement of a second messenger system and allow K^+ efflux. A1 activation also results in activation of ATP-dependent K^+ (K_{ATP}) efflux channels (Heurteaux *et al.*, 1995), reduction in cAMP and IP_3

Table 1: Adenosine receptor characteristics: affinity, location, mechanism of action and function. cAMP - 3'-5'-cyclic adenosine monophosphate; PLC - phospholipase C; IP_3 - inositol-1,4,5-trisphosphate (adapted from Dunwiddie and Masino, 2001).

Characteristics	A1	A2a	A2b	A3
Binding affinity for adenosine	70nM	150nM	5.1μM	6.5μM
Receptor distribution	cerebellum, hippocampus and cortex	striatum	astrocytes	wide
G-protein associated	G_{i1-3}, G_o	G_{Sc}, G_{olf}	G_{Sc}	G_{i3}, G_q
Function	↓cAMP, K^+ efflux, ↓IP_3	↑cAMP	↑cAMP ↑PLC	↓cAMP ↑PLC

production (Petcoff and Cooper, 1987; Dunwiddie and Fredholm, 1989) and inhibition of N-, P/Q- and other types of Ca^{2+} channels (Ambrosio *et al.*, 1997). By facilitating cation efflux, inhibiting cation influx and decreasing activity of adenylyl cyclase, A1 activation decreases neuronal activity by hyperpolarizing the cell (Dunwiddie and Masino, 2001). By decreasing the formation of cAMP and DAG, A1 receptor activation results in a decrease in protein kinase A and PKC activity respectively (Bouron, 1999). Stimulation of this receptor *in vitro* has also been shown to increase free radical scavenging in human endothelial cells (Maggirwar *et al.*, 1994). Administration of the A1 selective agonist N^6-(R)-phenylisopropyladenosine (R-PIA) caused significant increases in activity of CAT, GSHR, SOD and GSH-Px activity (Maggirwar *et al.*, 1994). This effect is blocked by the non-selective adenosine antagonist, theophylline, therefore indicating an adenosine receptor-mediated effect (Vitolo *et al.*, 1998). Taken all together, the above studies indicate that through the numerous inhibitory mechanisms, the adenosine A1 receptor plays an important role in the regulation of post-ischemic brain damage.

Activation of adenosine A1 receptors has been demonstrated for many years to be both cardioprotective (Heurteaux *et al.*, 1995; Linden, 2001) and neuroprotective (Fredholm, 1997; Sweeney, 1997; Blondeau *et al.*, 2000; Dunwiddie and Masino, 2001; Linden, 2001). Neuroprotective properties of adenosine A1 receptor activation have been shown in both *in vitro* (Logan and Sweeney, 1997) and *in vivo* (Blondeau *et al.*, 2000) ischemic models. Much of the research examining effects of A1 receptor activation have used A1 selective agonists such as N^6-cyclopentyladenosine (CPA) (Figure 6) at maximal concentrations of

10μM (Logan and Sweeney, 1997) and 100nM (Sanz *et al.*, 1996). CPA has been reported to have an affinity for A1 receptors of 0.3nM - 3nM and a selectivity for A1 receptors of 70-2000-fold over the A2 receptors (Bruns *et al.*, 1986; reviewed in Fredholm and Dunwiddie, 1988). A selective adenosine A1 receptor antagonist such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Figure 6) with a binding affinity at the A1 receptor of 0.5 - 2nM (Lohse *et al.*, 1987; reviewed in Fredholm and Dunwiddie, 1988) is also used. Many studies that used DPCPX employed a range of concentrations from 100nM (Sanz *et al.*, 1996; Logan and Sweeney, 1997) to 200μM (Vitolo *et al.*, 1998) in rat CGNs.

Adenosine and other A1 receptor agonists such as CPA, cyclohexyl-adenosine (CHA) and R-PIA have been shown to be neuroprotective against K^+ deprivation-induced apoptosis in CGNs (Vitolo *et al.*, 1998). Activation of A1 receptors has also been shown to decrease release of excitatory amino acids (Fredholm, 1997; Goda *et al.*, 1998). With depolarizing amounts of Ca^{2+} in the extracellular medium of cultured rat hippocampal synaptosomes, 100nM of CPA decreased the release of glutamate by 41% (Ambrosio *et al.*, 1997). CPA has been shown to decrease the amount of necrotic cell death induced by OGD by 87% in CGNs (Logan and Sweeney, 1997). Prior to the experiments described in this thesis, adenosine-mediated neuroprotection from ischemia-induced apoptosis had not been examined.

1.4.5.2 A2 receptor

There are 2 types of A2 receptors, A2a and A2b, which differ greatly from one another.

These receptors act in opposition to A1 by increasing adenylyl cyclase activity via Gs proteins (Fredholm, 1997) (Table 1). Over-activation of A2 receptors has been shown to be toxic in rat CGNs (Sei *et al.*, 1997), while inhibition is neuroprotective (Ongini and Schubert, 1998).

The adenosine A2a receptor is concentrated in the striatum, but is also present in the hippocampus and cerebral cortex (Fredholm, 1995). It has a relatively high affinity for adenosine (150nM) which indicates that this type of receptor could be activated during physiological events (Dunwiddie and Masino, 2001) and could therefore play a role in the purinergic tone of the brain. The receptor A2a has been shown to be linked to Gs_c activation which results in increased activity of adenylyl cyclase, opening of N-Ca^{2+} channels and facilitation of transmitter release (Fredholm and Dunwiddie, 1988; Fredholm, 1997; Dunwiddie and Masino, 2001).

Adenosine A2b receptor concentrations are low in all cells except for astrocytes. These receptors have a low affinity for adenosine (5.1μM), indicating that activation would occur only under non-physiological conditions, such as ischemia. The A2b receptor has also been linked to Gs_c protein and therefore activates adenylyl cyclase and phospholipase C (Dunwiddie and Masino, 2001). The A2b receptor has been demonstrated to be the major source of cAMP in during ischemia in astroglioma cells (Fiebich *et al.*, 1996). The A2b receptor has also been linked to IL-6 gene expression which would potentiate the damage caused by an immune response in an *in vivo* ischemic insult (Fiebich *et al.*, 1996).

1.4.5.3. A3 receptor

The adenosine A3 receptor has the lowest affinity for adenosine of the 4 receptors (6.5μM). The low affinity of this receptor indicates that it would not contribute to basal purinergic tone in the body. This receptor is associated with the G_{i3} and G_q G-proteins, which cause the activation of phospholipase C, the inhibition of adenylyl cyclase, and increase the release of Ca^{2+} from the mitochondria and ER (Dunwiddie and Masino, 2001). A3 receptors have only been discovered recently and are found to have a wide distribution in the body (Fredholm, 1997). These receptors would be activated during ischemia due to the increased concentration of adenosine. Activation of the A3 receptor has been shown to increase activity of SOD, CAT, GSH-Px and GSHR in rat basophilic leukemia cells (Maggirwar *et al.*, 1994).

1.5. Experimental model

To study the effects of adenosine A1 agonist pretreatment on cell death indicators following an *in vitro* simulated ischemia, primary cultures of rat CGNs were chosen as the experimental model. There are many advantages to using tissue culture as a model, notably the greater control over the experimental environment including pH, temperature, and concentrations of O_2, CO_2, glucose, Ca^{2+}, K^+ and astrocytic cell content. Using this method, CGN cultures have been characterized to have a 85-98% purity (Gallo *et al.*, 1982; Levi *et al.*, 1984; Kingsbury *et al.*, 1985; Juurlink and Hertz, 1993).

Using primary cell cultures of CGNs is also advantageous to this study as the cerebellum has high concentrations of both adenosine A1 receptors (Schousboe *et al.,* 1985; reviewed in Daval *et al.*, 1991; Fredholm, 1995) and glutamate receptors. Glutamate receptors play an important role in the development of ischemic damage, and their expression in CGN cultures is an important element in the present project. NMDA (Akazawa *et al.*, 1994), kainate (Petralia *et al.*, 1994), AMPA (Cambray-Deakin *et al.*, 1990) and metabotropic (Sanz *et al.*, 1996) glutamate receptors have also been detected in CGN cultures, in addition to GABA receptors A and B (Schousboe *et al.*, 1985).

Unfortunately, as with any model there are some significant disadvantages to using an *in vitro* model. Immune responses in ischemic injury play an important role in tissue damage accumulation and therefore the lack of immune cells in these cultures dictates the absence of immune-mediated damage. The absence of immune cells also causes concern in the quantification of cell death. Apoptotic cells do not normally lyse, but in the absence of phagocytosis from macrophages, secondary necrosis will occur (Lipton, 1999). Additionally, an ischemic infarct consists of both core and penumbral regions with varying intensities of insult between them. CGN cultures are uniformly exposed to an OGD insult in this model and therefore would lack the regions of damage associated with an *in vivo* ischemia. Some difficulties of neuronal tissue cultures include the requirement of strict aseptic conditions and the need for components of solutions to be made precisely to specification in the protocol, as cells are sensitive to inappropriate concentrations of medium components. Further concerns and support for this model are discussed in greater detail in section 4.6.1.

The use of CGN cultures was an appropriate model for the current project because both necrotic and apoptotic cell death have been reliably induced in these cells (Logan and Sweeney, 1997; Kalda *et al.*, 1998; Vitolo *et al.*, 1998). CGNs are commonly used to examine apoptotic cell death. A high K^+ concentration (24mM) in the growth medium of CGN cultures is required to maintain cell viability, as it mimics trophic support and opens L-type Ca^{2+} channels which increases intracellular Ca^{2+} concentration (Schulz *et al.*, 1996). When the concentration of K^+ is decreased (5mM), cells die by an apoptotic mechanism (Schulz *et al.*, 1999a) involving the activation of caspase-3 (Marks *et al.*, 1998).

Caspase-3 is one of the few common mediators of apoptosis and therefore activity of this enzyme is considered a reliable indicator for the quantification and qualification of apoptosis (Budihardjo *et al.*,1999). Caspase-3 is activated by excitotoxicity in CGNs (Nath *et al.*, 1998) and incubation of cerebro-cortico slice cultures with OGD medium (Nath *et al.*, 1998).

CGNs have also been used for many years to study necrotic cell death from a variety of stimuli including high concentrations of glutamate (Iorio *et al.*, 1993) and treatment with OGD insults (Juurlink and Hertz, 1993; Logan and Sweeney, 1997; Kalda and Zharkovsky, 1999) using release of the endogenous enzyme LDH as the indicator.

1.6. Hypothesis and specific objectives

In this investigation, the neuroprotective properties of the selective adenosine A1 receptor

agonist CPA were examined. Much of the neurodegeneration associated with ischemic damage is related to excitotoxicity in the cells (Obrenovitch and Urenjak, 1997). As described earlier, activation of A1 receptors can hyperpolarize neurons and inhibit glutamate release and the subsequent activation of post-synaptic glutamate receptors. Taken together, hyperpolarization would inhibit intracellular Ca^{2+} accumulation and increased ATP consumption, which are both associated with both apoptosis and necrosis. Additionally, adenosine A1 receptor activation is neuroprotective against both necrosis (Logan and Sweeney, 1997) after OGD and apoptosis induced after K^+ deprivation (Vitolo *et al.*, 1998). Therefore, the overall hypothesis of this project is that CPA will decrease both caspase-3 activity and LDH release in a concentration dependent manner in CGNs after simulated ischemia.

There were 4 main objectives to this project:

1) To examine caspase-3 activity and LDH release induced by a simulated ischemic insult of various lengths.

2) To examine neuroprotective properties of 1μM CPA (as measured by caspase-3 activity and LDH release) by treating CGNs for 1, 3 or 6 hours immediately prior to OGD insult.

3) To determine the most effective neuroprotective concentration of CPA.

4) To determine if concentration-dependent inhibition of CPA-mediated neuroprotection could be shown.

2.0. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemical additives for tissue culture growth media, experimental stroke and control media, polyvinyl alcohol with DABCO anti-fading agent, Triton X-100, acetone and lactate dehydrogenase were purchased from Sigma Aldrich (Oakville, ON). The minimal essential medium (MEM) which was obtained from Gibco (Burlington, ON). The CO_2 cylinders for the incubator were purchased from Island Oxygen (Charlottetown, PE) and the 95% N_2/5% CO_2 gas cylinders were purchased from Praxair (Charlottetown, PE). Assay kits for total protein and LDH detection were purchased from Diagnostic Chemicals Ltd. (Charlottetown, PE), the caspase-3 activity kits were obtained from Clontech (Palo Alto, CA) and the active caspase-3 enzyme was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Both primary antibodies (rabbit anti-glial fibrillary acidic protein [GFAP] polyclonal antibody and mouse anti-neuronal nuclear protein [NeuN] monoclonal antibody) and secondary (rhodamine labeled goat anti-rabbit and FITC labeled goat anti-mouse) antibodies were purchased from Chemicon Int. (Temecula, CA). Dry ice and halothane were obtained from Atlantic Veterinary College (AVC) Central Services and the AVC Pharmacy (Charlottetown, PE) respectively.

2.1.2. Equipment

Equipment used included a Forma Scientific water jacketed incubator (Fisher Scientific Ltd., Toronto, ON), a Forma Scientific laminar flow work station (Fisher Scientific Ltd., Toronto, ON), a Clay Adams centrifuge, a Corning pH meter 220 (Corning Scientific Products, Corning, NY) with a Lazar DO-166 dissolved oxygen probe (Lazar Research Ltd., Los Angeles, CA), a light microscope (Olympus America Inc., Melville, NY), a spectrophotometer (Amersham Pharmacia Biotech. Inc., Baie D'urfe, PQ), a rapid sampling pump (Amersham Pharmacia Biotech. Inc., Baie D'urfe, PQ), an Olympus confocal microscope (Olympus America Inc., Melville, NY), glassware (pipettes, Pasteur pipettes, Petri dishes) (Ultident Inc. St. Laurent, PQ), coverslips (Fisher Scientific Ltd. Toronto, ON), large and small scissors (Fisher Scientific Ltd. Toronto, ON), 15 and 50ml sterile tubes (Ultident Inc., Laurent, PQ,), 1.5ml microcentrifuge tubes (Ultident Inc., Laurent, PQ), spectrophotometric cuvettes (Ultident Inc., St. Laurent, PQ) and Deckglaser hemocytometer coverslips on a Spencer Bright-Line Neubauer hemocytometer (Sigma Aldrich, Oakville, ON).

2.2. Methods

2.2.1. Experimental animals

During the first year of this project, pregnant Wistar rats (10-14 days gestation) were

purchased from Charles River Inc. (Laval, PQ) and the following year rat breeding was initiated. The breeding animals were male and female Wistar rats (Charles River Ltd., Laval, PQ) which weighed 150-175g upon receipt. One to 3 females were paired with a male for 7 days and then separated into individual cages. All adult animals were housed in the North barn lab animal care facilities in the AVC in groups of 4-6, with access to food and water *ad libitum*. Rat pups matured to 6 or 7 days old prior to use in tissue culture. All protocols for this project were in accordance with the guidelines of the Canadian Council of Animal Care.

2.2.2. Petri dish and coverslip preparation for tissue culture

2.2.2.1. Sterilization

All CGN cultures, other than those designated for use with immunocytochemistry, were grown on pre-sterilized 35mm polystyrene Petri dishes. Cultures used in immunocytochemistry were plated on 20mm x 20mm glass coverslips. Coverslips were sterilized by incubation in 100% ethanol overnight at room temperature, after which ethanol was removed and coverslips were incubated at 60°C overnight and placed into sterile 35mm Petri dishes.

2.2.2.2. Maximization of attachment

To achieve adequate attachment of cells to Petri dishes or coverslips, surfaces were treated

with 0.1% poly-L-lysine in dH_2O. Briefly, 1.0ml of filter sterilized poly-L-lysine solution was added to each 35mm polystyrene Petri dish or on top of 22mm x 22mm coverslips in 35mm polystyrene Petri dishes. All Petri dishes and coverslips were then incubated at 37°C for a minimum of 30 minutes prior to solution removal and cell plating. The poly-L-lysine solution acts as a substrate for neurons to facilitate attachment of cells to the substrate and migration into groupings (Messer, 1977; Freshney, 1994). Increased cell attachment enhances formation and maintenance of neurite processes which increase the viability of CGN cultures (Messer, 1977).

2.2.3. Cell collection and dissociation

CGN cultures were prepared in batches using a total of 42 litters of 6-7 day old Wistar rat pups (ranging from 9-15 pups, both male and female). The pups were individually anesthetized in a staining jar using halothane and then decapitated with large scissors. The rostral portion of the skulls were excised using small scissors, cerebella were then removed using small curved forceps and placed into warmed sterilized solution A containing both glucose and bovine serum albumin (see appendix A). Glucose and bovine serum albumin were added in order to maintain viability of cells during collection and dissociation (Messer, 1977).

Meninges covering the surface of each cerebellum were removed and the neural tissue was disrupted with a sterilized razor blade. Cells in solution were then triturated with a sterile

Pasteur pipette to aid in dissociation and in the complete mixing together of the cerebella. Where more than one litter were used to create a batch, cerebella from different litters were pooled and thoroughly mixed together to negate possible differences between litters.

The cells in solution A were then divided between two sterile 15ml tubes and centrifuged at 300 x g in a Clay Adams centrifuge for 1 minute and the supernatant was discarded. In order to further dissociate cells, tissue clumps were resuspended in 8.0ml of solution B (Appendix A) (solution A plus 0.025% trypsin) and incubated at 37°C in a humidified 5% CO_2 incubator for 20 minutes with agitation every 5 minutes. The protease trypsin degrades protein connections between cells and therefore facilitates cell dissociation.

After incubation, 4.0ml of solution C (0.06% trypsin inhibitor and 0.006% DNAase) (Appendix A) was added to each tube. Due to cell lysis during dissociation, the presence of DNA in the media causes an increase in viscosity of the solution. This complicates separation of cells from the medium and causes cells to clump together, but by degrading the DNA with DNAse, the increase in viscosity is avoided (Freshney, 1994). Cells in solution were centrifuged in a Clay Adams centrifuge at 300 x g for 5 minutes and the supernatant was discarded. Cells were resuspended in 1.0ml of solution C, triturated with a sterile Pasteur pipette and left at room temperature for 5 minutes, allowing cell clumps to settle. The supernatant was then added to a sterile tube containing 3.0ml of pre-warmed minimal essential media #1 (MEM #1) containing fetal bovine serum, glucose, 24mM K^+ and essential amino acids (Appendix A). Clumped cells in each tube were then diluted in 1.0ml of

solution C, triturated and added to the MEM #1. Cells in MEM #1 were then centrifuged for 5 minutes at 300 x g. The supernatant was discarded and the pellet was resuspended in 1.0ml pre-warmed MEM #1.

The quantity of cells collected in the 1.0ml of MEM #1 was determined using an Olympus light microscope and a hemocytometer. Ten μL of the cell suspension was diluted 1:100 in 990μL MEM #1. Ten μl of this diluted solution was placed under a coverslip on a hemocytometer. Cells in each of the 5, $1mm^2$ grid sections were then quantified under light microscopy. Average cell counts from the squares were then divided by 5 for cells to be plated on coverslips, and by 10 for cells to be plated directly on Petri dishes. The difference between these formulae is to compensate for differences in area between the Petri dishes and coverslips. The result of the final calculation indicated the number of plates available from the quantified cells. Cells were plated at a concentration of 5×10^6 per coverslip in 300μL and at 7.0×10^6 in 600μL per Petri dish. As the cerebellum is made of 95% CGNs, and astrocytic content is low at this stage of development, quantification of visible cells is believed to be a reliable approximation (Messer, 1977).

On day 1 of culture, 2.0ml of sterile pre-warmed MEM #1 was added to the cultures. On day 2 of culture, MEM #1 was replaced by 3.0ml of pre-warmed MEM #2 containing horse serum and fluorodeoxyuridine on day 2 (Appendix A). Fluorodeoxyuridine is a mitotic inhibitor used to decrease mitosis of non-neuronal cells, such as astrocytes, in these cultures (Freshney, 1994). Culture media was not changed after day 2 in order to prevent glutamate-

induced damage resulting from medium changes (Driscoll *et al*, 1993). Throughout culture, cells remained at 37°C in a humidified incubator containing 5% CO_2.

2.2.4. Simulated ischemia

Krebs-Henseleitt solution containing 5.4mM K^+ (Appendix A) was used for both control and experimental media. To simulate a stroke *in vitro*, neurons were exposed to medium (OGD medium) that had been gassed with 95% N_2/5% CO_2 for approximately 30 minutes to make the solution hypoxic and lacked both glucose and essential amino acids (Logan and Sweeney, 1997; Ying *et al*., 1997; MacDonald and Stoodley, 1998; Tremblay *et al*., 2000). The non-OGD (control) medium contained 10mM glucose and essential amino acids and was gassed with 5% CO_2/95% ambient air in a incubator at 37°C for the duration of the treatment. Cultures were maintained in humidified incubators at 37°C with 5%CO_2/95% ambient air for the duration of the experiment. This protocol differed from those described above as cultures used in the above mentioned articles were exposed to an anaerobic environment during the ischemic insult rather than the transient hypoxic environment used in these experiments. The change in the protocol used in these experiments was done to enable the study of the effects of CPA on both apoptotic cell death. The OGD medium described above was used throughout the studies in this thesis and was considered less severe an insult than media described previously by Logan and Sweeney (1997).

The pO_2 values of both media (OGD and non-OGD) were measured using a pH meter with

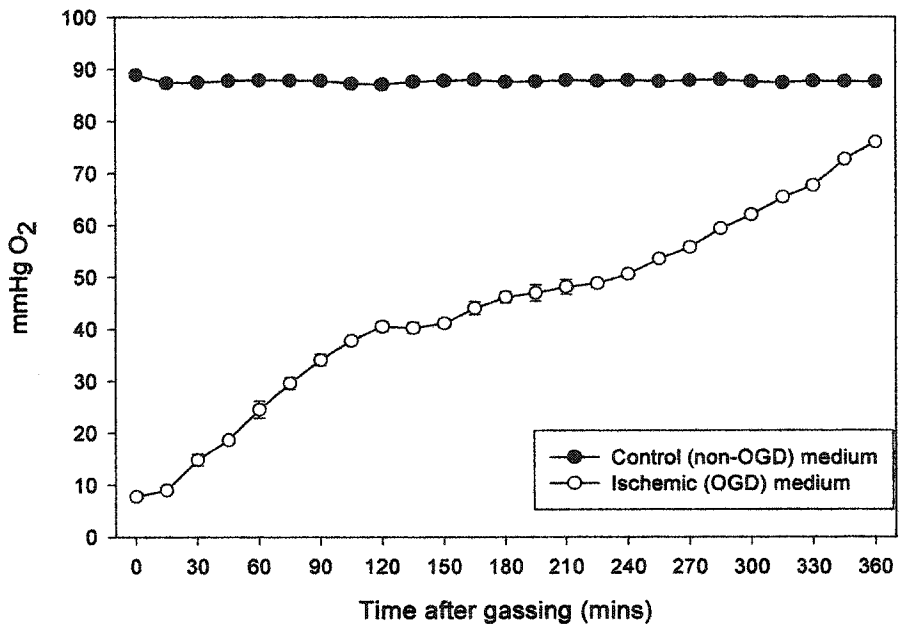
dissolved oxygen probe. The ischemic solution was gassed until the pO_2 level was 7.1-8.3mm Hg compared to a pO_2 of 88mm Hg in the non-OGD (control) medium (Figure 8). Problems occurred in measuring oxygen tension within the medium. It should be noted that while the oxygen tension in the OGD medium was much lower than that in the non-OGD, the absolute values reported in Figure 8 probably do not reflect pO_2 in the cultures. This is because measurements were made in a large flask. This is problematic as the depth of the medium in the flask is much greater than that of the Petri dish and rate of oxygen saturation would be much less at that depth. Therefore both the actual extent and length of the hypoxic insult would be significantly different than that shown in Figure 8.

The pH of both OGD and non-OGD solutions was determined with the same instrument. Findings indicated that the pH of non-OGD and OGD media were 7.44 ± 0.12 and 7.39 ±0.19, respectively. Following gassing, the average pH value for OGD was 7.36 ± 0.12, which was within the accepted physiological range defined at the beginning of this project.

2.2.5. Sample collection and analysis

Forms of necrotic and apoptotic cell death were measured using assay kits that measure caspase-3 activity in the cytosol and LDH in the extracellular medium. Samples for the caspase-3 assay (1.5ml of media and cells) and the LDH assay (1.5ml of medium only) were collected separately in 1.5ml microcentrifuge tubes. Caspase-3 samples were centrifuged

Figure 8: Effect of incubating OGD medium in a humidified environment containing 95% ambient air/5% CO_2 at 37°C for 6 hours after gassing with 95% N_2 / 5% CO_2 until pO_2 measured 7.11 to 8.35 mmHg (mean ± s.e.) (n=3). pO_2 values for OGD medium increased over a 6 hour incubation, while pO_2 values for non-OGD (control) medium (n=3) remained unchanged. Measurements of pO_2 for non-OGD medium were significantly ($p<0.05$) higher than that of OGD medium at all times during this experiment.



at 300 x g for 10 minutes, frozen on dry ice and stored at -80°C for 1 to 5 days until analyzed.

2.2.5.1. Caspase-3 assay

The caspase-3 assay used in this project was based on the principle that the proteolytic enzyme, caspase-3, selectively cleaves aspartate-glutamate-valine-aspartate (DEVD)-ρ-nitroanilide (ρNA). ρNA is the chromophore in the assay and absorbs light at 405nm. Activated caspase-3 removes ρNA from DEVD-ρNA, resulting in release of the colorimetric molecule and a corresponding increase in the absorbance of the sample. By utilizing a kit which only measures the activity of the caspase-3 enzyme, expression and pro-caspase content of the cells does not interfere with the assessment of cell stress.

Briefly, the caspase-3 assay involved incubating cells for 10 minutes in 100μL of cell lysis buffer on ice and extracting the cytosol by centrifugation at 400 x g at 4°C for 10 minutes. The 100μL of cytosol extract was divided into 2-50μl samples and each was added to 50 μl of reaction buffer containing 6mM dithiothreitol, used to obtain optimal activity of the enzyme. Five μl of DEVD-pNA substrate was added to only one tube as the other served as a control for background absorbance of the sample. Samples were incubated in a 37°C water bath for 1 hour and absorbance measurements were taken using a rapid sampling pump attached to a quartz cuvette in a spectrophotometer. The absorbance at 405nm of samples without substrate was then subtracted from absorbance measurements of samples with substrate. In order to calculate the number of units of caspase-3 activity, a ρNA standard

curve was generated (Appendix B). Absorbance measurements for 0, 25, 50, 100 and 200μM ρNA were taken and the slope of this curve was used in the equation:

caspase-3 units/hr = Δabs/hr x 1/(slope of the ρNA curve).

2.2.5.2. LDH assay

LDH catalyzes the conversion of L-lactate and NAD^+ to pyruvate and reduced nicotinamide adenine dinucleotide (NADH) + H^+. NADH causes an increase in absorbance of light at 340nm and the rate of increase in NADH is proportional to the amount of LDH present.

To perform the LDH assay, 50μl of medium was added to 2.5ml of LDH reaction buffer, which contained lactate and NAD^+. This solution was pre-warmed to 37°C and following addition of media sample, was incubated for a further 1.5 minutes prior to absorbance readings. Absorbance readings were then taken 0, 0.5, 1.0, 2.0 and 3.0 minutes after placing samples into the spectrophotometer. Units of LDH were determined by using the following formula as recommended by the manufacturer:

$$\text{LDH units/L} = \frac{\Delta\text{Abs/min x assay volume (ml) x 1000}}{\text{6.22 x light path (cm) x sample volume}}$$

The assay volume was 2.5ml, 6.22=absorbance coefficient of NADH at 340nm, the light path was 1cm and the sample volume was 25μL.

2.2.5.3. Protein assay

The total protein assay was based on the Biuret reaction which is based on the ability of protein to complex with copper in an alkaline reagent to form a blue-violet colored molecule. The assay was performed by resuspending cell remnants from the caspase-3 assay in protein reagent and incubating the sample for 10 minutes at room temperature. The samples were then placed in the spectrophotometer and changes in absorbance were measured at a wavelength of 540nm. Units of protein were then calculated by the following formula:

$$\text{Total protein g/L} = \frac{\text{abs (sample)}}{\text{abs (standard)}} \times \text{concentration of standard}$$

Weight of protein was used to normalize all caspase-3 and LDH measurements to control for the number of cells on the plates.

2.2.6. Determination of glial population in CGN cultures

To determine the proportions of neurons and astrocytes in the cultures, quantitative immunofluorescence using a neuron-specific antibody (NeuN) and a glial-specific antibody (GFAP) was performed. Briefly, cultured CGNs were fixed with cold (3°C) acetone for 3 minutes and rinsed twice with phosphate buffered saline (PBS). Cells were then incubated for 1 hour at room temperature with PBS containing 2% horse serum and 0.3% triton X-100 (PBS-HS-T). Horse serum was included to block non-specific binding, and triton X was included to permeabilize cells, thereby allowing antibodies access to the intracellular antigens NeuN and GFAP.

Cultures were incubated overnight with the primary antibodies, rabbit anti-GFAP and mouse anti-NeuN diluted 1:500 and 1:50, respectively in PBS-HS-T. Following incubation, plates were rinsed 3 times in PBS-HS-T and incubated for 2 hours with secondary antibodies diluted to 1:100 in PBS-HS-T (rhodamine tagged goat anti-rabbit for GFAP and Fluorescein Isothiocyanate [FITC] tagged goat anti-mouse for NeuN). Coverslips were then rinsed 3 times with PBS-HS-T and dipped in distilled water to rinse off any excess salts. Water was removed from coverslips using an absorbant tissue, a single drop of polyvinyl-alcohol mounting medium was added to facilitate proper mounting of the coverslips and coverslip edges were sealed with nail polish. This mounting medium contained DABCO, an antioxidant agent which was used to prevent loss of fluorescence associated with time. This chemical decreases ROS produced by the excitation of a fluorescent tag, thereby preventing fading.

Slides were viewed using the blue and green filter cubes of a laser scanning confocal microscope which allowed the imaging of the FITC and rhodamine tags respectively. Images for both antibodies were then captured using FluoView software (Olympus America Inc., Melville, NY). A 50 square grid was superimposed on the magnified images (200x), and 10 random computer generated numbers were chosen between 1 and 50 to determine which of these squares would be counted. Neurons were quantified in each of the 10 selected squares and the sum was multiplied by 5 to give an approximate total neuronal cell count. Glial cells were then quantified throughout the image. The number of glial cells was then divided by total cell count and multiplied by 100 to give a percentage contamination of the cultures

(n=5). The average amount of contamination and standard error of the mean were calculated.

2.2.7. Experimental protocols

Six replicates (plates) were used in all the experiments. This number of replicates is generally held to provide sufficient statistical power for detection of significant differences between treatments. Plates were randomly assigned to treatment group such that, in almost all the experiments, batches were represented equally across groups (i.e. one plate per batch was found in each treatment group). However, in the case of 6 treatment groups, the 6 plates within each group were derived from only 5 batches of cells.

2.2.7.1. Determination of maximal cell death

In an attempt to assess the severity of the OGD insult used in this project, measurements of maximal caspase-3 activity and LDH release measurements were made using stimuli known to induce only apoptosis or necrosis in this model. Maximal caspase-3 activity was induced by replacing control growth medium (MEM #2) containing 24mM K^+, with growth medium containing only 5mM K^+. Cells were then incubated for 24 hours prior to sample collection (Galli *et al.*, 1995; Logan and Sweeney, 1997; Vitolo *et al.*, 1998).

Maximal necrotic cell death was induced by exposing neurons to 20mM glutamate in Krebs' solution for 20 minutes prior to sample collection of the mean. LDH contained in the

remaining intact cells after glutamate insult was extracted by the incubation of cells with cell lysis buffer. LDH measurements were made from both media and cytosol samples to determine the percentage of LDH released by the glutamate incubation. Protein measurements from cultures exposed to glutamate were then compared to those of untreated cells to give an to estimate of the percentage cell death.

2.2.7.2. OGD medium incubation lengths and sampling times

To determine the most effective incubation length of OGD medium to induce both caspase-3 activity and LDH release, cultures were incubated with OGD medium for 1,3 or 6 hours (Figure 9A). Additionally, to determine the optimal time for sample collection, cells were collected 0, 1, 3, 6, 12 or 24 hours after replacement of OGD medium with control growth medium (Figure 9A). The length of cell incubation with OGD medium which induced significant caspase-3 activity and LDH release, and the time of sampling which provided the best measurement of both cell death indicators were chosen for use in all subsequent experiments.

2.2.7.3. Duration of CPA treatment

The effect of various lengths of 1μM CPA incubations prior to OGD insult were examined to determine the relationship between CPA incubation time and the amount of neuroprotection. Cells were incubated with 1μM CPA either during the OGD insult or for

Figure 9: Experimental designs for each investigation. A) To determine most effective protocol for OGD-induced cell damage. B) To examine the effect of CPA treatment on OGD-induced damage. C) To determine efficacy of CPA for both caspase-3 activity and LDH release. D) To determine the efficacy of DPCPX-induced inhibition of A1 receptor mediated neuroprotection. ☐ Denotes cells incubated in growth medium (MEM #2) while ▒ denotes cells incubated with OGD or non-OGD medium.

A)

Cells used were 6 or 7 days old	1, 3, or 6 hour treatment	Cells were then collected immediately, or 1, 3, 6, 12 or 24 hours after treatment.

B)

Cells used were 6 or 7 days old	Treatment with 1μM CPA for 1, 3 or 6 hours	Treatment of some cells with 1μM CPA. OGD/non-OGD treatment was performed as determined in experiment A.

C)

Cells used were 6 or 7 days old	Cells were treated with vehicle (PBS) or 1μM DPCPX for 30 minutes prior to CPA	Vehicle (PBS) or 0.01, 0.1, 0.3 or 1μM CPA treatment for duration determined in B. Treatment occurred 0, 1, 3, 6 or 24 hours prior to OGD/non-OGD treatment	OGD/non-OGD treatment was performed as determined in

D)

Cells used were 6 or 7 days old	Vehicle (PBS) or 0.01, 0.03, 0.1, 0.3 or 1μM DPCPX treatment for 30 minutes prior to 1μM CPA administration	OGD/non-OGD treatment was performed as defined in experiment A.

1, 3, or 6 hour durations prior to administration of OGD medium (Figure 9B). These data were used to determine the duration of CPA treatment that gave the greatest amount of neuroprotection against both caspase-3 activity and LDH release. The most effective treatment length was used in all subsequent experiments.

2.2.7.4. CPA concentration response determinations and effect of A1 antagonist

In order to verify that any neuroprotection demonstrated in the previous experiment resulted from activation of adenosine A1 receptors, concentration-response curves were generated and the effect of a selective adenosine A1 antagonist, DPCPX, on CPA-mediated neuroprotection was examined. DPCPX (1μM) or vehicle (PBS) was added to control growth medium on cultures for 30 minutes before CPA or vehicle administration. CPA was added to the medium in concentrations of 0.01, 0.1, 0.3 or 1μM and cells were then incubated for a predetermined time (see section 2.2.7.3). Control cell cultures were incubated with the vehicle (PBS) alone. At the end of the treatment, the medium was replaced with fresh control growth medium and cells were incubated for 0, 1, 3, 6 or 24 hours prior to treatment with OGD medium (Figure 9C). EC_{40} values were calculated by assuming that maximal neuroprotection corresponded to 0 units of caspase-3 activity and LDH release. LDH and caspase-3 values from cells exposed to the simulated ischemic insult but administered only PBS were used as the 0% inhibition values.

2.2.7.5. Concentration response determinations of A1 receptor antagonist

To further examine if CPA-induced neuroprotection was mediated by A1 receptors, a concentration response curve for the selective A1 receptor antagonist DPCPX was generated. Vehicle (PBS) or DPCPX in concentrations of 0.01, 0.03, 0.1, 0.3 and 1μM was administered 30 minutes prior to CPA (1μM) or vehicle (PBS) addition. Cells were then incubated for the duration determined in section 2.2.7.3. The chosen length of time between drug treatment and incubation of cells with OGD medium was determined by the time of CPA pretreatment which mediated the greatest amount of neuroprotection in section 2.2.7.4.

2.2.8. Statistical analysis

Plates were the statistical units of concern. Raw data of caspase-3 and LDH measurements were analyzed by 2-way analysis of variance (ANOVA) to determine if there were any significant differences between groups. Not all data conformed to the assumptions of ANOVA. All groups were independent and had homogeneity of variance, but not all had Gaussian distribution. Those data which were not normal underwent a 1-way ANOVA (non-parametric) and statistical differences detected did not differ from that of a 2-way ANOVA. The small number of replicates per treatment group made it impossible to test for a batch effect (heterogeneity of variance). However, a batch effect is unlikely because batches were equally represented across most of the groups. The only exceptions, as mentioned in section 2.2.7, were six treatment groups within each of which, two of the six plates (replicates) came

from the same batch of cells. When data from the duplicate plates were removed from the relevant analyses (some of which were significant), there were no changes in significance. This indicates that the likelihood of a batch effect in those experiments, was low.

All ANOVA and Tukey's tests were performed using the software Minitab 12® (Minitab Inc., State College, PA). Statistical differences were defined as having p-values of less than 0.05. An example of the ANOVA output tables is given in Appendix C. Linear regression and coefficient of variation were calculated for all of the standard curves using Sigma Plot® versions 6 and 7 (SPSS Science, Chicago, IL). Line graphs which had regression coefficients of greater than 0.98 were considered straight lines.

3.0. Results

3.1. Assessment of tissue culture purity

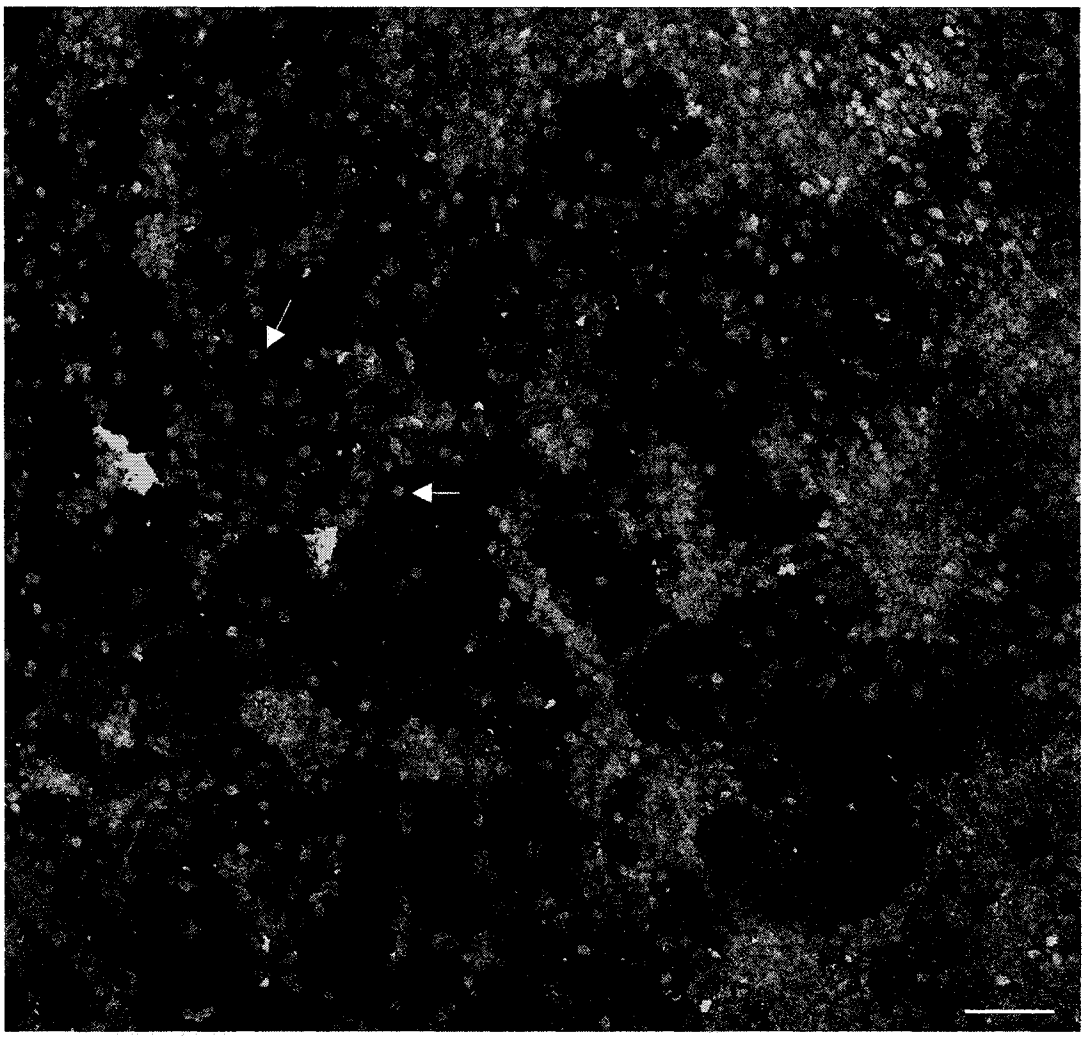
Using immunocytochemistry and confocal microscopy, granule neurons and glial cells were visualized. Cambray-Deakin (1990) reported rat CGN cell somas to have diameters of 10-12μm. CGN cell bodies are known to consist mainly of nuclei (Messer, 1977) and therefore structures detected by NeuN should be similar in size. Cells staining positively for the rat anti-NeuN antibody (Mullen *et al.*, 1992) in this study, were similar in size and form to the description of cultured CGNs in the literature (Messer, 1977) (Figure 10A).

The glial cells detected were morphologically similar to those described in the literature; namely, having cell bodies 20-30μm in diameter with diverse astrocytic processes (Daniels and Brown, 2001). Also, no fluorescence was seen in the nuclei of glial cells which was expected as GFAP is a cytoskeletal protein (Choi and Kim, 1984) (Figure 11A). Fluorescence was not detected in any of the negative control images (secondary antibody only) when examined with either the wavelength used for FITC or rhodamine (Figures 10B and 11B).

The mean of the neuron cell count (± s.e.) was 2010.8 ± 117.1 cells per image (representing 1/800 of total area of the coverslip) (Figure 10A), compared to an mean glial cell count of 29.2 ± 1.9 cells per image (Figure 11A). Therefore, an estimation of the glial content in

Figure 10: Representative images of antibody labeling of neuronal cells in cerebellar granule cultures. A) primary mouse-anti-NeuN and secondary FITC-labeled anti-mouse antibodies. Scale bar = 50μm. B) secondary FITC-labeled anti-mouse antibody only. Arrows indicate quantifiable neurons. Scale bar = 100 μm.

A)



B)

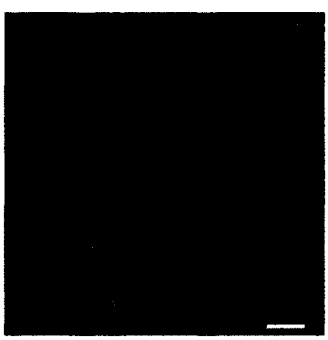
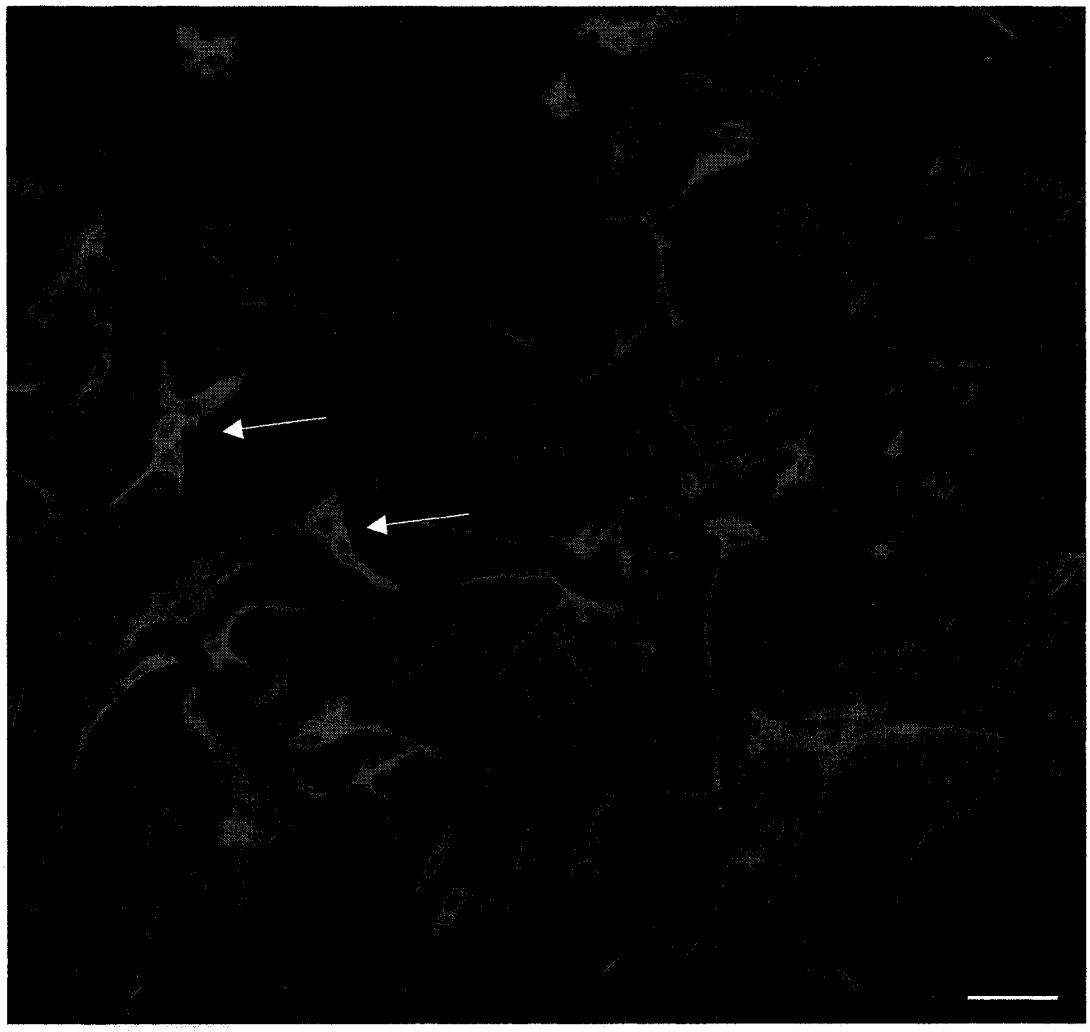
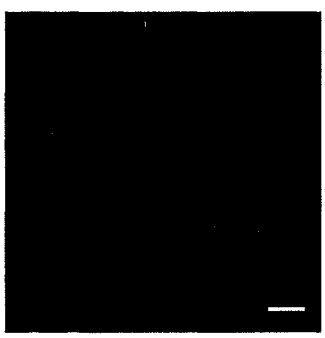


Figure 11: Representative images of antibody labeling of glial cells in cerebellar granule cultures. A) primary rabbit-anti-GFAP and secondary rhodamine-labeled anti-rabbit antibodies. Scale bar = 50μm. B) secondary rhodamine-labeled anti-rabbit antibodies only. Arrows indicate quantifiable glial cells. Scale bar = 100μm.

A)



B)



these cultures is 1.4% of total cell number.

3.2. Maximal caspase-3 and LDH measurements

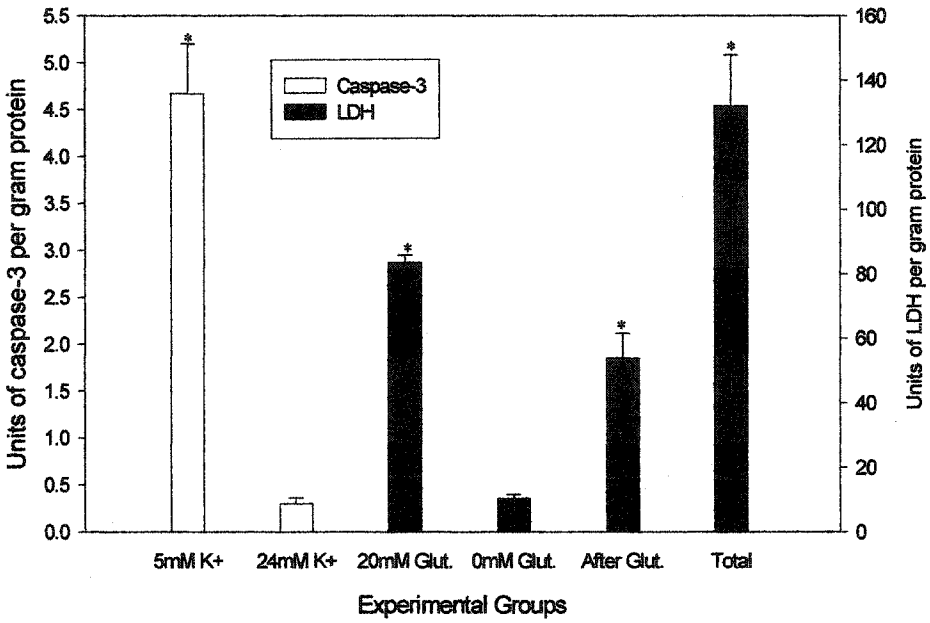
3.2.1 Apoptotic cell death

To induce apoptotic cell death, cells were incubated for 24 hours with medium containing only 5mM K^+ which greatly differed from the control medium which contained 24mM K^+. Cells exposed to the K^+ deprived medium had a mean (± s.e.) of 4.67 ± 0.53 units of caspase-3 per gram protein compared to 0.30 ± 0.06 for cells incubated with control medium (Figure 12). The amount of caspase-3 activity induced by K^+ deprivation was considered maximal and was used to calculate the percentage of maximal caspase-3 activity in subsequent experiments.

3.2.2 Necrotic cell death

After incubation of cells for 20 minutes in Krebs' solution containing 20mM glutamate, a mean (± s.e.) of 83.53 ± 2.25 units of LDH per gram protein were released into the extracellular medium (Figure 12). A further 53.89 ± 7.59 units of LDH per gram protein were released following incubation with cell lysis buffer. In comparison, in cells exposed to control solution (Krebs', 0mM glutamate) for 20 minutes, only 10.32 ± 1.23 units LDH per gram protein were measured in the medium. When these cells were lysed, 132.13 ±

Figure 12: Effect of a 24 hour incubation of cells with K^+-deprived (5mM K^+) medium on caspase-3 activity and a 20 minute incubation of 20mM glutamate on LDH release. K^+-deprived medium increased caspase-3 activity over that of cells exposed to control (24mM K^+) medium. Incubating cells with glutamate induced LDH release above that of control, but further LDH was released by cell lysis. * denotes significant differences ($p<0.05$) from the respective control. Values are expressed as mean ± s.e. n=6 for all groups.



15.79 units of LDH per gram protein were found in the medium, indicating a total LDH content of approximately 142.45 units per gram protein. The amount of LDH released as a result of glutamate incubation (83.53 ± 2.25 units of LDH per gram protein) was used as the maximal LDH release.

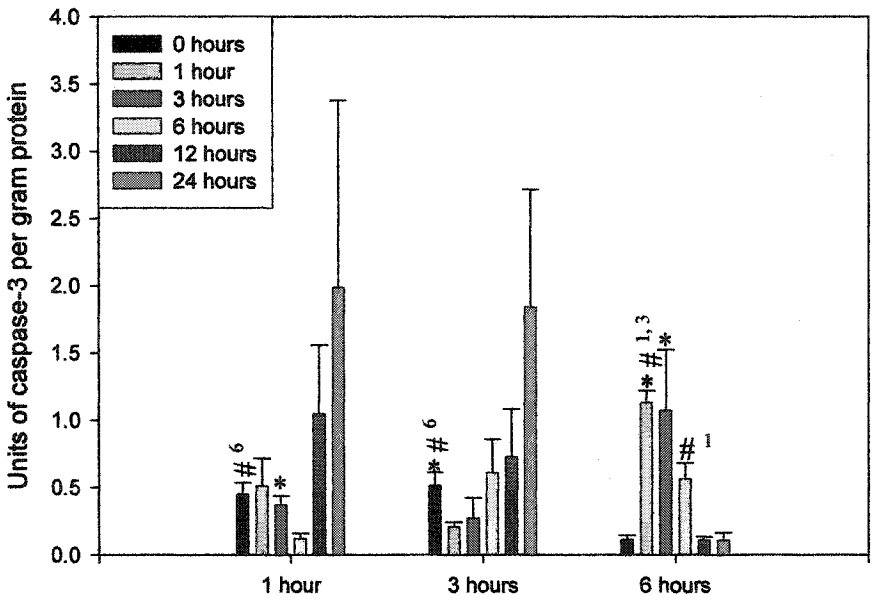
3.3. OGD induced both caspase-3 activity and LDH release

To determine the effect of varying lengths of OGD insult and times of sample collection on cell death markers the following experiment was performed. CGNs were incubated with OGD or non-OGD medium for 1, 3 or 6 hours and samples were collected 0, 1, 3, 6, 12 or 24 hours following replacement of experimental media with control growth medium (see Figure 9A). Caspase-3 activity in the cytosol and LDH release in the medium were then measured. Changes in caspase-3 activity or LDH release in cells exposed to OGD insults were compared to the activity seen in complementary control cells. A complementary control group was defined as having been exposed to the same duration of experimental medium and having the sample collected at the same time after insult.

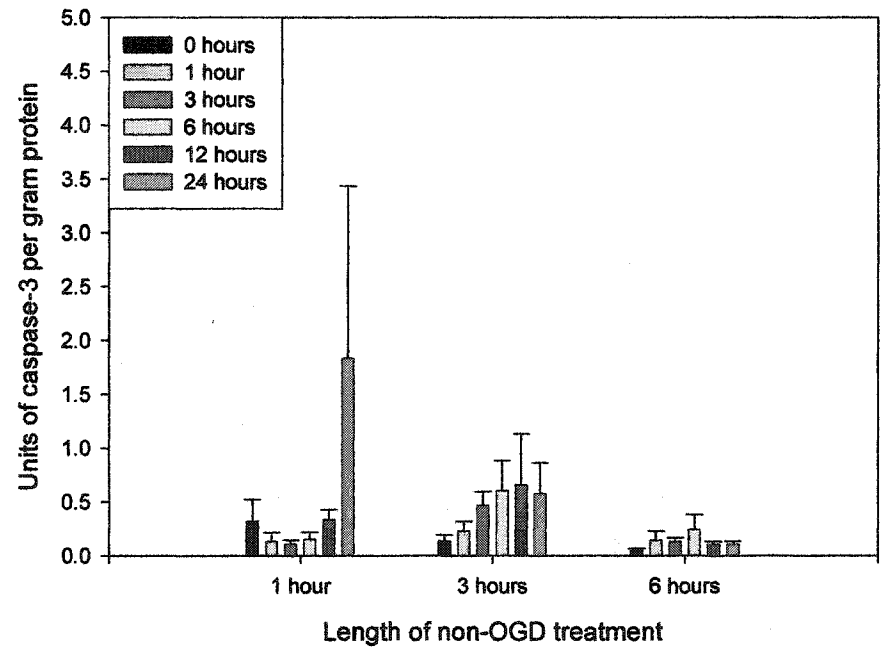
All durations of OGD insult increased caspase-3 activity. However, the times that peak activity occurred were dependent on the length of time that cells were exposed to the OGD medium (Figure 13A). Three hours after cells were exposed to a 1 or 6 hour OGD insult, caspase-3 activity increased over the complementary control. The increased caspase-3 activity immediately after a 3 hour OGD insult was also higher than the values of the

Figure 13: Effect of OGD (A) or non-OGD (B) treatment on caspase-3 activity in cells treated for 1, 3 or 6 hours and samples collected 0, 1, 3, 6, 12 or 24 hours after treatment. All lengths of OGD treatment increased caspase-3 activity was increased with the most significant increases after a 6 hour treatment. Data are expressed as a percentage of non-OGD values. Values are expressed as mean ± s.e. (n=6). # denotes a significant ($p < 0.05$) difference, for a given time of sample collection, a significant difference from the length of treatment indicated by the superscript. * denotes a significant difference ($p<0.05$) between OGD (Fig. 13A) and non-OGD (Fig. 13B) incubation lengths at the same time of sample collection.

A)



B)



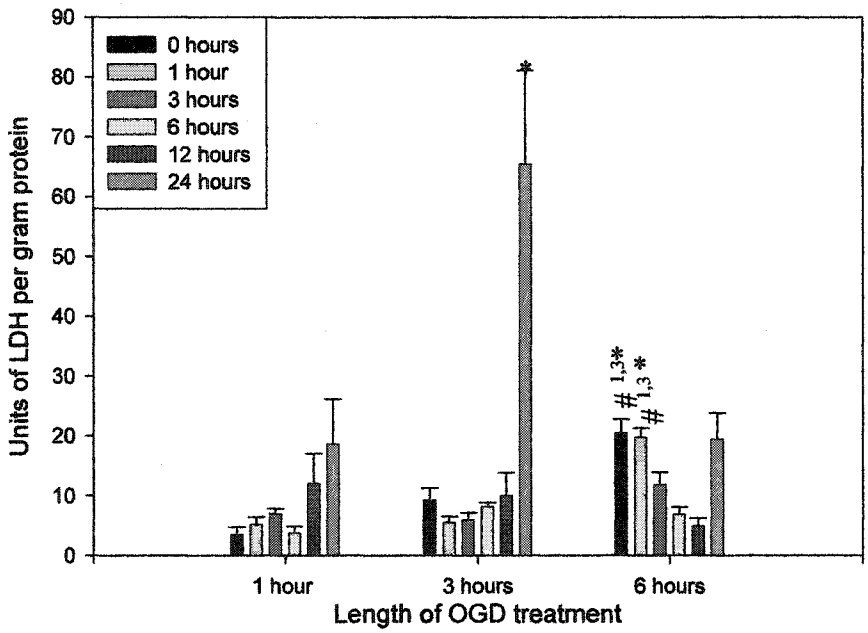
complementary control cells. A 6 hour treatment of cells with OGD medium produced the most significant increases in caspase-3 over that of control. One hour after OGD insult, caspase-3 activity increased over both the complementary control values and those of cells incubated with OGD medium for either 1 or 3 hours. Six hours after cells were exposed to a 6 hour OGD insult, caspase-3 activity was higher than the values for cells incubated with OGD for 1 hour. Incubation of cells with non-OGD medium for 1, 3 or 6 hours did not affect caspase-3 activity (Figure 13B).

A 1 hour incubation of OGD medium did not induce LDH release, above complementary control, at any time after insult (Figure 14A). Twenty-four hours after a 3 hour OGD insult LDH release was increased over control and was the highest measurement of LDH release in this experiment. Immediately, and 1 hour after cells were treated with OGD medium for 6 hours, LDH release was higher than that of complementary control measurements (Figure 14A). The increases in activity seen at these times were also higher than cells exposed to either 1 or 3 hour OGD insults. Surprisingly, 24 hours after a 1 hour incubation of non-OGD medium LDH release was significantly higher than that of cells incubated with non-OGD medium for 6 hours (Figure 14B).

The data presented in Figures 13 and 14 were then transformed to express caspase-3 activity or LDH release as a percentage of the non-OGD measurements. This was done in order to negate the variations in the control samples and the resulting values are presented in Figure 15.

Figure 14: Effect of OGD (A) or non-OGD (B) treatment on LDH release in cells treated for 1, 3 or 6 hours and samples collected 0, 1, 3, 6, 12 or 24 hours after treatment. Only cells treated for 6 hours OGD medium demonstrated increased LDH release. Data are expressed as a percentage of non-OGD values. Values are expressed as mean ± s.e. (n=6). # denotes a significant ($p < 0.05$) difference, for a given time of sample collection, a significant difference from the length of treatment indicated by the superscript. * denotes a significant difference ($p<0.05$) between OGD (Fig. 14A) and non-OGD (Fig. 14B) incubation lengths at the same time of sample collection.

A)



B)

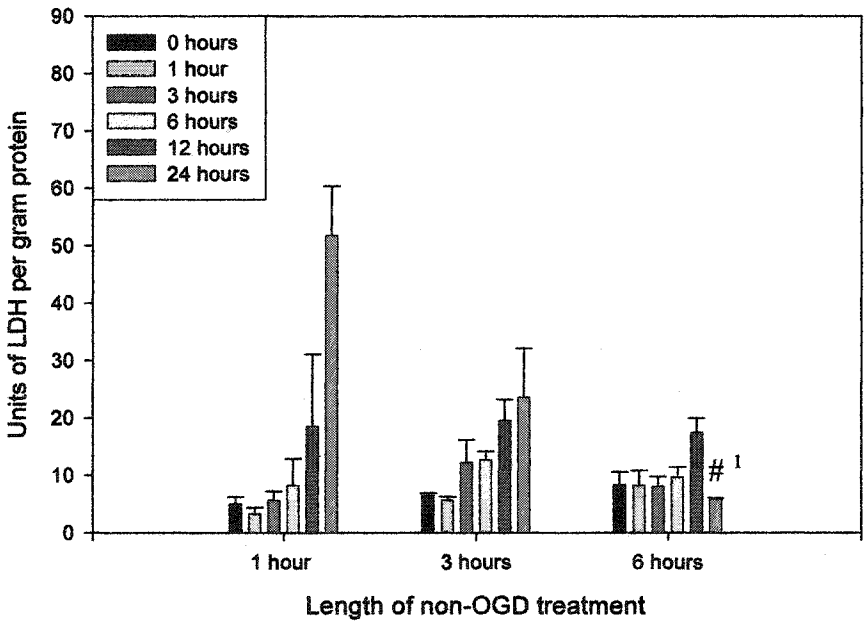
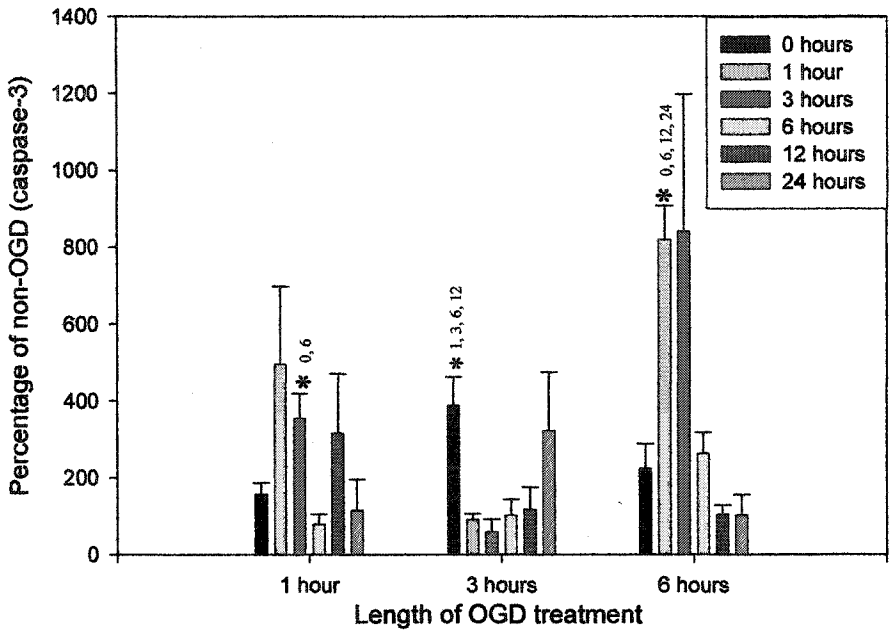


Figure 15: Effect of the OGD treatment length on the percentage increase in caspase-3 activity (A) and LDH release (B) over control. Both cell death indicators increased with longer incubations of OGD. Data are expressed as a percentage of non-OGD values. Values are expressed as mean ± s.e. (n=6). * denotes a significant ($p < 0.05$) difference from the sample collected at the hour(s) indicated in superscript.

A)



B)

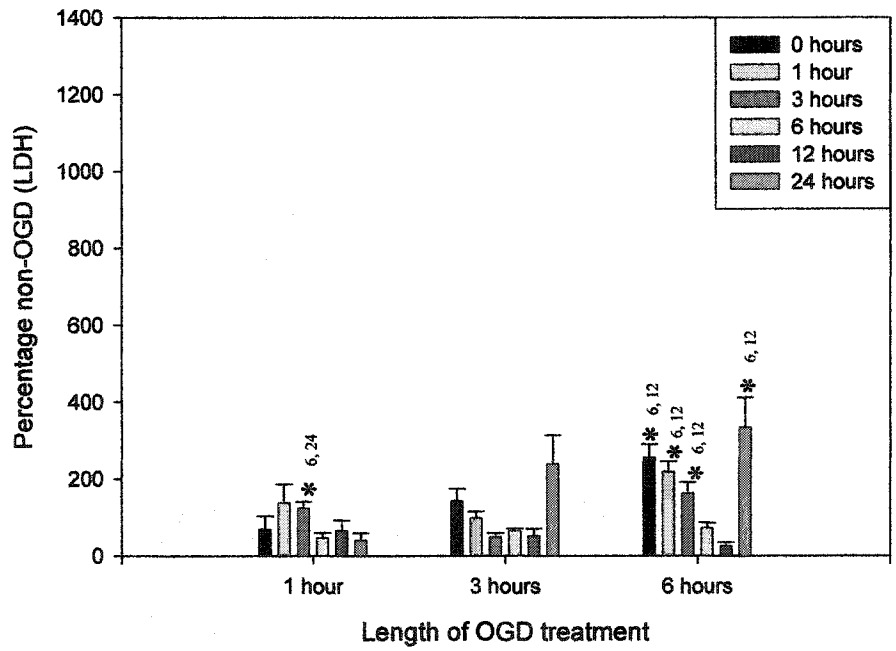


Figure 15 demonstrates that there are variations in measurements of caspase-3 activity and LDH release within each incubation time, independent of control values. When expressing the data in this way, some of the significant differences between sampling times indicated in Figures 13 and 14 are no longer present. Caspase-3 activity peaked 3 hours after cells were incubated with OGD medium for 1 hour (Figure 15A). At this time, values were significantly higher than the activity measured both immediately and 6 hours after insult. Treating cells with OGD for 3 hours induced a peak in caspase-3 activity immediately after insult, which was higher than that of samples collected 1, 3, 6 or 12 hours after insult. Caspase-3 activity peaked 1 hour after cells were incubated with OGD for 6 hours and this value was higher than measurements taken immediately or 6, 12 and 24 hours after insult.

Three hours after cells were treated with OGD medium for 1 hour, LDH release was increased over measurements taken 6 and 24 hours after the insult (Figure 15B). There were no significant increases in LDH release after a 3 hour OGD insult. A 6 hour incubation of OGD medium induced significant increases in LDH release which peaked immediately after insult and was sustained for 3 hours. LDH release decreased in samples taken 6 and 12 hours after the 6 hour OGD insult, but increased again 24 hours after treatment. Both caspase-3 activity and LDH release were increased 1 hour after cells were treated with OGD medium for 6 hours. Therefore, in all subsequent experiments, simulated ischemia was induced by a 6 hour incubation of OGD and samples were collected 1 hour after insult.

3.4. CPA-mediated neuroprotection

To examine the possible neuroprotective effects of adenosine receptor activation, the selective adenosine A1 receptor agonist CPA (1μM) was administered either during OGD incubation or 1, 3, or 6 hours immediately prior to the insult. All CPA treatments decreased OGD-induced caspase-3 activity and LDH release. At maximum, values of caspase-3 activity were reduced by a mean (± s.e.) of 98.1 ± 1.5% and LDH release by a mean (± s.e.) of 80.2 ± 1.7% after treatment with CPA for three hours prior to OGD (Figure 16). A 3-hour CPA treatment also produced greater neuroprotection against caspase-3 activity than treatment during the OGD insult (Figure 16). These data support a role for CPA-mediated neuroprotection against simulated ischemic cell death as measured by caspase-3 activity and LDH release. A 3-hour CPA treatment was chosen for use in all subsequent experiments as it provided significant neuroprotection against caspase-3 activity and LDH release.

To further explore CPA-mediated neuroprotection, cultures were pretreated with CPA at various concentrations and at different times prior to OGD insult (Figure 9C). In cells exposed to CPA pretreatment immediately, or 1, 3,6 or 24 hours before OGD insult, only the 1μM concentration of CPA-induced significant neuroprotection against caspase-3 activity at all times of treatment (Figures 17-21A). One and 0.3μM concentrations of CPA decreased caspase-3 activity when administered 6 hours before cells were treated with OGD medium (Figure 20A). At all times of pretreatment, LDH release was only inhibited by the 1μM concentration of CPA (Figures 17B-21B).

Figure 16: Effect of various durations of 1μM CPA treatment on caspase-3 activity and LDH release. CPA decreased both cell death indicators at all treatment lengths. All values are expressed as a percentage inhibition of caspase-3 activity or LDH release of the 0μM group. * denotes significant ($p < 0.05$) difference from 0μM CPA group. # denotes a significant difference from samples exposed to the length of OGD indicated as a superscript. n=6 for all groups.

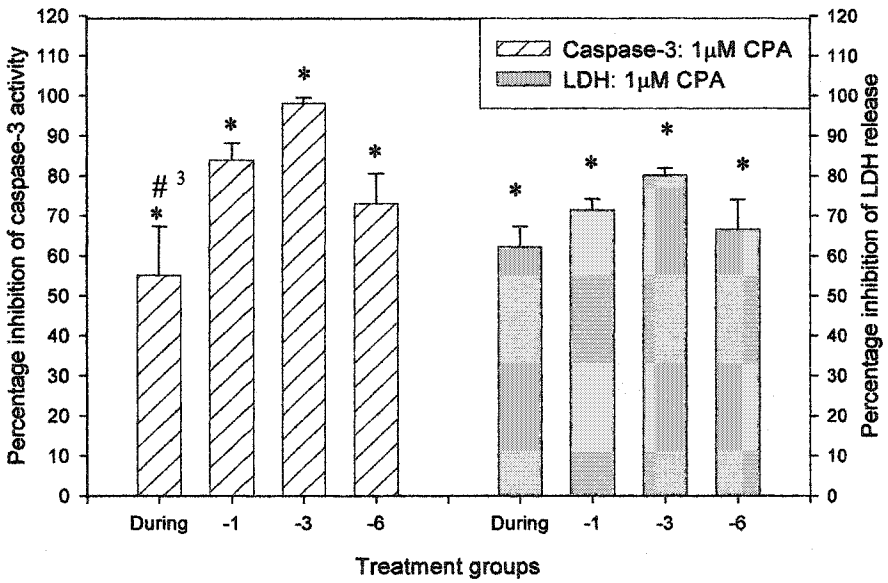
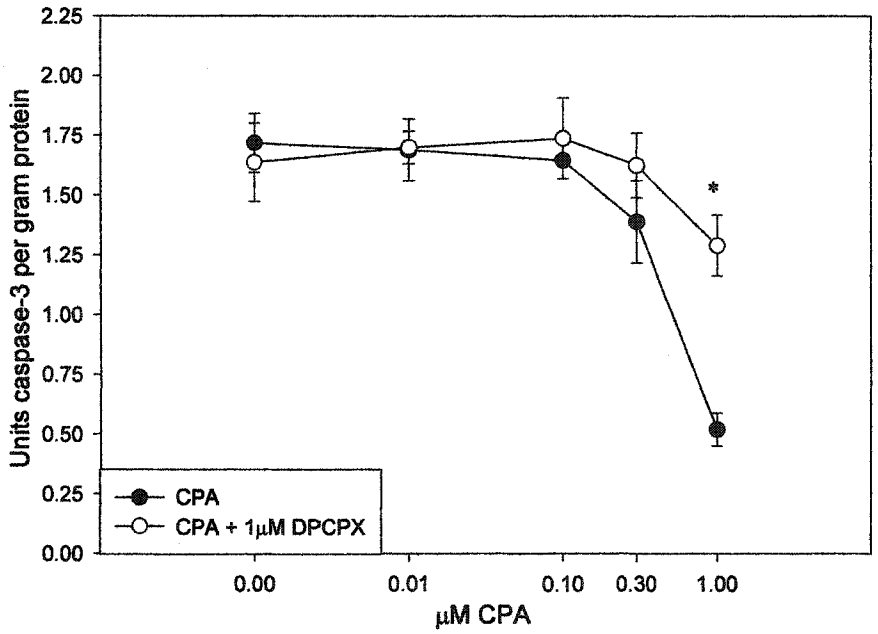


Figure 17: Effect of a 3 hour treatment of various concentrations of CPA (log scale) on both caspase-3 activity (A) and LDH release (B) when administered immediately prior to an OGD insult. The effect of DPCPX (1μM) when administered 30 minutes prior to CPA/vehicle was also examined. Neuroprotection induced by 1μM CPA was reversed by DPCPX. All values are given as mean ± s.e. (n=6). * denote significant differences from CPA in the absence of DPCPX.

A)



B)

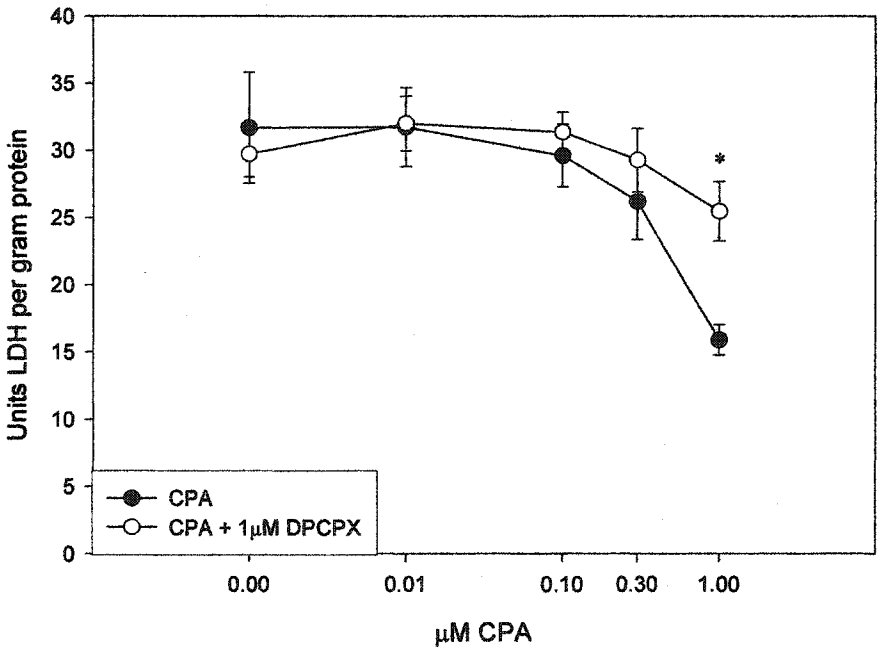
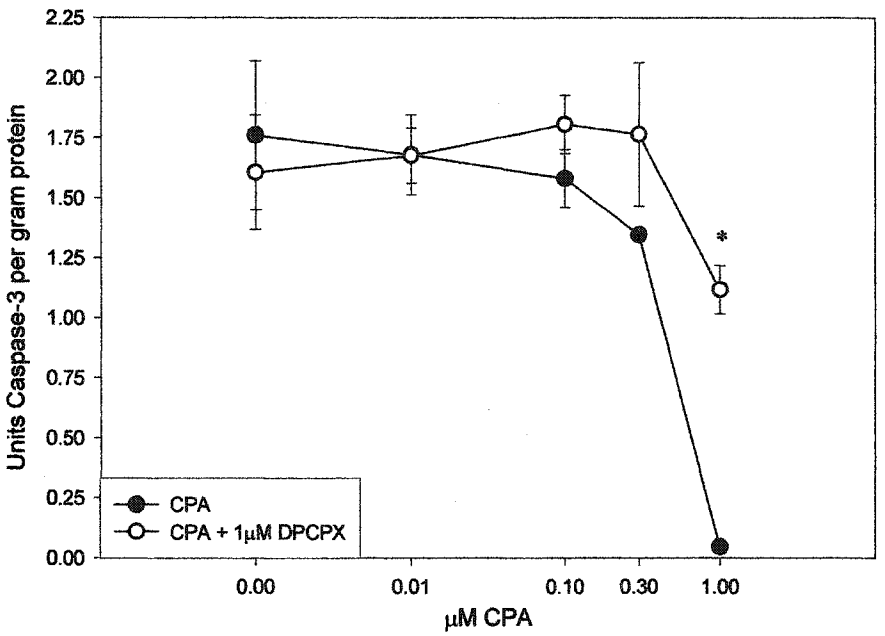


Figure 18: Effect of a 3 hour treatment of various concentrations of CPA (log scale) on both caspase-3 activity (A) and LDH release (B) when administered 1 hour prior to an OGD insult. The effect of DPCPX (1μM) when administered 30 minutes prior to CPA/vehicle was also examined. Neuroprotection induced by 1μM CPA was reversed by DPCPX. All values are given as mean ± s.e. (n=6). * denote significant differences from CPA in the absence of DPCPX.

A)



B)

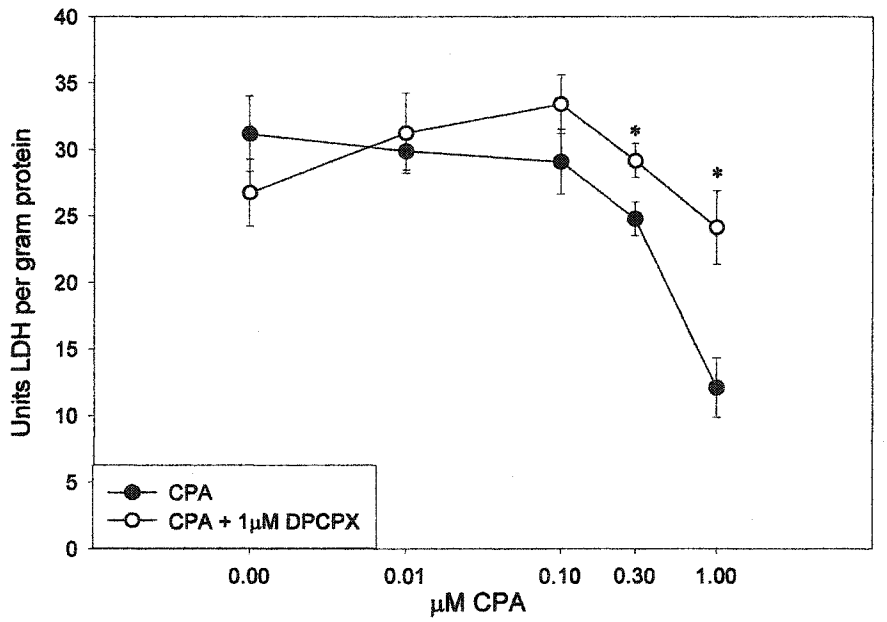
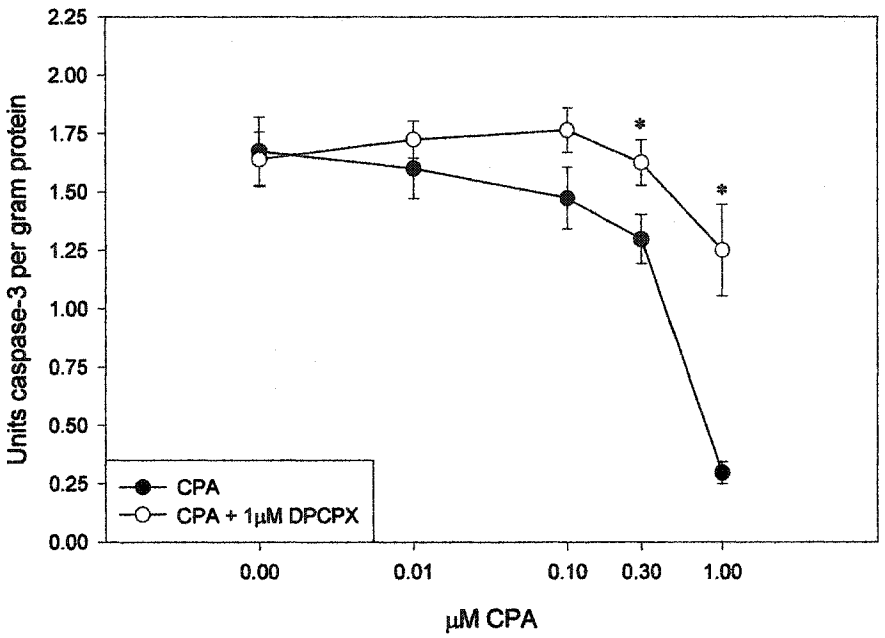


Figure 19: Effect of a 3 hour treatment of various concentrations of CPA (log scale) on both caspase-3 activity (A) and LDH release (B) when administered 3 hours prior to an OGD insult. The effect of DPCPX (1μM) when administered 30 minutes prior to CPA/vehicle was also examined. Neuroprotection induced by 0.3 and 1μM CPA was reversed by DPCPX. All values are given as mean ± s.e. (n=6). * denote significant differences from CPA in the absence of DPCPX.

A)



B)

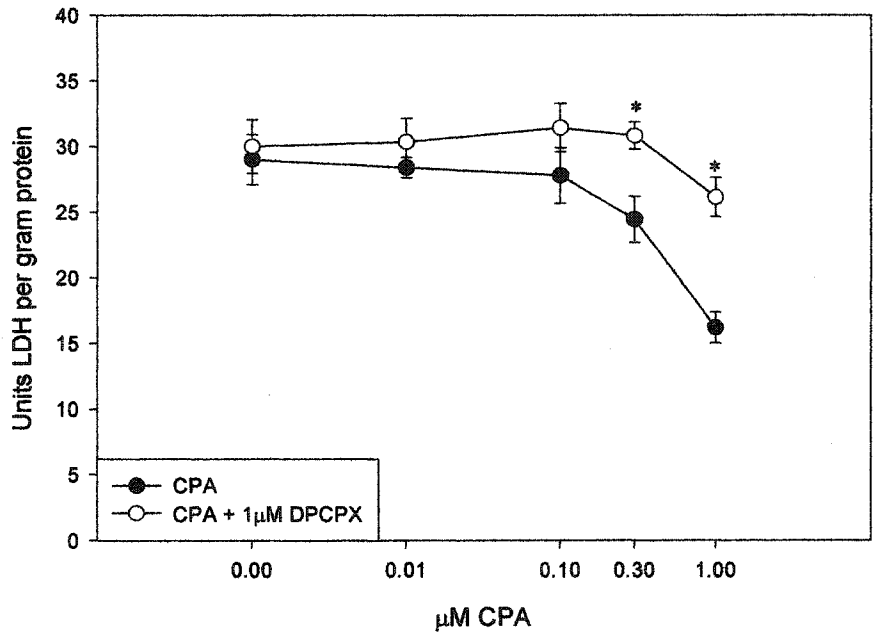
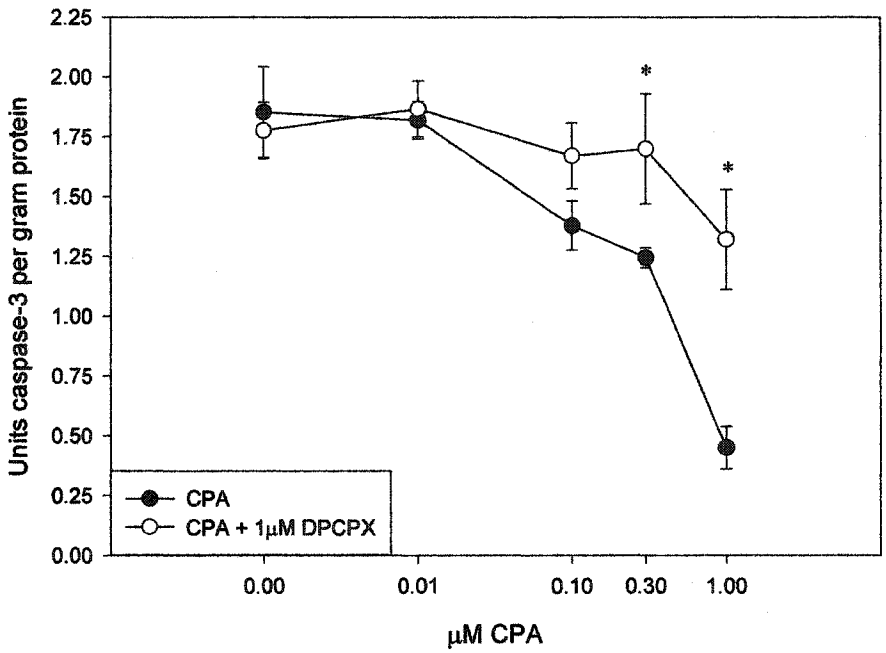


Figure 20: Effect of a 3 hour treatment of various concentrations of CPA (log scale) on both caspase-3 activity (A) and LDH release (B) when administered 6 hours prior to an OGD insult. The effect of DPCPX (1μM) when administered 30 minutes prior to CPA/vehicle was also examined. Neuroprotection induced by 1μM CPA was reversed by DPCPX. All values are given as mean ± s.e. (n=6). * denote significant differences from CPA in the absence of DPCPX.

A)



B)

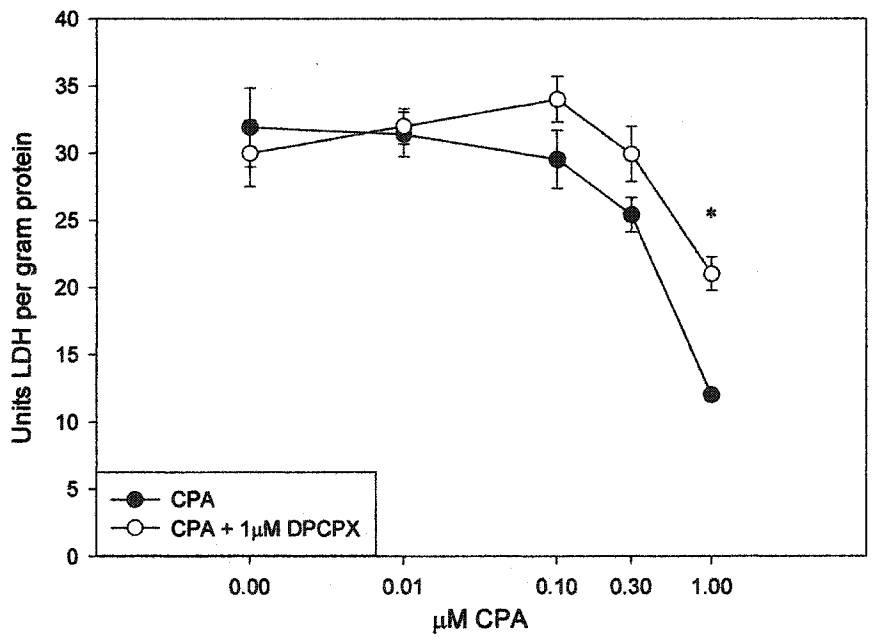
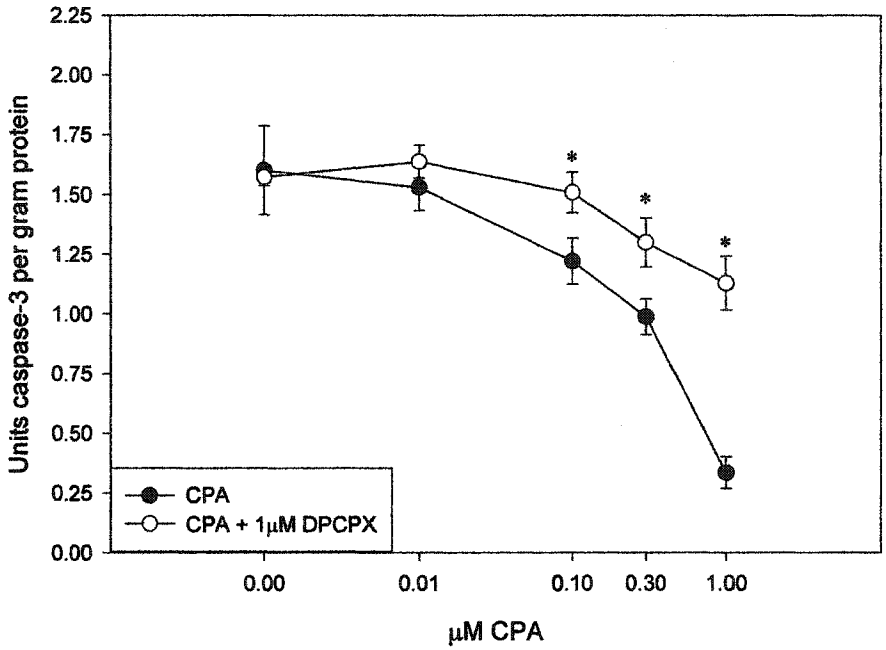
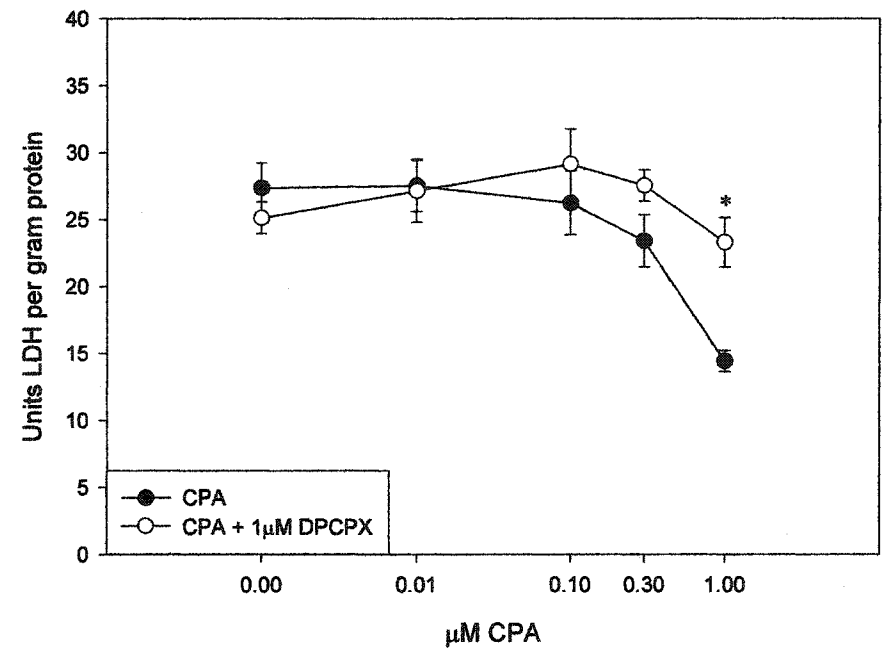


Figure 21: Effect of a 3 hour treatment of various concentrations of CPA (log scale) on both caspase-3 activity (A) and LDH release (B) when administered 24 hours prior to an OGD insult. The effect of DPCPX (1μM) when administered 30 minutes prior to CPA/vehicle was also examined. Neuroprotection induced by 1μM CPA was reversed by DPCPX. All values are given as mean ± s.e. (n=6). * denote significant differences from CPA in the absence of DPCPX.

A)



B)



Because the maximum concentration of CPA tested did not elicit a 50% reduction in LDH values, EC_{40} values were calculated. The EC_{40} values for caspase-3 ranged from 0.38 to 0.49μM, and those for LDH ranged from 0.52 to 0.84μM (Table 2). This demonstrates CPA is slightly more potent at inhibiting caspase-3 activity than LDH release in this model, but as Figure 22 demonstrates, the efficacy is quite different.

Surprisingly, concentration response curves were similar regardless of the time of CPA pretreatment. In summary, Figures 22A and 22B show that it is only at the 1μM concentration of CPA that consistent neuroprotection is observed at all times of pretreatment. The highest amount of protection against caspase-3 activity was found when cells were treated with CPA 1 hour before OGD insult, where the greatest decrease in LDH release was seen in cells treated either 1 or 6 hours before OGD insult. In the cells treated with CPA 1 hour before OGD insult, caspase-3 activity was decreased by 98% and LDH release by 65% relative to 0μM CPA groups (Figures 22A,B).

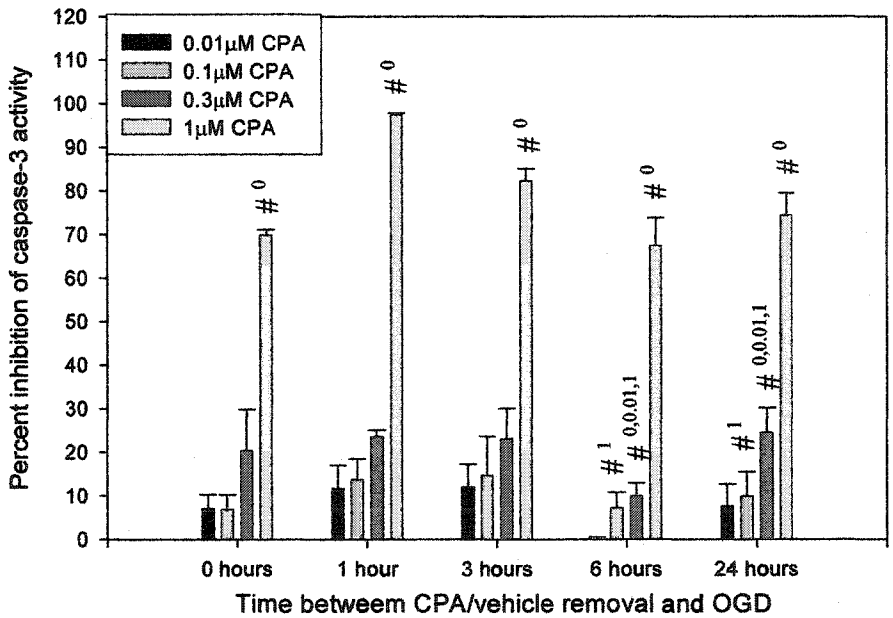
Interestingly, the concentration-dependent relationship of CPA-mediated inhibition of caspase-3 activity was only apparent when CPA treatment occurred 6 and 24 hours before OGD insult. Also, there was no trend towards a decrease in the amount of neuroprotection with an increase in time between removal of CPA and the beginning of OGD (Figure 22). CPA treatment did not have an effect on cells exposed to non-OGD medium (Figure 23). However, 1μM DPCPX increased both caspase-3 activity and LDH release, and was partially reversed by the presence of 1μM CPA.

Table 2: EC_{40} values for CPA mediated inhibition of caspase-3 activity and LDH release for cells that received treatment at various times prior to OGD insult. Values were calculated 0 units of caspase-3 or LDH per gram protein to be the maximal effect.

Time between CPA removal and OGD insult	LDH	Caspase-3
0 hours	0.69μM	0.49μM
1 hour	0.54μM	0.46μM
3 hours	0.84μM	0.44μM
6 hours	0.52μM	0.38μM
24 hours	0.77μM	0.44μM

Figure 22: Effect of altering time of CPA treatment on caspase-3 activity (A) and LDH (B) release. CPA induced neuroprotection at all times of administration. Values are expressed as percent inhibition of caspase-3 activity or LDH release of cells treated with 0μM CPA ± s.e. n = 6. * denote significant difference ($p<0.05$) between the 1μM CPA concentrations, while # denotes significant differences between concentrations within the same time of pretreatment.

A)



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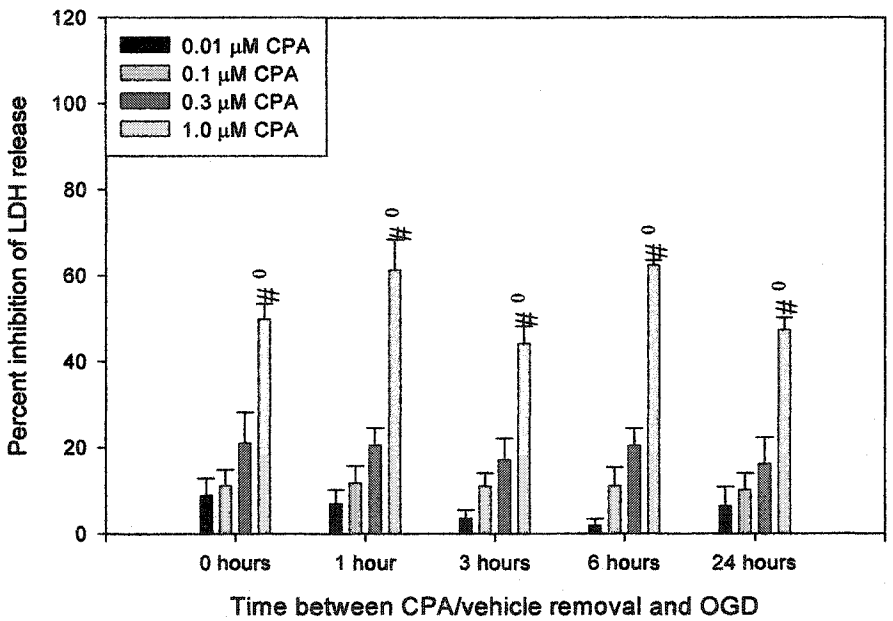
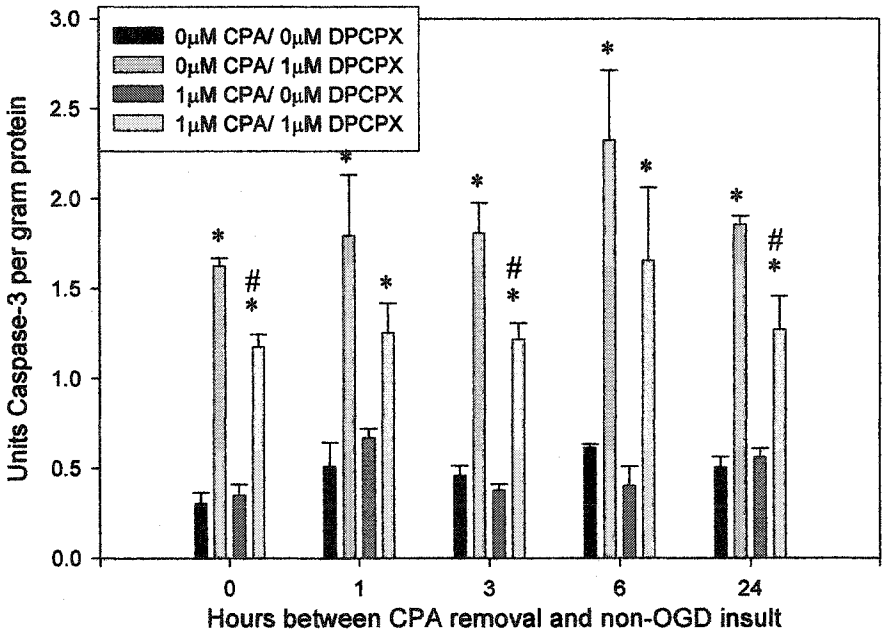


Figure 23: Effect of DPCPX (1μM) alone or in the presence of 1μM CPA on both caspase-3 activity (A) and LDH release (B) in cells treated with non-OGD (control) medium. CPA reversed DPCPX induced cell damage at all times of administration. Values are given as mean ± s.e. with an n=6 for each group. * denote significant differences ($p<0.05$) from 0μM CPA/0μM DPCPX values. # denote significant differences ($p<0.05$) from 1μM DPCPX/0μM CPA.

A)



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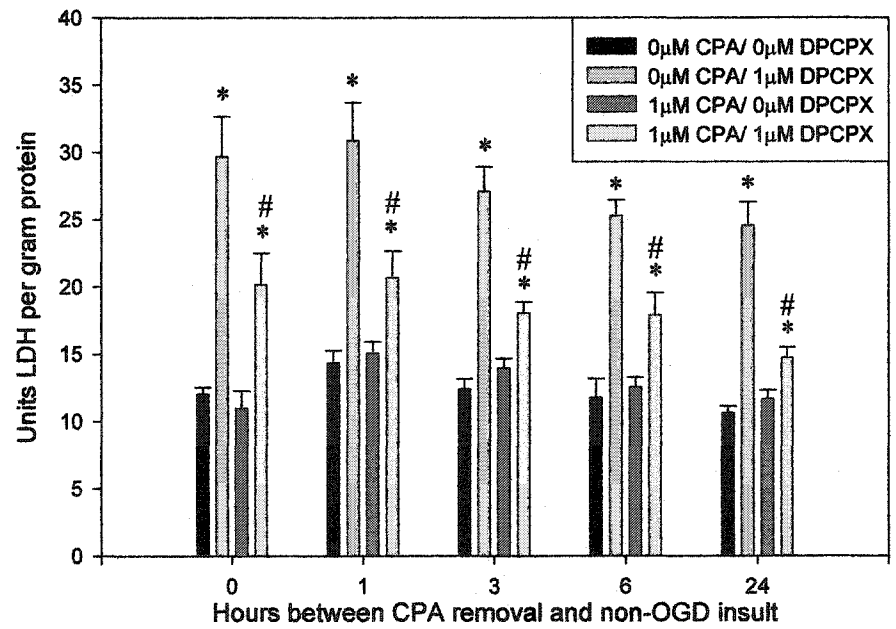


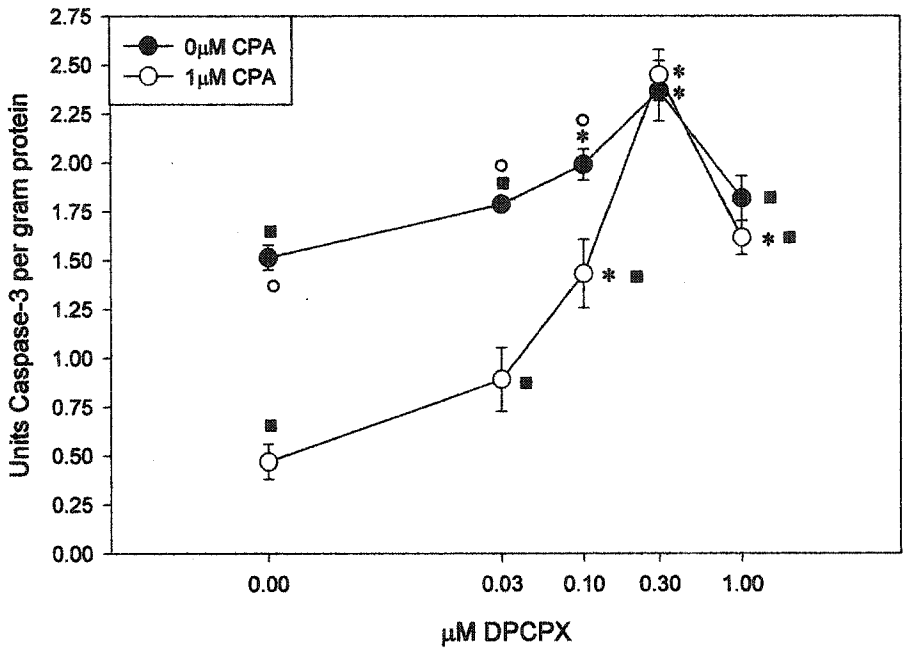
3.5. Inhibition of CPA-mediated neuroprotection by an A1 receptor antagonist

Pretreatment of cells with 1μM DPCPX for 30 minutes prior to CPA administration inhibited any CPA-mediated neuroprotection, independent of pretreatment time (Figures 17-21). This is not surprising considering the K_i of DPCPX is 0.46nM for the A1 receptor (Fredholm, 1997) (ie. 2000-fold less than the concentration used in these experiments).

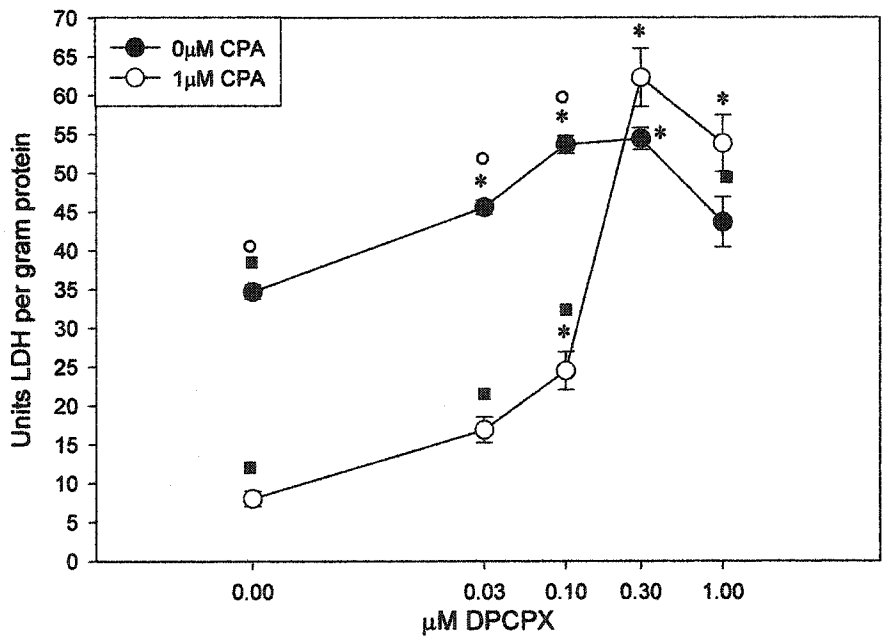
As a result of this finding, an additional experiment was performed using 5 concentrations of DPCPX (0.01, 0.03, 0.1, 0.3, 1μM) in the presence or absence of 1μM CPA. The results demonstrated that DPCPX could reverse CPA-mediated neuroprotection against both caspase-3 activity and LDH release in a concentration dependent manner. DPCPX alone increased both caspase-3 activity and LDH release in a concentration dependent manner (Figure 24). The effect of DPCPX on CGNs appears to be biphasic as the peak increases in both caspase-3 activity and LDH release, and the reversal of CPA-mediated neuroprotection, is seen at 0.3μM concentration. At a concentration of 1μM, DPCPX alone does not affect caspase-3 activity and LDH release induced by incubating cells in OGD medium. DPCPX-mediated reversal of CPA-induced neuroprotection against caspase-3 activity was significantly less at the 1μM concentration than in the 0.3μM concentration. This effect was not seen when examining the LDH release data.

Figure 24: Effect of various concentrations of DPCPX (log scale) on caspase-3 activity (A) and LDH release (B) in the presence or absence of 1μM CPA. DPCPX concentration-dependently increased both caspase-3 activity and LDH release. Values are expressed as units per gram protein, mean ± s.e. * denotes differences ($p<0.05$) from 0μM DPCPX, ○ denote differences ($p<0.05$) from the 1μM CPA group, ■ denote differences ($p<0.05$) from the 0.3μM DPCPX concentration within cells exposed to the same concentration of CPA.

A)



B)



4.0. Discussion

4.1. Culture purity

Primary cultures of CGNs are a widely used model in neuroscience research, primarily due to the characteristic high purity (85-98%) of such cultures (Messer, 1977; Juurlink and Hertz, 1993; Logan and Sweeney, 1997; Kalda *et al.*, 1998; Vitolo *et al.*, 1998). Purity of cultures was determined by calculating the percentage of glial cells in representative cultures. Immunocytochemistry, using fluorescently labelled neuron-specific and glial-specific antibodies, was used to calculate percentage glial cell content.

NeuN is a neuron-specific nuclear protein found in all neurons in the cerebellum except Purkinje cells (Mullen *et al.*, 1992). Because CGNs make up 95% of the neuronal content in cerebella, quantification of NeuN positive cells is considered an accurate method for determination of neuronal cell content (Mullen *et al.*, 1992). GFAP is a cytoskeletal glial specific protein found in oligodendrocytes (Choi and Kim, 1984) and both type 1 and type 2 astrocytes (Freshney, 1994). GFAP is not detected in glial progenitor cells or microglia (Freshney, 1994). Astrocytes are the major type of glial cell found in CGN cultures (Messer, 1977), and therefore, quantification of GFAP positive cells is considered an accurate measurement of glial contamination.

The high concentration of neurons in cultures resulted in thick weblike neurite formations

which grew over glial cells. This is similar to the findings of Holt *et al.* (1996), who described glial cells in CGN cultures as a flat cell layer on which neurons grew. This layering of cells caused problems when trying to image neurons and glial cells together. In order to image both types of cells, confocal images were taken on two separate planes within the same field of view. Cells within each of the images were then quantified. Counting cells was difficult at times primarily due to large groupings of neurons. In cases where cell bodies were not clearly distinguishable, the cell(s) were disregarded.

The CGN cultures used were found to contain 1.4% astrocytic contamination. The small standard error for both neuron and glial mean cell counts demonstrated the consistency of both plating densities and percentage of contamination. This level of glial content is similar to that reported by Levi *et al.* (1984) and lower than most published values (Messer, 1977; Schousboe *et al.*, 1985; Kingsbury *et al.*, 1988; Juurlink and Hertz, 1993; Logan and Sweeney, 1997). However, the presence of other potential contamination sources, such as Purkinje, basket or Golgi neurons, and other types of glial cells was not investigated. As CGNs comprise the majority of neuronal cells in the cerebellum, and fluorodeoxyuridine was added to cultures to inhibit the proliferation of glial cells, the measurements taken in this experiment are believed to be an accurate estimation of culture purity.

According to estimates during plating, approximately 5×10^6 neurons were cultured per coverslip. However, when the neuronal cell counts from this experiment were extrapolated to approximate total number of cells plated per coverslip, the mean approximate cell count

was 1.6×10^6 neurons. Given that there are a number of layers of neurons on each coverslip and only 1 layer was quantified, it is possible that the neuronal counts are an underestimate of actual population size. When the glial counts were extrapolated, there were approximately 2.4×10^4 glial cells per coverslip. Unlike the neurons, there was only 1 plane in which glial cells could be detected. Therefore, when the number of neuronal layers are taken into consideration, the actual percent of glial cells in these cultures is likely much lower than the 1.4% calculated. The small percentage of glial cells in these cultures indicates that measurements of caspase-3 activity and LDH release, and results observed with OGD and CPA reflect effects on neurons rather than astrocytes.

The methods used in this study differ from those used in some published reports, as the immunocytochemistry circumvented the use of biochemical or morphological methods to determine the level of glial contamination (Messer, 1977; Juurlink and Hertz, 1993; Logan and Sweeney, 1997). Immunocytochemistry was chosen for the quantification of cell numbers over biochemical methods because of several offered advantages. One particular complication encountered when using biochemical methods to measure purity is ensuring total neuronal cell death. Some reports in the literature use the percentage of protein remaining after glutamate-induced neuronal cell death as an indication of glial cell content (Juurlink and Hertz, 1993; Logan and Sweeney, 1997). The experiments in this investigation found 9% protein remaining after a high concentration of glutamate, in spite of the 1.4% calculated glial content. It is possible however, that the small number of glial cells contain 9% of total protein, given the larger size of these cells (Logan and Sweeney, 1997). In

addition, biochemical methods give a percentage of purity, whereas the immunocytochemical methods used in this project provide cell population estimates. Morphological methods for assessing culture purity are subjective, whereas methods used in this project are objective, in that cells either stain positively or negatively for an antibody and fall into one of two discrete categories.

4.2. Maximal caspase-3 activity and LDH release

To assess the severity of the OGD insult used in this investigation, experiments that induced maximal measurements of caspase-3 activity and LDH release were performed. To this end, cells were incubated for 24 hours with K^+ deprived (5mM) medium to induce apoptosis and incubated for 20 minutes with 20mM glutamate to induce necrosis.

The 15.6-fold increase in caspase-3 activity after 24 hours of K^+ deprivation over control (24mM K^+) (section 3.2.1.) was similar to, or slightly higher than, values in current literature. A 14-fold increase in caspase-3 activity was measured after 22 hours (Marks *et al.*, 1998) and a 7.4-fold increase after 24 hours of K^+ deprivation in rat CGNs (Moran *et al.*, 1999). The values for the activity of caspase-3 after a 24 hour incubation with K^+ deprived medium in these experiments were considered maximal measurements. The maximal caspase-3 activity was then used to calculate percentage of caspase-3 induced by the incubation of cells with OGD medium.

Maximal caspase-3 measurements in this experiment were taken at the end of a 24 hour incubation of K^+ deprived medium. However, this sampling time may not have been the optimal time to measure caspase-3 activity. Moran *et al.* (1999) found a 10-fold increase over control after 8 hours of K^+ deprivation, but only a 7.4-fold increase after 24 hours in rat CGNs. Similarly, Nath *et al.* (1998) found a 19-fold increase in caspase-3 activity in CGNs after an 8 hour K^+ deprivation. In contrast, Marks *et al.* (1998) found a 16-fold increase after 13 hours of K^+ deprivation which did not significantly decrease when the insult was extended to 24 hours in rat CGNs. If incubation of cells with K^+ deprived medium induces peak caspase-3 activity at a time other than 24 hours, the measurements of maximal caspase-3 activity may be less than the actual maximal value. However, because of the contradictions in the literature, a conclusion cannot be made as to the optimal length of K^+ deprivation required to induce maximal caspase-3 activity. The variability in the literature may be due to a number of factors including the methods used to measure caspase-3 or sample collection.

LDH release induced by glutamate incubation was expressed as a percentage of the total LDH measurement described above. LDH release as a result of glutamate incubation comprised 61 ± 2% of total LDH with 9 ± 1 % protein remaining. The release of only 61% of total LDH after a 20 minute incubation with 20mM glutamate (section 3.2.2) was similar to the literature. Juurlink and Hertz (1993) found 60% LDH release after glutamate incubation with <10% protein remaining in mouse CGNs. Also, Logan and Sweeney (1997) demonstrated that 76 ± 10% was the maximum LDH release measured after 60 minutes

incubation of 10mM glutamate with less than 5% protein remaining. Because glutamate selectively kills neurons, the LDH remaining in cells after glutamate incubation is believed to be contained in astrocytes (Juurlink and Hertz, 1993; Logan and Sweeney, 1997). The 1.4% astrocyte content, as determined in section 3.1., may have comprised the 9% of protein remaining after glutamate insult due to the larger size of astrocytes compared to the CGNs. Therefore, LDH remaining after glutamate incubation was most likely from astrocytes.

4.3. Comments on experimental model

By mimicking the decrease in oxygen, glucose and amino acids that occurs in an *in vivo* ischemic insult, an OGD insult should induce similar damage to that occurring in a stroke. Rat CGN cultures have been used to study excitotoxicity (Marini *et al.*, 1999), hypoxia (Logan and Sweeney, 1997) and substrate deprivation (Logan and Sweeney, 1997), all of which are associated with ischemia. Similar to *in vivo* ischemic damage, mild OGD induces both necrosis and apoptosis (Kalda *et al.*, 1998) and OGD-induced damage is inhibited by administration of an A1 receptor agonist (Logan and Sweeney, 1997; Sweeney, 1997) and calcium buffering mechanisms (Abdel-Hamid and Tymianski, 1997; Tymianski and Wallace, 1993), similar to neuroprotection *in vivo*.

As with any experimental model there are several limitations and criticisms of the stroke model used in this project. When compared to *in vivo* ischemia, this *in vitro* model lacks some of the characteristics associated with ischemic damage. This model has no penumbra

or core, but rather all neurons are exposed to an identical stimulus. Anoxic depolarizations, which are associated with the core, have not been demonstrated in any *in vitro* model and therefore are not believed to contribute to *in vitro* ischemic cell death. There are also differences in the types and prevalence of cells in CGN cultures, when compared to an *in vivo* environment. As glial cells normally outnumber neurons by 10-fold in the brain, the lack of astrocytes, which normally provide extracellular glutamate and K^+ uptake *in vivo*, would increase the severity of excitotoxicity. Immune cells which would potentiate an increase in ROS concentrations *in vivo,* are absent in these cultures. The lack of some ischemic mechanisms involved in damage accumulation, as described above, may be one explanation for the requirement of longer duration of ischemia *in vitro* (Lipton, 1999).

Much like an *in vivo* ischemic insult, in this model the length of OGD insult positively correlates with the severity of the cell damage (Logan and Sweeney, 1997). The incubation time with OGD medium is analogous to the decrease in blood flow which occurs *in vivo* and results in the induction of similar mechanisms of cell damage some of which are described below. A sustained lack of ATP results in loss of ion homeostasis and accumulation of Ca^{2+} in the mitochondria which disrupts the mitochondrial membrane and further inhibits oxidative phosphorylation (Lipton, 1999). The reversal of the Na^+ dependent glutamate transporter (Dingledine and McBain, 1999), constant depolarization of the neuron (Meldrum and Garthwait, 1990) and a lack of astrocytes to aid in glutamate uptake (Dingledine and McBain, 1999) would promote cell damage. The metabolism of ATP to xanthine results in increased ROS production (Sussman *et al.*, 1989) (Figure 2). Increased ROS concentrations

cause increased mitochondrial damage, including release of cytochrome C (Lipton, 1999). Apoptosis induced by ischemia is believed to be caused by increased production of ROS, increased glutamate release and increased intracellular Ca^{2+} concentration, similar to that of an *in vivo* ischemic insult (Kalda *et al.*, 1998).

Following a 6 hour incubation, the pO_2 of the OGD medium was still significantly lower ($p<0.05$) (76.0 ± 0.5mm Hg) than the non-OGD medium (87.6 ± 0.4mm Hg). However, when examining these results complications in measuring pO_2 values must be taken into consideration (see section 4.6.1.). There have been a number of studies which indicate that mild ischemic insults (short duration or milder hypoxia) result in more apoptotic than necrotic cell death and the ratio of necrosis to apoptosis increases with the intensity of an insult (Kalda *et al.*, 1998). The results of this experiment support this theory (section 3.2.1.). Cells incubated with OGD for 1 hour had increased caspase-3 activity only 3 hours after the insult, and LDH release was not increased over control. Immediately after cells were exposed to 3 hours of OGD insult caspase-3 activity was increased over control. Only samples collected 24 hours after a 3 hour OGD insult had higher amounts of LDH release than those of control cells. These results suggest that treating cells with OGD for 1 hour induced too mild an ischemic insult to cause LDH release, as only caspase-3 activity was increased. The 3 hour OGD insult was believed to be more severe than a 1 hour incubation of OGD medium, as it induced both caspase-3 activity and LDH release. Incubating cells for 6 hours with OGD medium induced significant increases in both caspase-3 activity and LDH release.

Induction of apoptosis has been shown to result from excitotoxicity (Nath *et al.*, 1998). Therefore, as the concentration of extracellular glutamate increases due to ischemia, with few astrocytes to facilitate glutamate uptake, excitotoxicity would result. In this study, 38% of maximal caspase-3 activity was detected 1 hour after a 6 hour OGD insult (Figure 13A) which is high when compared to that of Kalda and Zharkovsky (1999). These authors found that 24% of cells had condensed fragmented chromatin following a 90 minute OGD stimulus in rat CGNs. However, Namura *et al.* (1998) described a 14-fold increase over control in caspase-3 activity following unilateral carotid ligation and 30 minutes of reperfusion in mice. The 8-fold increase over control in caspase-3 activity measured in this experiment is low in comparison. This may be because of the differences between this model and the *in vivo* model used by Namura. Cells undergoing apoptosis do not proceed through apoptotic pathways at the same rate (Marks *et al.*, 1998; Nath *et al.*, 1998; Moran *et al.*, 1999), so that all cells may not have activated caspase-3 at the time of sample collection.

The 19% of maximal LDH release detected 1 hour after a 6 hour of OGD insult is low when compared to Logan and Sweeney (1997) who measured a 40% of maximal LDH release after a 6 hour OGD insult in CGNs. A discrepancy between these experiments was expected, as the severity of the respective OGD insults differed greatly from each other. Compared to the sustained anoxia used by Logan and Sweeney (1997), the transient hypoxic OGD insult used in these experiments was expected to induce a much less severe simulated ischemia. A less severe ischemic insult allowed for the assessment of CPA-mediated neuroprotection against both apoptosis and necrosis, where more severe insults are known to induce necrosis alone.

To our knowledge, this was the first experiment to describe OGD-induced caspase-3 activity in cultured CGNs. Many projects using OGD in these cultures utilize DNA fragmentation and/or propidium iodide staining as measurements of apoptotic cell death. These methods, however, are controversial due to the internucleosomal cleavage of DNA has been observed in necrotic, as well as apoptotic, cells injured by ischemia (Sei *et al.*, 1994). The measurement of OGD-induced caspase-3 activity adds an important aspect of biochemical measurements of apoptosis to the current literature.

One concern regarding LDH measurements was that a portion of LDH release induced by OGD was from astrocytes. To address this concern, results of this project were compared to that of Logan and Sweeney (1997), who demonstrated an increase in LDH release from neurons following a 30 minute incubation of OGD in rat CGNs. An 18 hour OGD incubation was required to induce significant LDH release from cultured astrocytes, demonstrating the higher tolerance of astrocytes for ischemia. When comparing the findings of Logan and Sweeney (1997) to this project, differences in the OGD models, as described above, must be considered. When the tolerance of astrocytes for OGD, and the less severe OGD stimulus in these experiments compared to those of Logan and Sweeney (1997) are taken into consideration, LDH release from astrocytes is not believed to confound the LDH measurements of this project.

Non-OGD medium contained oxygen, glucose and amino acids, but contained only 5.4mM K^+ compared to 24mM K^+ in the growth medium which is normally used to keep CGNs

depolarized in culture. However, Marks *et al.* (1998) demonstrated that a 4 hour incubation of CGNs in medium containing 5mM K^+ did not induce caspase-3 activity when measured 18 hours after replacement of 25mM K^+ medium. Additionally, these authors demonstrated that a 13 hour incubation of K^+ deprived medium induced a 30% cell loss. Therefore, the lack of depolarizing concentrations of K^+ in the non-OGD medium for the 1, 3 or 6 hour incubations was not believed to confound the caspase-3 measurements. This is supported by the lack of a significant increase in caspase-3 activity induced by 1, 3 or 6 hour non-OGD treatments at any time of sample collection.

Osmolarity of the OGD and non-OGD media was measured to determine if there was a difference due to the absence of glucose and amino acids (Appendix A). The removal of amino acids and glucose did not greatly affect the osmolarity of the OGD (285mOsm) and non-OGD (300mOsm) media. This decrease in osmolarity during OGD is not sufficient to induce osmotic stress on exposed cells and therefore would not be the cause of the increased LDH release seen in cells incubated with non-OGD medium (Sun *et al.*, 2001). Apoptosis and necrosis in control cells may be attributed to manipulation of the cells during the many media changes, as each manipulation of the cells elicits release of glutamate (Driscoll *et al.*, 1993). These manipulations may be the cause of the dramatic, although highly variable, increase in LDH release 24 hours after cells were incubated with non-OGD medium for 1 hour.

4.4. CPA-mediated neuroprotection

Adenosine A1 receptor-mediated neuroprotection is believed to occur in two phases. The first phase, which lasts for 2 hours after treatment, is mediated by the immediate neuroprotective effects of receptor activation. These mechanisms have been demonstrated to involve both the G(i/o) proteins and PKC through the inhibition of ATP-binding to K_{ATP} channels (Terzic *et al.*, 1994). The opening of K_{ATP} channels allows for K^+ efflux from the cell or movement of K^+ from the cytosol into the mitochondria (Cohen *et al.*, 2000). By decreasing the cytosolic K^+ concentrations, resting membrane potential is decreased and therefore, the cell becomes hyperpolarized (Schubert *et al.*, 1994). Activation of adenosine A1 receptors by CPA induces hyperpolarization of the neurons (Dunwiddie and Fredholm, 1989; reviewed in Dunwiddie and Masino, 2001) which inhibits depolarization and therefore decreases excitotoxicity. Additionally, Brundege and Dunwiddie (1996) demonstrated that adenosine release by one neuron can act on itself as a retrograde messenger and inhibit 80% of its own excitatory responses. Activation of A1 receptors also decreases Ca^{2+} influx through N-, P-, Q- and other types of Ca^{2+} channels, again inhibiting excitotoxicity and Ca^{2+}overload (Ambrosio *et al.*, 1997) which can cause both apoptosis and necrosis (Kure *et al.*, 1991; Castilho *et al.*, 1998). Another mechanism by which activation of A1 receptors decreases neuronal activity is the inhibition of adenylyl cyclase. Decreasing adenylyl cyclase decreases the production of cAMP (Dunwiddie and Fredholm, 1989) which has been associated with decreased OGD-induced apoptosis in rat CGNs (Kalda and Zharkovsky, 1999).

The second phase of preconditioning has been shown to be mediated by adenosine receptor activation and is dependent on activation of K_{ATP} channels (Reshef *et al.*, 1998; Plamondon *et al.*, 1999; Cohen *et al.*, 2000; Lee and Emala, 2001), PKC activation (Lee and Emala, 2001), mitogen-activated protein kinases (Cohen *et al.*, 2000) and NFκB (Blondeau *et al.*, 2001). The second phase is also dependent on protein synthesis, but has been shown to decrease activation of c-fos (Das *et al.*, 1999). If genes expressed by c-fos activation would increase cellular damage, decreasing c-fos activation would have a neuroprotective effect. Preconditioning also induces the activation of GSH-Px, heme oxygenase, SOD and CAT (Das *et al.*, 1999) all of which would scavenge the ROS resulting from ischemia and inhibit damage.

The transcription factors shown to be involved in preconditioning-induced neuroprotection have many diverse target genes. NFκB can induce the expression of a variety of genes including the pro-apoptotic proteins Bax and Bcl-Xs, the anti-apoptotic protein Bcl-2 and Bcl-Xl (reviewed in Mattson and Camandola, 2001), Ca^{2+} binding protein (Cheng *et al*, 1994), pro-inflammatory proteins (Christman *et al.*, 2000) and the adenosine A1 receptor protein (Nei *et al.*, 1998).

The 1μM concentration of CPA was chosen as the highest concentration tested because it has been previously reported to produce neuroprotection against both K^+ deprivation-induced apoptosis (Sanz *et al.*,1996) and OGD-induced necrosis (Logan and Sweeney, 1997). This investigation demonstrated for the first time that activation of adenosine A1 receptors

decreases indicators of both necrosis and apoptosis induced by a simulated ischemic insult in cultured rat CGNs. By administering 1μM CPA during OGD insult or for durations of 1, 3 or 6 hours before the insult, both caspase-3 activity and LDH release were decreased. The pretreatment of cells with CPA for 3 hours prior to OGD insult more effectively inhibited caspase-3 activity than that of CPA treatment during OGD incubation. Cells exposed to 3 hours of pretreatment may have undergone a higher degree of hyperpolarization which would better protect them from excitotoxicity. These cells may have also experienced greater neuroprotection due to the combination of mechanisms involved in the primary and secondary phases of preconditioning.

One complication with this experiment was that length and time of CPA pretreatment were combined. For example, the 1 hour CPA pretreatment began 1 hour before OGD insult and the 3 hour CPA pretreatment began 3 hours before OGD insult. It may be that the results shown here are due to the time of CPA treatment rather than, or in addition to, the length of incubation. This suggestion is supported by Reshef *et al.* (2000a,b) who found that a 15 minute pretreatment of rat cortical cultures with 1μM of the adenosine A1 receptor agonist R-PIA induced significant neuroprotection.

CPA pretreatment was more effective in inhibiting caspase-3 activity than LDH release in all experiments (Figure 16 and 22). This may be due to the induction of 38% of maximal caspase-3 activity and only 19% of maximal LDH release. These differences may also be due to the short period in which cells enter an irreversible pathway leading to necrotic cell

death and to the lack of intrinsic inhibitory mechanisms (Hale *et al.*, 1996). Apoptosis occurs over a longer period and the beginning of the pathway is reversible. There is therefore a longer amount of time before commitment to cell death occurs and it has many mechanisms that allow cell rescue even after initiation of the process (Dirnagl *et al.*,1999). Therefore, CPA may provide greater neuroprotection against caspase-3 activity simply due to the larger window of opportunity and the greater number of mechanisms for protection.

When examining the results from the CPA concentration response curves, it was surprising that only the 1μM concentration of CPA induced neuroprotection against caspase-3 activity and LDH release at all times of pretreatment. These results differ from those of Logan and Sweeney (1997) who showed that CPA inhibited LDH release after OGD insult in a concentration dependent manner. The differences in the potency of CPA between the results of this investigation and those of Logan and Sweeney (1997) may be the result of the differences in the severity the OGD insults. Logan and Sweeney (1997) induced 45% of maximal LDH after OGD insult in comparison to the 19% induced in this OGD model. If a greater percentage of cells were undergoing apoptosis and necrosis, there would be greater possibility of inhibiting caspase-3 activity and LDH release. Some measurements had large standard errors and an increase in sample size, to increase statistical power, may result in significant differences between groups. Power calculations between treatment groups only demonstrated a power of 50.2% and as 80% is an acceptable value, increasing the sample size to increase the statistical power may be an effective way to elucidate differences between treatment groups. Given the variances of the groups, the sample size to achieve 80% power

was found to be 12 plates per treatment group.

EC_{40} values for CPA-mediated inhibition of LDH release varied greatly between the times of pretreatment, differing as much as 300nM. The EC_{40} values for decreases in caspase-3 activity fell within a 110nM range. The EC_{40} values of inhibition of caspase-3 activity tended to decrease until 6 hours and then increase slightly. Unfortunately, statistical analysis of EC_{40} values was not possible as concentration response curves could not be generated from the same cells. Due to the lack of consistent concentration-dependent responses, EC_{40} values should be considered estimates.

Taking into consideration the high concentration of CPA (1μM), the lengthy 3 hour treatment time and the lack of concentration-dependent responses, desensitization of adenosine A1 receptors was a concern. A 2 hour incubation with CPA (10μM) nearly abolished post-synaptic receptor activation, but had no effect on the pre-synaptic receptors until 12 hours of treatment in cultured rat hippocampal neurons (Wetherington and Lambert, 2002). The concentration of CPA used in this experiment is 10-fold lower and therefore the rate of desensitization would be significantly lower. If desensitization of post-synaptic receptors did occur, it would have inhibited CPA-mediated activation of inward rectifying K^+ channels. By diminishing the efflux of K^+, hyperpolarization would not occur and neuroprotection from excitotoxicity would be inhibited. Therefore, if desensitization did occur, it must not have been severe, as all times of pretreatment induced significant neuroprotection. Additionally, the CPA treatment in this investigation was as effective as Reshef *et al.* (2000a,b) who used

a 15 minute incubation of 1μM R-PIA. As a 15 minute incubation would not induce desensitization (Wetherington and Lambert, 2002) and the results of this investigation with a 3 hour CPA treatment are similar, it is doubtful that receptor desensitization occurred.

Our measurements for the inhibition of LDH release were similar to those of Reshef *et al.* (2000a,b), who found a 50-60% decrease in LDH release mediated by 1μM R-PIA in rat cortical neurons exposed to chemical ischemia. Similar to our findings, the efficacy of R-PIA-mediated protection did not decrease even when pretreatment occurred 24 hours before ischemic insult (Reshef *et al.*, 2000a,b). However, the inhibition of caspase-3 activity and LDH release did significantly increase. In this project, neurons that received CPA pretreatment 1 hour before OGD insult were afforded greater neuroprotection against caspase-3 activity than cells pretreated 0, 6 or 24 hours before OGD insult. There were significant increases in the percent inhibition of LDH in cells pretreated 1 and 6 hours before insult, above all other groups. The increased efficacy in cells pretreated 1 hour before OGD insult may be due to the combination of primary and secondary phases of preconditioning.

The first phase of preconditioning, which is known to last 2 hours, would have occurred during the full length of the 3 hour CPA treatment. At the time of OGD administration 4 hours would have passed since the beginning of the CPA treatment and mechanisms involved in the secondary phase of preconditioning may be taking effect. The A1 receptors which were stimulated at the end of the treatment, would have utilized mechanisms of the primary phase and would still have been functioning 1 hour after the beginning of the OGD insult.

The increased efficacy in cells treated with CPA 1 hour before incubation with OGD medium would not have been seen in the results of Reshef *et al.* (2000a,b) as R-PIA was on the cells for 15 minutes. Therefore, it is possible that a combination of primary and secondary preconditioning mechanisms is responsible for the increased neuroprotection in cells that received CPA treatment 1 hour before OGD.

Ending CPA treatment 3 hours before treating cells with OGD induced less neuroprotection than where cells had only 1 hour between CPA treatment and OGD administration, but was similar to the results from cells exposed to OGD immediately after CPA (section 3.4.). The change in the amount of neuroprotection seen in cells that were exposed to OGD 1 hour after the end of CPA treatment compared to cells exposed for longer durations is likely due to the lack of any primary phase mechanisms, because a 3 hour delay between CPA treatment and OGD incubation would have prevented involvement of the primary mechanisms. Therefore, neuroprotection seen in this group would have been solely dependent on secondary mechanisms. The efficacy of inhibition of caspase-3 activity in this group was similar to cells that were exposed to OGD insult both 6 and 24 hours after the end of CPA treatment. The efficacy of LDH inhibition was similar to that in cells exposed to OGD 24 hours after CPA treatment. The similarities between the groups mentioned above are likely due to the dependence on the sustained secondary phase mechanisms.

Surprisingly, in cells exposed to OGD medium 6 hours after CPA treatment, neuroprotection against LDH release was increased (section 3.4.). The greater amount of protection from

LDH release may have been a result of changes in gene expression which, unlike some other secondary mechanisms, require a period of time before they have an effect. These cells began CPA treatment 9 hours before being exposed to OGD medium which may have allowed a sufficient amount of time to produce and incorporate the new proteins induced by preconditioning. The increased expression of both anti- and pro-apoptotic proteins may have been responsible for the maintained, rather than increased, efficacy of inhibition of caspase-3 activity in cells exposed to OGD insult 6 hours after CPA treatment. Increased amounts of Ca^{2+} binding protein may have inhibited necrotic cell death more than apoptotic and therefore have caused the increased efficacy in inhibiting LDH release. Activation of adenosine A1 receptors induces increased A1 receptor mRNA and A1 agonist binding sites after 24 hours (Basheer *et al.*, 2000) similar to NFκB-induced increases in A1 receptors (Nei *et al.*, 1998). If the peak increase in adenosine A1 receptor binding was seen 24 hours after the stimulus there may have been an increased amount of A1 receptors at the time of the OGD insult in cells that received pretreatment 6 and 24 hours before insult.

4.4.1. Non-OGD results

An effect of a 3 hour incubation of cells with non-OGD medium on caspase-3 activity or LDH release was not detectable at any time of administration. 1μM DPCPX, when administered before a non-OGD insult, induced significant increases in both caspase-3 activity and LDH release. Antagonizing A1 receptors prior to change of the medium would be especially harmful to cells, as it would prevent the endogenous neuroprotective effects of

adenosine during culture manipulation (Driscoll *et al*, 1993). Unlike cells exposed to OGD medium, cells that were administered non-OGD medium would only undergo glutamate-induced excitotoxicity if endogenous neuroprotective mechanisms, such as A1 receptor activation were decreased. As discussed above, the high concentration of DPCPX may have also led to non-selective binding to A2b receptors and would suggest that if the concentration of DPCPX were lowered there would be higher amounts of caspase-3 activity and LDH release than that measured. The presence of 1μM CPA reversed DPCPX-mediated increases in caspase-3 activity and LDH release. These results for caspase-3 activity and LDH release were independent of pretreatment time.

4.5. Inhibition of CPA-mediated neuroprotection by an A1 receptor antagonist

The initial concentration of DPCPX (1μM) was chosen because of its ability to inhibit A1-mediated effects (Hettinger *et al.*, 1998; Wetherington and Lambert, 2002). DPCPX-mediated antagonism of CPA-induced neuroprotection against caspase-3 activity and LDH release, demonstrated that the effects of CPA are mediated by adenosine A1 receptors. Surprisingly, 1μM DPCPX in the absence of CPA did not increase either caspase-3 activity or LDH release. When a concentration response curve was generated, both caspase-3 activity and LDH release were increased by DPCPX in a concentration-dependent manner. The decreased amount of damage induced by DPCPX at the 1μM concentration compared to the 0.3μM concentration is likely due to non-selective binding of DPCPX at A2b receptors, as the selectivity of DPCPX for A2b is only 10 - 200 times less than for A1 receptors

(Fredholm, 1995). Activation of A2b receptors causes excitation of the neuron, which would be harmful during ischemia. However, if DPCPX inhibited the binding of adenosine to this receptor, it would inhibit cellular damage and therefore decrease caspase-3 activity and release of LDH.

4.6. Technical considerations

4.6.1. Simulated stroke model

There are many *in vitro* and *in vivo* stroke models (Lipton, 1999). Some *in vitro* models include dissociated cortical neurons, hippocampal slice cultures and dissociated hippocampal neurons. *In vivo* models include transient middle cerebral artery occlusion, which results in severe tissue damage and unilateral carotid occlusion which induces a mild global ischemia (Lipton, 1999). As described briefly in section 4.3., there are many differences between the *in vitro* model used in this project and *in vivo* models used elsewhere and, as with any experimental model, there are advantages and disadvantages.

The many advantages of using primary CGN cultures for simulated stroke include the control given to the investigator over the neuronal environment, including regulation of oxygen tension and control over glucose, amino acid, Ca^{2+}, glutamine, and K^+ content in the growth and experimental media. The purity of the CGN cultures is beneficial as results are known to reflect an effect of the experiment on CGNs rather than glial cells. Also, the access that the investigator has to the extracellular medium allows for sample collection with little or

no disturbance of the cells. CGN cultures have been used for many types of neuroscience research and as a result these cultures have been well characterized for receptor population and morphological features. As with many *in vitro* models, the decreased number of animals used to acquire adequate replications in an experiment helps to reduce animal use.

Unfortunately, there are also many disadvantages to using an *in vitro* model. The most significant differences between this model and an *in vivo* model stem from the isolation of CGNs from their natural environment. By inhibiting astrocyte proliferation and having less than 2% of astrocytes compared to the 90% found normally in the brain, glutamate and K^+ uptake would be drastically inhibited. By maintaining cells in an environment which does not have blood flow or immune cell access, lysed apoptotic cell fragments and particles from cells which have undergone necrosis remain in the extracellular medium and lead to further cell damage (Lipton, 1999). Immune cells in an *in vivo* ischemic insult increase ROS production and the lack of immune cells in this model would dictate a decreased severity of the insult when compared to *in vivo* ischemia (Lipton, 1999). Key characteristics of ischemic damage are the presence of core and penumbra regions after an *in vivo* ischemia. In this model however, all cells are exposed to identical stimuli and therefore regions with varied intensities of cell damage and types of cell death is lacking. During an *in vivo* ischemic insult a key mechanism of spreading depression and excitotoxic cell death is anoxic depolarization (Kristian and Siesjö, 1997). Anoxic depolarization has yet to be demonstrated in an *in vitro* model and therefore is not believed to contribute to the damage in this OGD model. Even in the absence of an immune response, the lack of astrocytes and anoxic depolarization, the

CGN model is believed to be a good model for examination of protection against simulated ischemic damage (Sweeney *et al.*, 1995; Juurlink and Sweeney, 1997; Logan and Sweeney, 1997; Kalda and Zharkovsky, 1999).

Decreasing oxygen tensions in the medium around cells over 90% (from 90mm Hg to 7-8mm Hg) and removing organic sources of ATP production (glucose and amino acids) induced a mild to moderate ischemia in this model. To determine if there were differences in osmolarity between the non-OGD and OGD medium due to the removal of the glucose and amino acids osmolarity was measured. Measurements for non-OGD and OGD media were 300 and 285 mOsm respectively, indicating that osmotic shock would not contribute to cellular damage induced by OGD medium.

Measurements of oxygen tension in non-OGD medium were lower (90mm Hg) than that reported in literature (150mm Hg) (Freshney, 1994). Also, the measured rate of re-oxygenation of the OGD medium was believed to be lower than the actual due to the measurement of pO_2 from the bottom of a large (500mL) flask. The rate of re-oxygenation at such a depth in a 500 mL flask would be much lower than that of the thin layer of medium (3mL) on the surface of a 35mm petri dish, indicating that cells exposed to the OGD medium would remain hypoxic for a shorter period of time than that indicated in Figure 8. Initial values would have been consistent however, and therefore the severity of hypoxia is expected to have been approximately equal for all experiments.

4.6.2. Measurements of apoptotic and necrotic indicators

Measurements of caspase-3 activity or LDH release for each individual culture plate were divided by the grams of protein collected from cell remnants of each plate in an attempt to negate for variations in cell content between culture plates. One concern was that the protein content in cells exposed to OGD and non-OGD media would differ due to the greater amount of necrotic cells, which would have released proteins into the extracellular medium during lysis. However, protein from cells exposed to OGD compared to non-OGD in the absence of any drug did not differ significantly.

Activity of caspase-3 is believed to be a reliable indicator of apoptotic cell death (Armstrong *et al.*, 1997; Gottron *et al.*, 1997). This enzyme is one of the few common mediators of cell death in apoptosis (Cohen, 1997; Eldadah *et al.*, 2000). Caspase-3 activity is also involved in the latter portion of the cell death process and therefore, the possibility of cell survival after caspase-3 activation is less than if the indicator was involved earlier. However, a second method for quantifying apoptotic cells should have been utilized to ensure that cells were not recovering after caspase-3 activation. An additional measurement could have been performed by a Hoechst stain or TUNEL stain to detect chromatin condensation and DNA fragmentation respectively. The morphological changes associated with apoptosis induced by ischemia are controversial; however using another method in addition to caspase-3 activation would have been advantageous.

The method used to quantify caspase-3 activity utilized DEVD as a substrate. This substrate has been shown to be highly selective for caspase-3 cleavage, therefore cleavage by other caspases is not likely. The time of collection of caspase-3 samples was believed to be the most effective, as apoptosis was shown to peak 30 minutes after reperfusion following left common carotid occlusion in mice (Namura *et al*, 1998).

LDH release, as measured by LDH activity in the extracellular medium is believed to be a reliable method for quantifying necrosis (Juurlink and Sweeney, 1997; Sweeney *et al.*, 1995). As LDH would not be released from cells unless the cell membrane was disrupted, there should be little or no LDH present in the extracellular medium from healthy, undisturbed cells.

4.6.3. General criticisms

If the experiments of this investigation were to be repeated there are a number of aspects which should be altered. When using CGNs the investigator should attempt to minimize the number of media changes that each culture plate is exposed to. In this experiment, cells were not washed after drug removal in order to decrease the number of cell manipulations. The lack of rinsing is not believed to have complicated the findings of this investigation; however if the study were to be repeated and if cells were to be manipulated less during the experiment, they should be rinsed after drug removal.

The limitations of this study are common to all *in vitro* experiments as there are many differences between *in vivo* environments and culture conditions. It is therefore difficult to extrapolate *in vitro* results to *in vivo* predictions. In addition, as with many experimental models, the extrapolation to humans of results seen in rodents must be made cautiously. Although there are no indications that adenosine A1 receptors present in CGN cultures differ from those found in humans, interspecies differences always provide possible complications to applying the results of an experiment performed on a non-human species to humans.

One of the major criticisms of this project was the use of single markers for apoptosis and necrosis. Although caspase-3 is an effector enzyme which occurs late in the apoptotic cascade and results in DNA cleavage, cell rescue may still be possible. Ideally, a number of measurements or methods to quantify apoptotic cell death should have been utilized to ensure that cells which had increased caspase-3 activity did not survive. Similarly, this investigation has shown that CPA can decrease caspase-3 activity for a prolonged period of time. However, rather than inhibiting apoptotic cell death, CPA may only be inhibiting caspase-3 activity alone and cells may die by another apoptotic mechanism. If measurements of maximal caspase-3 activity were to be repeated, various lengths of K^+ deprivation should be used to determine which would have induced the maximal effect. Similarly, maximal LDH measurements should be done using various concentrations and durations of glutamate to determine which would elicit the highest release of LDH.

In the CPA concentration response curve, concentrations should have been increased above

1μM to determine what the maximal effect of CPA against both caspase-3 activity and LDH release is. However, given the affinity of CPA for the A1 receptors, both desensitization and non-selective binding would have to be taken into account. By adding the higher dose, concentration response curves may have demonstrated concentration-dependent responses and would have allowed for more accurate calculation of EC_{50} values.

Despite the above limitations, the objectives outlined at the beginning of this project were all met. In examining caspase-3 activity and LDH release following OGD insult, we found that a 6 hour insult and 1 hour reperfusion was sufficient to induce increases in both indicators of cell death. When the neuroprotective properties of CPA were tested, it was found that a 1 hour treatment at a concentration of 1μM was the most effective. Lastly, the specific A1 receptor antagonist, DPCPX inhibited CPA-mediated neuroprotection in a concentration-dependent manner.

4.7. Future directions

The experiments of Reshef *et al.* (2000a,b) have demonstrated consistent neuroprotection until 72 hours of separation between R-PIA treatment and chemical ischemia; however these experiments examined LDH release alone. It should be determined if a similar length of protection can be seen against caspase-3 activity and LDH release. Additionally, it should be attempted to replicate these results in an *in vivo* model.

It has been well demonstrated that adenosine A1 receptor-mediated preconditioning requires protein synthesis (Das *et al.*, 1999). Therefore, by using DNA microarray technology, the assessment of changes in gene expression as a result of both the OGD stimulus and the CPA treatment may aid in determining a molecular mechanism of adenosine A1 receptor-mediated preconditioning.

The selectivity CPA demonstrates for A1 adenosine receptors and the inability of the body to metabolize it, makes this agonist preferred to adenosine itself. Although the neuroprotection seen in this investigation shows the promise of CPA as a therapeutic agent, various cardiovascular effects associated with adenosine A1 receptor activation must also be considered when attempting to continue this study to *in vivo* application. If cardiovascular effects of A1 receptor activation are not so severe as to inhibit pharmacologic use of CPA for neuroprotection from ischemia, it could be used for patients who have already experienced, or who are at high risk for having a cerebral ischemic event. By maintaining a pretreatment dose in the patient, should an ischemia occur, CPA should decrease overall cell damage. If a single dose of CPA mediates sustained neuroprotection for 24 hours, staggered doses may maintain neuroprotection without desensitizing A1 receptors.

If similar results could be seen *in vivo*, damage resulting from stroke could be drastically decreased. Inhibiting apoptosis by 98% would drastically reduce the amount of delayed cell damage, which has been demonstrated to be apoptotic (Northington *et al.*, 2001). By decreasing necrosis by 65%, the size of the infarct core and therefore the amount of

spreading depression would also be reduced. Additionally, as the severity of glutamate-induced excitotoxicity would be lessened by the presence of CPA, the intensity and therefore damage induced by the spreading depression should also be lessened. Taken together, pretreatment of susceptible patients with CPA may significantly decrease stroke-mediated cell damage. Essentially, the potent neuroprotection induced by CPA pretreatment may make the difference between life and death, paralysis and mobility or independence and permanent hospitalization.

5.0. REFERENCES

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APPENDIX A

SOLUTIONS

Phosphate buffered saline (PBS):

137mM NaCl, 7.75mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl

DAILY SOLUTIONS FOR TISSUE CULTURE			
Solution A /100ml	Solution B /20ml	Solution C /20ml	Solution D /20ml
1mM Mg2+	0.025% Trypsin	0.0012g Dnase	0.005g poly-L-lysine
0.3% w/v Bovine Serum Albumin		0.012g Trypsin inhibitor	
13mM glucose			

CONTENTS OF MINIMUM ESSENTIAL MEDIA (MEM) WITH EARLE'S SALTS	
INORGANIC SALTS (mg/L)	
$CaCl_2$ (200)	KCl (400)
$MgSO_4$ (98)	NaCl (6800)
NaH_2PO_4 (140)	
OTHER COMPONENTS (mg/L)	

phenol red (6)	sodium succinate (100)
succinic acid (75)	D-glucose (1000)
AMINO ACIDS (mg/L)	
L-cytine - 2HCl (31)	L-histidine HCl - H_20 (42)
L-phenylalanine (32)	L-leucine (52)
L-methionine (15)	L-lysine-HCL (73)
L-isoleucine (52)	L-threonine (48)
L-tryptophan (10)	L-tyrosine (36)
L-valine (46)	L-arginine- HCl (126)
VITAMINS (mg/L)	
D-Ca Pathothenate (1)	choline Bitartrate (1.8)
folic acid (1)	i-inositol (2)
Niancinamide (1)	pyridoxal HCl (1)
riboflavin (0.1)	thiamine HCl (1)

MEDIA COMPONENTS	**MEM # 1/ L**	**MEM #2/ L**
MEM	860ml	840ml
Fetal Bovine serum (FBS)	100ml, 10% vol/vol	----------
Horse Serum (HS)	----------	100ml, 10% vol/vol
Glutamine	0.1168g, 0.8mM	0.1168g, 0.8mM
Glucose	25ml of 1.2M, 30mM	25ml of 1.2M, 30mM
KCl	10ml of 2.4M, 24mM	10ml of 2.4M, 24mM
Penicillin/Streptomycin	5.0ml, 50 IU penicillin/ 50µg Streptomycin	5.0ml, 50 IU penicillin/ 50µg Streptomycin
Fluorodeoxyuridine	20ml of 1.2M	----------

SIMULATED ISCHEMIA

INGREDIENTS	CONTROL KREBS'	ISCHEMIC KREBS'
NaCl	111mM	111mM
$NaHCO_3$	26.2mM	26.2mM
NaH_2PO_4	1.2mM	1.2mM
KCl	5.4mM	5.4mM
$CaCl_2$-$2H_2O$	1.8mM	1.8mM
$MgCl_2$	1.8mM	1.8mM
Glutamine	0.15g	0.15g
Glucose	10mM	----------
Amino Acids	20mL	----------

APPENDIX B: Validation of spectrophotometric assays for caspase-3, LDH and protein.

Methods

In order to test the accuracy of the LDH kits, a LDH standard curve (n=4) was performed using defined concentrations of LDH (0, 25, 50, 75, 100 and 125 units LDH/L) added to the LDH reagent.

Evaluation of the accuracy of the protein assay involved the development of a standard curve using known concentrations of bovine serum albumin (0, 2, 4, 6, 8, 10, and 12g/L). Bovine serum albumin solutions were assessed for amount of protein as measured by the kit. These values were then compared to those of the actual amount of protein administered. Linear regression was performed on the concentration vs. administered protein curve and coefficients of variation for units calculated vs. units administered curve was calculated.

The validity of the caspase-3 assay was assessed by 3 methods. Standard curves for ρNA and activated caspase-3 enzyme were performed, and a caspase-3 selective inhibitor was used to determine the specificity of the kit. The optical density of increasing concentrations of ρNA in cell lysis buffer was measured and the slope used to calculate the units of caspase-3, as recommended in the kit schematics. A comparison of the administered vs. calculated units of caspase-3 activity was used to determine if the slope of the ρNA curve was giving

accurate measurements. To do this, a standard curve with 0, 2, 4, 6, 8, 10 and 12 units of activated caspase-3 enzyme was derived. The optical density values were then compared to those for with known amounts of caspase-3.

To demonstrate specificity of caspase-3 for DEVD-ρNA substrate, 0.5μL of the selective caspase-3 inhibitor DEVD-fmk was used. Samples were incubated both in the presence and absence of DEVD-fmk, a caspase-3 inhibitor, to determine the percentage inhibition as an indication of method specificity.

Total sample volume from the caspase-3 kit is 100-105μL. This small volume made the use of a sipper pump attached to a quartz spectrophotometric cuvette essential to accurately measure absorbance of the samples. To determine if the chosen pump setting (1.4 seconds) was adequate to move the sample into the cuvette for an accurate reading, 100μL volumes of various dilutions of ρNA (0, 0.5, 1.0, 1.5, 2.0 and 3.0μM) were used to evaluate the consistency of the pump. Triplicate measurements were taken on separate days, each after disassembling and reassembling of the pump. The coefficient of variation and linear regression coefficient were then calculated.

Results for validation of the caspase-3 assay.

The results shown in Figure 25A indicate a correlation between administered units of

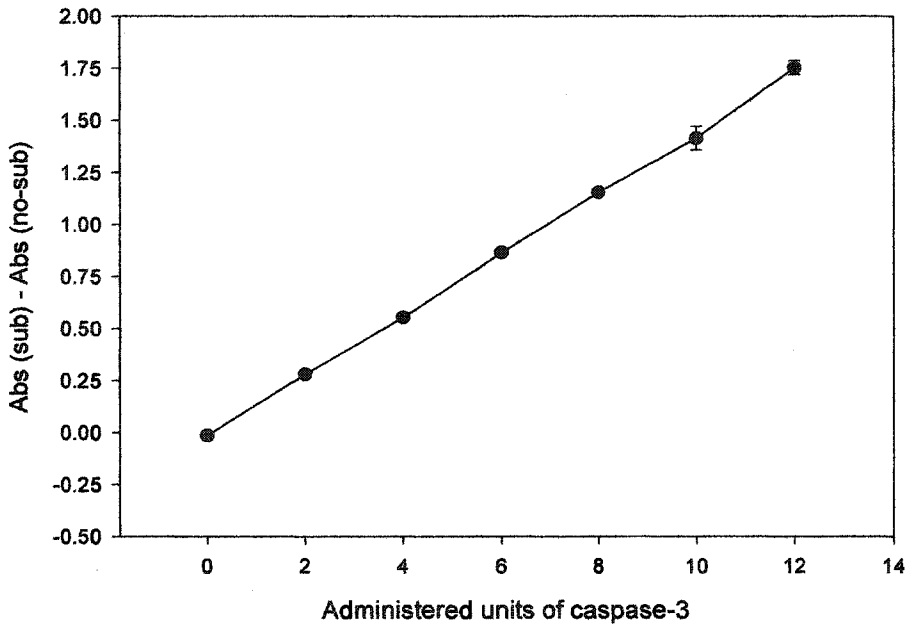
caspase-3 and change in absorbance between samples with and without substrate, indicating an increase in caspase-3 activity ($R^2 = 0.999$). The pooled coefficient of variation was 3.17%, and the small standard error of 0.58% indicates that this kit gives accurate and reproducible results (Figure 25B).

A second standard curve was generated to assess the reliability of the sipper pump. The duration of time required to move a sample into the spectrophotometric cuvette was found to be 1.4 seconds. In order to verify that measurements would be consistent between uses an absorbance curve was made using 100μL samples of various concentrations of the colorimetric molecule from the caspase-3 kit (ρNA). Measurements were taken on 3 different days, after removal and re-connection of the sipper. Linear regression demonstrated that the data lay on a straight line ($R^2 = 0.999$); the coefficient of variation was 2.1 ± 0.3% (Figure 26A). The small coefficient of variation indicates that the sipper pump will give accurate and consistent measurements of 100μL samples.

An increase in unbound ρNA, by caspase-3 induced cleavage of DEVD-ρNA demonstrates that an increase in caspase-3 activity would result in increased absorbance of a sample using this kit (Figure 26A). Additionally, a specific caspase-3 inhibitor DEVD-fmk was used to determine if cleavage of DEVD-ρNA was dependent on caspase-3 activity (Figure 26B). The presence of DEVD-fmk decreased activity of caspase-3 by 95% (from 1.72 ± 0.12 U/g to 0.10 ± 0.01 U/g) of uninhibited samples from the same cells. Therefore increases in -3.

Figure 25: Standard curves for caspase-3 A) measurements of caspase-3 activity with varying amounts of administered enzyme; B) Comparison of administered and measured units of caspase-3. Data are expressed as mean ± s.e.

A)



B)

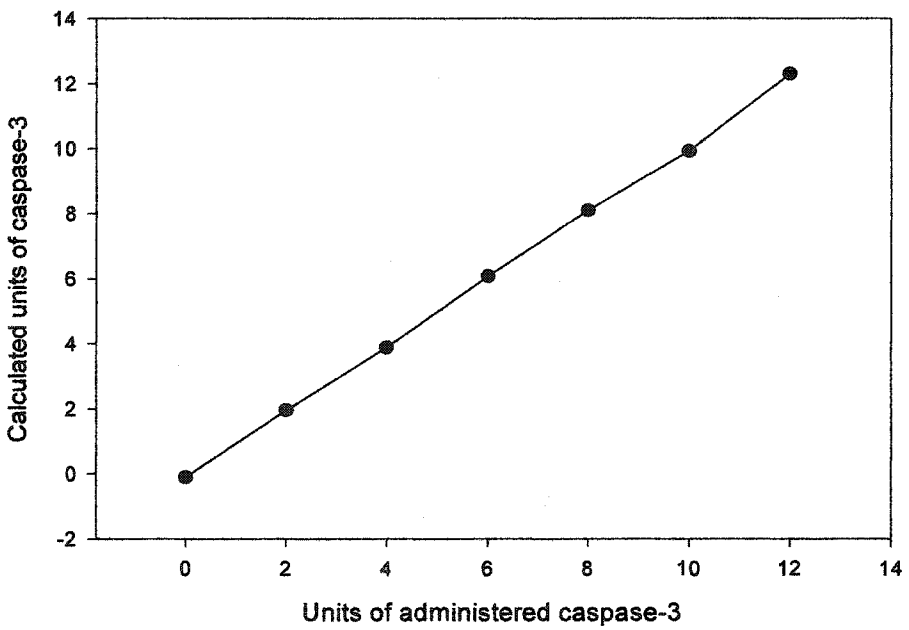
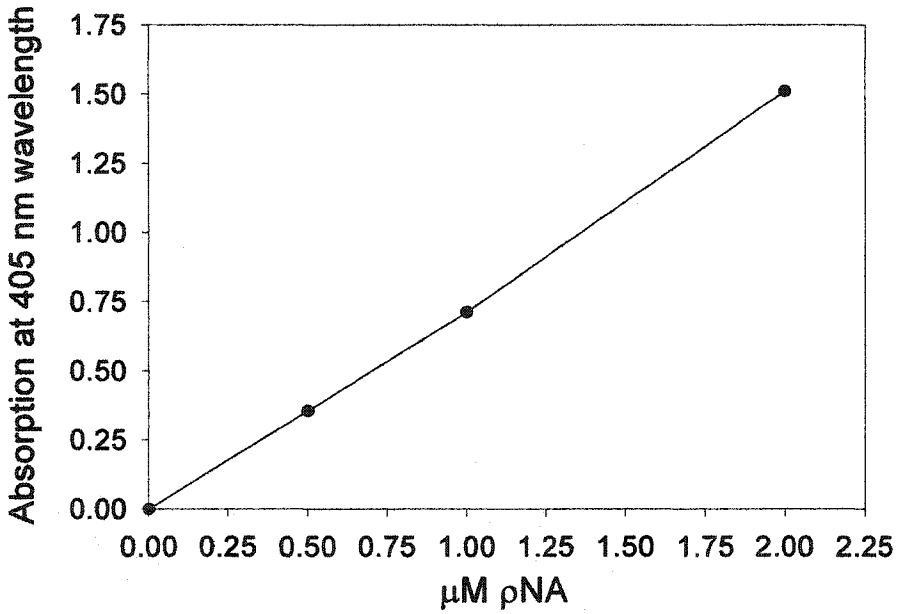
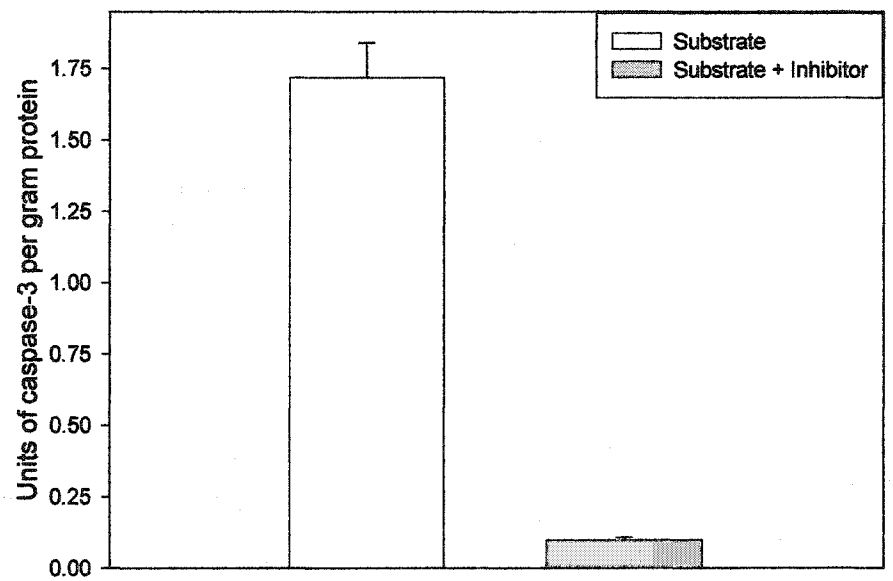


Figure 26: A) Standard curve of ρNA absorbance using sipper apparatus (mean ± s.e.) (n=4). B) Effect of caspase-3 inhibitor on measured activity of caspase-3 (mean ± s.e.) (n=4).

A)



B)



absorbance of pNA can be directly linked to the activity of caspase

Results for validation of the LDH assay.

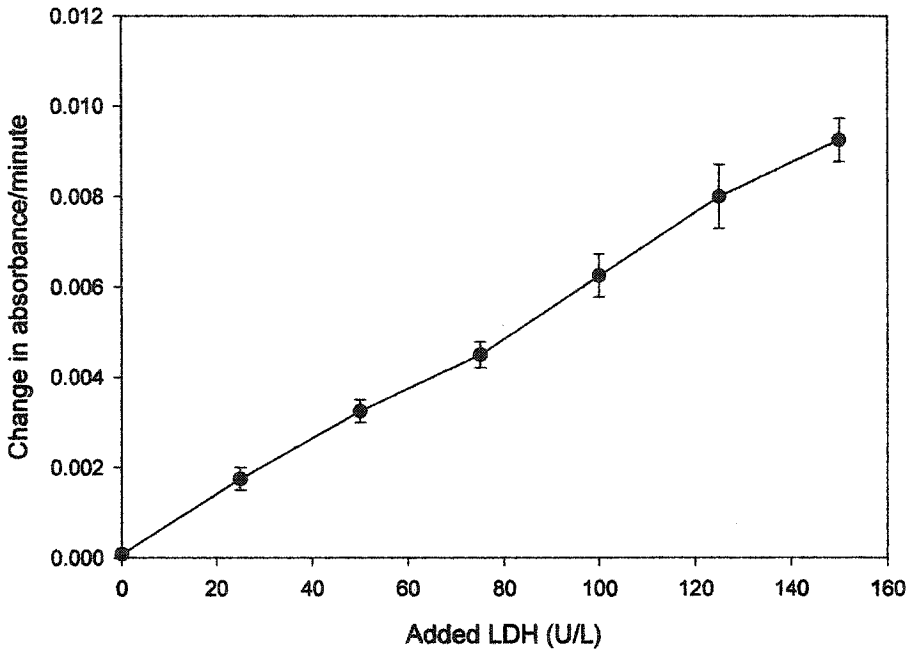
Increasing concentrations of LDH caused a concentration-dependent increase in detected activity using the LDH kit (Figure 27A). This was linear since the correlation coefficient R^2 for the known administered amounts of LDH and the computed values was 0.998 with a coefficient of variation of 8.0 ± 1.2% (Figure 27B). When absorbance was converted to U/L there was no significant difference between the number of administered units and calculated units as determined by a 1-way ANOVA with post-hoc Tukey's ($p>0.05$). These data verify that the formula for calculation of LDH activity recommended in the kit schematic is accurate.

Results for validation of the protein assay.

To test the accuracy of the total protein assay, a concentration curve was generated using Bovine serum albumin dissolved in PBS. Absorbance of the sample at 540nM increased with an increased concentration of administered protein (Figure 28A). Linear regression analysis indicates that this is a linear relationship, ($R^2=1$) and the coefficient of variation was 4.63 ± 1.23 %, which demonstrates the reproducibility of measurements from the assay (Figure

Figure 27: A) Standard curve demonstrating changes (mean ± s.e.) in absorbance with varying concentrations of LDH (n=4). B) Comparison of added amounts of LDH to calculated amounts of LDH (mean ± s.e.) (n=4).

A)



B)

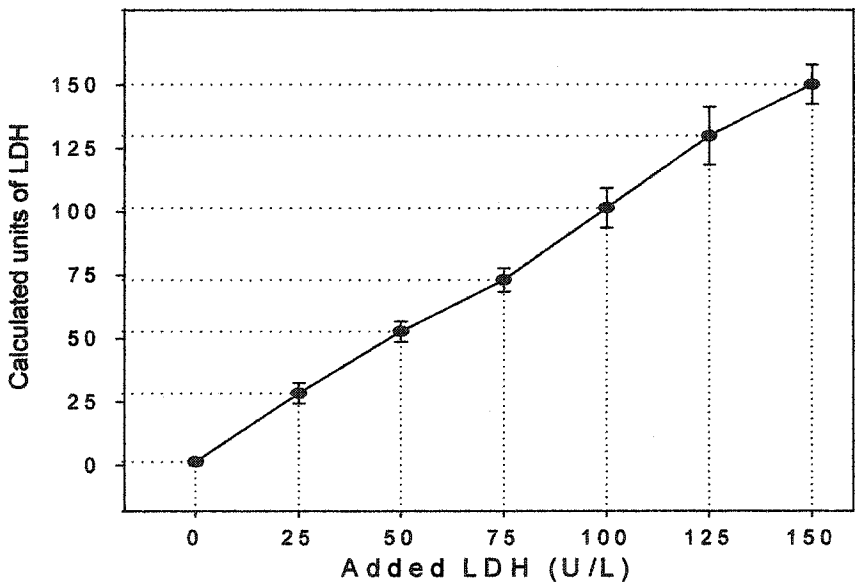
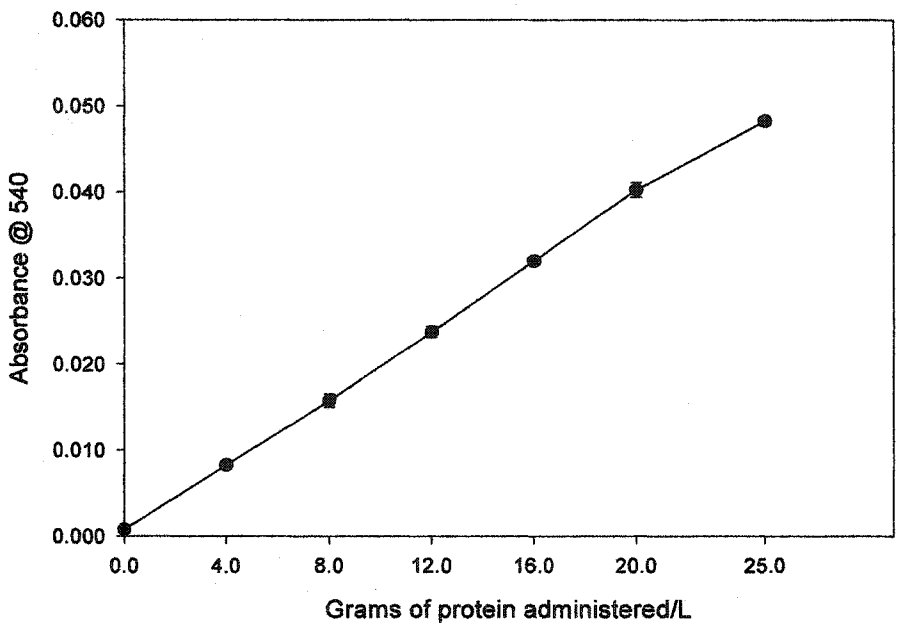
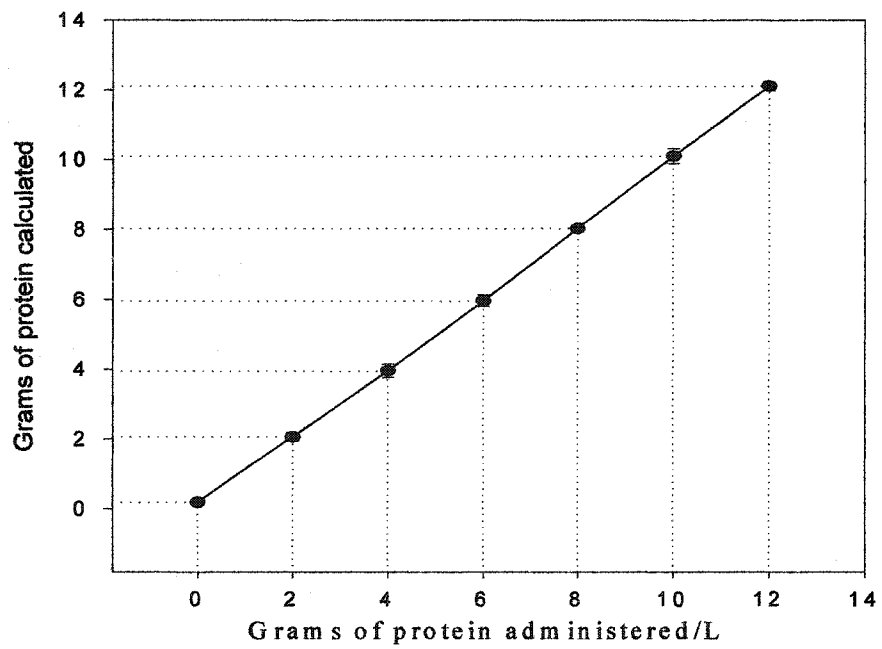


Figure 28: A) Standard curve showing the effect of added protein weight on absorbance (mean ± s.e.) (n=4); B) Standard curve showing the relationship between added and calculated protein values (mean ± s.e.) (n=4).

A)



B)



28B). Each time the assay was used, absorbance of a standard was measured (n=27) at 540 nm wavelength. This gave a mean of 0.231 ± 0.000g/L with a coefficient of variation of 0.66 %. Additionally, when absorbances were converted to weight protein, there were no significant differences between administered and measured amounts of protein. The small coefficients of variation for both the standard curves and measurements indicate that the protein assay gave reliable measurements. Additionally, the linearity of the standard curve indicates the accuracy of the assay (Figure 28B).

APPENDIX C : Example ANOVA table generated from experimental data. Table is from Results Section 3.4. and compares the effects of different concentrations of CPA on caspase-3 activity.

Tukey's Multiple comparisons test	Mean Difference	q	P value	95% confidence interval of difference
0.01μM CPA vs. 0.1μM CPA	-0.8694	4.504	P < 0.05	-1.671 to -0.06727
0.01μM CPA vs. 0.3μM CPA	-1.106	5.728	P < 0.01	-1.908 to -0.3036
0.01μM CPA vs. 1μM CPA	-1.086	5.628	P < 0.01	-1.889 to -0.2844
0.01μM CPA vs. vehicle	-1.199	6.211	P < 0.01	-2.001 to -0.3968
0.1μM CPA vs. 0.3μM CPA	-0.2364	1.224	P > 0.05	-1.038 to 0.5657
0.1μM CPA vs. 1μM CPA	-0.2171	1.125	P > 0.05	-1.019 to 0.5850
0.1μM CPA vs. vehicle	-0.3295	1.707	P > 0.05	-1.132 to 0.4725
0.1μM CPA vs. 1μM CPA	0.1927	0.9985	P > 0.05	-0.7828 to 0.8214
0.3μM CPA vs. vehicle	-0.9319	0.4828	P > 0.05	-0.8953 to 0.7089
1μM CPA vs. vehicle	-0.1125	0.5826	P > 0.05	-0.9146 to 0.6896

ANOVA TABLE	SS	df	MS
Treatment (between columns)	**5.795**	**4**	**1.449**
Residual (within columns)	**5.589**	**25**	**0.2236**
Total	**11.38**	**29**	