POTENTIAL THERAPIES AND NEUROPROTECTIVE CASCADES IN ANOXIA TOLERANT FRESHWATER TURTLE *TRACHEMYS SCRIPTA ELEGANS*

By

Gauri Nayak

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This dissertation was prepared under the guidance of the candidate's dissertation advisor, Dr. Howard Prentice, College of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

SUPERVISORY COMMITTEE:

_____
Howard Prentice, Ph.D.
Dissertation Advisor

_____
Sarah Milton, Ph.D.

_____
Jang Yen Wu, Ph.D.

_____
Kathleen Dorey, Ph.D.

_____
Rodney Murphey, Ph.D.
Chair, Department of Biology

_____
Jianning Wei, Ph.D.

_____
Gary Perry, Ph.D.
Dean, The Charles E. Schmidt College of Science

_____
Barry Rosson, Ph.D.
Dean, Graduate College

_____
Date

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ABSTRACT

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Mammalian neurons exhibit extreme sensitivity to oxygen deprivation and undergo rapid and irreversible degeneration when oxygen supply is curtailed. Though several neuroprotective pathways are activated during oxygen deprivation, their analyses are masked by the complex series of pathological events which are triggered simultaneously. Such events can be analyzed in the anoxia tolerant fresh water turtle, which can inherently survive the conditions of oxygen deprivation and post-anoxic reoxygenation without brain damage. It is likely in such a model that modulation of a particular molecular pathway is adaptive rather than pathological. The major objective behind this study was to analyze the intracellular signaling pathways mediating the protective effects of adenosine, a potential neuromodulator, and its effect on cell survival by influencing the key prosurvival proteins that prevent apoptosis. *In vivo* and *in vitro* studies have shown that adenosine acts as a neuroprotective metabolite and its action can

be duplicated or abrogated using specific agonist and antagonists. Stimulating the adenosine receptors using selective A1 receptor agonist N6-cyclopentyladenosine (CPA) activated the presumed prosurvival ERK and P13-K/AKT cascade promoting cell survival, and suppression of the receptor using the selective antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine) activated the prodeath JNK and P38 pathways.

The complex regulation of the MAPK's/AKT signaling cascades was also analyzed using their specific inhibitors. The inhibiton of the ERK and AKT pathway increased cell death, indicating a prosurvival role, whereas inhibiton of the JNK and p38 pathway increased cell survival in this model. *In vitro* studies have also shown a high Bcl-2/BAX ratio during anoxia and reoxygenation, indicating a strong resistance to cell death via apoptosis. Silencing of the anti-apoptotic Bcl-2 gene using specific siRNA upregulated levels of prodeath BAX, thus altering the Bcl-2/BAX ratio and elevating cleaved Caspase-3 levels leading to increased cell death. Another promising neuroprotective target which we analyzed was Neuroglobin, which was induced during oxygen crisis and silencing this gene indicated that its plays a major role in modulation of ROS. This study strongly emphasizes the advantages of an alternate animal model in elucidating neuroprotective mechanisms and revealing novel therapeutic targets which could eventually help clinicians to design new stroke therapies based on naturally tolerant organisms.

DEDICATION

I wish to dedicate this thesis to my parents (Mr. Hari Nayak and Mrs. Jayalaxmi Nayak), my elder brother (Mangesh Nayak) and my aunt (Swapna Pai) without whom my dream to achieve my doctoral degree would not have been possible. Their love, emotional support and care have made me succeed in each and every ustep I took in my life. Above all, this thesis is for my loving and caring husband Girish Barot who stood besides me in thick and thins and always kept me smiling and motivated. He being a graduate student in the Chemistry department has always been an advantage for spending sleepless nights working on the time course experiments and also for having fun at different departmental celebrations or parties. It was impossible to realize this thesis without him.

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INTRODUCTION

Pathological processes associated with ischemia such as heart diseases and strokes are leading causes of death and serious adult disability in most industrialized countries. Stroke accounts for more than 15 million deaths worldwide every year. The total estimated direct and indirect cost of stroke is 68.9 billion dollars (American Heart Association. Heart Disease and Stroke Statistics—2009 Update).

Normal Cerebral Blood flow (CBF) of 50 to 60 ml/100g/ min is considered to be to a prerequisite and major critical factor for maintenance of normal functional activity of the mammalian brain. Ischemic conditions are due to a reduction in this flow rate. Decrease in the blood supply of less than 10ml/100g/min culminates in permanent loss in the neuronal integrity.

Cerebral Ischemia

The vertebrate brain is considered to be the organ that is most susceptible to damage by compromised oxygen availability (hypoxia) or blood flow (ischemia) due to its high metabolic rate and obligate aerobiosis. Although the brain occupies only 2% of the body weight it exhibits enormous energy demands. Proper functional activity of the brain depends upon an uninterrupted supply of a significant 20% (50 ml/min) of the resting oxygen, 15% (800 ml/min) of the cardia output and 25% (310 μmol/min/kilogram of brain tissue) of glucose thus exhibiting great susceptibility to ischemic insults. Clinical

studies have shown that 5 sec of complete anoxia causes apparent functional impairment of the central nervous system, 8-12 sec of complete anoxia leads to total loss of consciousness (Oehmichen and Meissner, 2006) and death results within 10 mins of total oxygen deprivation (Nedergaard and Dirnagl, 2005). The occurrence of these rapid devastating events clearly indicates the extreme sensitivity of neurons to any interruption in the brain's blood supply. Though the response is rapid, it constitutes an orchestrated activation of a cascade of events at the physiological and molecular level alters neuronal function and trigger cell death.

The anoxic or ischemic event in a mammalian neuron is characterized by depletion of energy reserves that results in imbalance of the transmembrane ion flow, increased anaerobic glycolysis, acidosis due to lactate production, increased calcium influx, and the massive release of neurotransmitters including glutamate leading to excitotoxicity (Lipton, 1999; Lee et al., 2000; Mehta et al., 2007; Rami et al., 2008). Increased levels of cellular calcium result from over activation of the NMDA receptor caused by glutamate (Lipton, 1999; Moro et al., 2005; Rami et al., 2008). This calcium influx leads to the activation of proteases, lipases, and endonucleases which exacerbates cell injury leading to cell death (Choi et al., 1993, Amir et al., 2005). The major hallmark event during anoxic brain failure is the collapse of the mitochondrial membrane potential that triggers the activation of the pro and anti-apoptotic proteins which regulate the mitochondrial pathway of apoptosis (Lipton, 1999, Milton and Prentice, 2007, Dirnagl and Meisel, 2008). However, a strong proportion of tissue injury that accompanies ischemia is not only due to lack of oxygen supply but also due to reoxygenation/reperfusion after brief period of ischemia. This damage is mainly caused

by the post-ischemic release of oxygen radicals (Berger et al., 2002). ROS (Reactive oxygen species) formation causes direct damage to the cellular components by oxidizing essential cellular proteins, lipids and nucleic acids (Hashimoto et al., 2003) as well as stimulating the stress-related signaling pathways like the MAP kinases (Das and Maulik, 2003, Dirnagl and Meisel, 2008) which culminates by activating the death cascade.

Glucose is the sole metabolite used by the brain via aerobic respiration; however, cerebral ischemia results in the instant switch in the mode of glucose utilization. Anaerobic glycolysis results in lactate accumulation (about 20-fold compared to normoxia) which lower the cellular pH and leads to acidosis (Hata et al., 2000). The energy produced by this alternate mechanism yields only $1/18^{th}$ the energy conventionally produced (Chih and Roberts, 2003). Severe acidosis during cerebral ischemia results in several pathological effects including lipid peroxidation, histological and metabolic injury (Siesjo et al., 1985, 1993), worsening of cerebral infraction (Vornov et al., 1996) and inhibition of antioxidant capacity (Ying et al., 1999). However, mild acidosis is seen to be protective (Simon et al., 1993; Vornov et al., 1996).

A significant reduction in the oxygen and glucose supply to the brain results in the mal-functioning of the major components of the mitochondrial electron transport chain which directly affects ATP synthesis (Kreisman et al., 1981; LaManna et al., 1984; Dirnagl and Meisel 2008). The enormous energy demands of the brain are not met by generation of ATP by anaerobic glycolysis and this leads to the failure of the ATP dependent Na^+/K^+ ion pumps which eventually leads to membrane depolarization and inhibition of neuronal activity (Allen et al., 2005). The total imbalance in the ionic conductance's leads to the accumulation of extracellular K^+ which triggers the release of

neurotransmitters including glutamate, dopamine, GABA (γ-amino butyric acid), glycine etc. However major damage is caused by the massive release of excitatory amino acids like glutamate (Lee et al., 2000; Haberg and Sonnewald, 2006). The increased levels of glutamate cause disruption of the neuronal membrane potential via the overstimulation of the NMDA receptor, which culminates in calcium influx, as well as the activation of metabotropic glutamate receptor, a G-protein coupled receptor (GPCR) (Lipton, 1999). The overstimulation of the glutamate receptors and failure of the uptake mechanisms causes a steep increase in the intracellular calcium levels (Lipton, 1999, Lee et al., 2000; Philis et al., 2001). The alteration in calcium homeostasis is the primary cause for neuronal damage following ischemia or brain trauma or during development of several neurodegenerative diseases (Schubert et al, 1997) which further activate several enzymes including proteases, lipases, DNases and nucleases which damage the integrity of the cytoskeleton as well as leading to neurotoxicity and ultimately resulting in cell death (Choi et al., 1993; Amir et al., 2005; Perlman, 2006). Excessive intracellular calcium results in the increased production of reactive oxygen species (ROS) via the activation of Phospholipase A2 (Ouyang and Giffard, 2004). Additionally, toxic amounts of ROS are generated during recovery from anoxia.

Reactive Oxygen Species

A major destructive phase encountered by the brain is during reoxygenation due to massive release of ROS by the mitochondria (Nita et al., 2001; Lewen et al., 2000; Chan, 2001). The mammalian brain is exquisitely susceptible to ROS damage due to its high lipid content and low anti-oxidant capacity (Floyd and Carney, 1992, Leutner et al., 2001). The massive burst of ROS overwhelms the innate antioxidant and scavenging

capacity of the brain which consists of constitutive antioxidant enzymes including catalase, glutathione-S-transferases, and the superoxide dismutases (SOD) and non-enzymatic antioxidants like vitamin E, vitamin C and glutathione (GSH) and leads to irreversible damage to the neuronal integrity (Seis, 1993; Chan, 2001). Treatment with various antioxidant compounds has been shown to decrease free radical accumulation and cell death both *in vivo* and *in vitro* following oxygen deprivation/reoxygenation (Sheng et al., 2002; Wang et al., 2003). The generation of ROS in excess of the antioxidant mechanisms are also caused by glutamate excitotoxicity which disrupts the mitochondrial membrane potential leading to release of ROS (Kahlert et al., 2005), dopamine (Collier et al., 2003), and elevated intracellular calcium (Sharikabad et al., 2004; Nagy et al., 2004). A number of studies have shown that exposure to increased glutamate levels resulted in marked increase in ROS (Wang et al., 2003; Chi et al., 2005) and that these effects can be abrogated by pretreatment with exposure to NMDA antagonists (Sharp et al., 2005).

Although the mitochondrion is the site for liberation for ROS as side products of oxidative energy metabolism, this organelle also exhibits high susceptibility to ROS. Maintenance of redox homeostasis is a critical factor in preventing cell injury and a massive ROS release can act as mediator to activate a deleterious cascade of events regulating cell death, senescence, and pathogenesis of several diseases (Droge, 2002). The downstream damage in response to a massive ROS release is caused by the activation of the mitochondrial pathway of apoptosis, which is tightly regulated through the control by the balanced activity of the pro and antiapoptotic members of the Bcl-2 family (Ouyang and Giffard, 2004; Mehta et al., 2007). A shift in the levels of expression of these proteins is central to the overall cellular response to an apoptotic signal (Green

and Reed, 1998). The collapse of the mitochondrial membrane potential due to excessive ROS leads to the opening of the mitochondrial membrane potential transition pore (MPTP), (Shimizu et al., 1999; Sharpe et al., 2003; Tsujimoto, 2003). Consequently the opening of the mitochondrial permeability transition pore leads to the release of apoptogenic proteins such as cytochrome C into the cytoplasm which contributes to the activation of the caspase cascade (Shimizu et al., 1999, Lucken-Ardjomande and Martinou, 2005, Dirnagl et al., 2008). The complex downstream events occurring during cell death are also regulated by a complex series of mediators which include the family of proline directed serine/threonine kinases known as the mitogen activated protein kinases (MAPK's) MAPK's and AKT(protein kinase B) (Mehta et al., 2007; Dirgnal and Meisel, 2008).

Signaling pathways in the ischemic brain

In ischemia neurons respond to the compromised oxygen availability by triggering a dynamic network of signaling pathways including the mitogen activated protein kinases (MAPK's) and phosphatidylinositol 3-kinase (PI3K/AKT). The MAPK's consist of a superfamily of proline directed serine/threonine kinases which include the Extracellular signal regulated kinase (ERK) and the Stress activated protein kinase (SAPK's) which include c-Jun N-terminal kinases/stress-activated protein kinase 1 (JNK/SAPK1) and p38/stress-activated protein kinase 2 (p38/SAPK2). The activation these complex cascades in response to cell surface receptors or external stimuli control several intracellular events (for review see Pearson et al., 2001; Martindale and Holbrook, 2002). The activation of these pathways takes place by transmitting extracellular signals through membrane or cytoplasmic receptors to the nucleus and

ultimately bringing about changes in gene expression and cell function by post-translational modification of proteins via phosphorylation (Laderoute and Webster, 1997). Activation of the MAPK's and PI3K/AKT takes place in response to several stress conditions including ischemia, oxidative stress, thermal/osmotic stress and anoxia (Lennmyr et al., 2002; Clerk et al., 1998; Greenway and Storey, 2000; Kamada et al., 2007). The activation of pro-survival pathways and suppression of pro-death signaling cascades is critical for cell survival in the face of anoxia/ischemia (Xia et al., 1995; Zablocka et al., 2003).

The ERK signaling cascade is activated during pathological conditions including anoxia/ischemia in response to alteration in calcium homeostasis, glutamate excitotoxicity and ROS (Chu et al., 2004; Hetman and Gozdz, 2004, Sawe et al., 2008). The activation of the ERK pathway in such conditions triggers phosphorylation of a wide range of cellular substrates and activates transcription of cAMP response element binding protein (CREB) which in turn activates the prosurvival Bcl-2, and suppresses the apoptotic proteins such as Bad, Bim and caspases (Sgambato et al., 1998; Hetman and Gozdz, 2004, Sawe et al., 2008). Activation of ERK has been found to be neuroprotective in several rat ischemia model systems (Xia et al., 1995; Han and Holtzman, 2000; Irving et al., 2000; Park et al., 2004) and also in pathogenesis following ischemia (Wang et al., 2004). Activation of the ERK pathway is thus implicated in both neuronal survival and death; the specific response may be due to differences in the triggering, dynamics and duration of its activation (Martindale and Holbrook, 2002; Ho et al., 2007) potentially in combination with its subcellular signaling and translocation (Hetman and Gozdz, 2004). ERK activation in response to ROS and inflammatory cytokines leads to cell death

(Zhuang and Schnellmann, 2006; Sawe et al., 2008). Rapid and transient activation of ERK can be pro-survival (Guyton et al., 1996a; Guyton et al., 1996b; Li et al., 2002) but in contrast prolonged elevation of ERK causes increased cell death (Wang et al., 2004). The overall outcome of ERK signaling indicates that activation of the ERK pathway can play a crucial role in determining the fate of the cell during oxygen crisis and the overall response depends upon cellular stress conditions, and cell model systems used.

Activation of JNK has been implicated in promoting cell death (Mielke et al., 1999; Guan et al., 2006) however; it was reported to provide protection in cardiac myocytes under conditions of adequate ATP availability (Dougherty et al., 2002; Engelbrecht et al., 2004). P38 has been found to be pro-apoptotic in a rat neuronal ischemic model system as well as in cardiac myocytes (Barone et al., 2001; Engelbrecht et al., 2004). However a recent study in cardiac myocytes indicates that a protective role in delayed preconditioning is triggered by p38 and ERK (Beguin et al., 2007).

AKT (protein kinase B), a serine threonine kinase activated by PI-3 kinase signals to key important compartments of the cell promoting cell survival (Datta et al., 1999). AKT regulates gene expression, cell growth and differentiation as well as cellular responses during stress (Scheid and Woodgett, 2003). AKT is found to be anti-apoptotic in several study models (Jin et al., 2002; Yamaguchi et al., 2001; Hirai et al., 2004; Kilic et al., 2006). These protective roles are thought to be in part via its interactions with the Bcl-2 family of proteins. Studies have shown that AKT phosphorylates Bad- a proapoptotic protein thus preventing its translocation to the mitochondria and inhibiting the release of apoptogenic proteins including cytochrome c, and by this means preventing the opening of the mitochondrial membrane permeability pore (Chan et al., 2004;

Kamada et al., 2007). Recent studies have shown that the activation of the pro-apoptotic stress-activated protein kinase (SAPK'S) cascade is prevented by the pro-survival phosphatidylinositol 3-kinase/AKT pathway in cerebral ischemia by inhibiting Bad (Kamada et al., 2007). Studies reveal that a high degree of cross talk exists between the respective signal transduction pathways, however, and alteration of one pathway will affect other pathways and thus influence the outcome after ischemia. However several candidate factors that are activated during onset of hypoxia/anoxia including adenosine (Dunwiddie and Masino, 2001; Dirnagl et al., 2003; Canals et al., 2005), HIF-1 (Hypoxia inducuble factor) (Semenza, 2007), AMP kinase (Cheung and Hart, 2008), K_{ATP} channels (Watanabe, 2008), and mTOR (Mammalian target of rapamycin) (Chong and Maiese 2007; Swiech et al., 2007) directly or indirectly trigger the substantial signal transduction cascades. Additionally, ROS levels also have a effect on activation of these cascades (Rodrigo and Standen, 2005). The activation of the ischemic defense mechanism is strongly linked to the purine adenosine which increases during hypoxia due to breakdown of ATP, the energy currency of the cell (Dunwiddie and Masino, 2001; Wardas, 2002).

Adenosine as a Neuromodulator

In mammals a reduction in blood flow to the brain during hypoxia/ischemia causes a steep elevation in the extracellular levels of adenosine derived from breakdown of ATP due to depletion of energy reserves (Dunwiddie and Masino, 2001; Canals et al., 2005). Adenosine plays a endogenous neuroprotective role in mammals during and following cerebral ischemia (Gupta et al., 2002; Kulinsky et al., 2001) mainly via suppressing synaptic activity and inhibiting the release of excitatory neurotransmitters (Rudolph et al., 1992). Studies have suggested a crucial role of adenosine in repairing

brain tissue through neuropharmacological intervention. Mediators such as adenosine are a part of normal physiology and their activity can be altered successfully using specific agonists and antagonists. This might help to restore calcium homeostasis or to promote protective pathways that can lead to regeneration (Rebeiro, 2005).

Several studies to date have confirmed adenosine receptors as therapeutic targets in treating cerebral ischemic diseases, sleep disorders and inflammatory reactions (Jacobson and Gao, 2006). It has been shown that adenosine receptor stimulation is critical for neuronal survival during global cerebral ischemia (Kulinsky et al., 2001). Adenosine A1 receptor activation in the mammalian brain inhibits neurons presynaptically by decreasing vesicular release and postsynaptically by modulating transmembrane ion flow (Sciotti et al., 1992; Cooke et al., 1999, Haas and Selbach, 2000). Adenosine A1 receptor stimulation suppresses intracellular calcium influx (Rudolphi et al., 1992) and inhibits synaptic transmission by preventing glutamate release (Zhu and Krnjevic 1993, Wardas, 2002) thus avoiding glutamate toxicity (Dunwiddie and Masino, 2001). The neuroprotective effect of CCPA (cyclopentyladenosine), an A1 adenosine receptor selective agonist during hypoxia and ischemia can be reversed by DPCPX in highly enriched primary cultures of neurons as well as in heart (Logan and Sweeney, 1997, Safran, 2001). Similarly Adenosine A_{2A} receptor stimulation attenuated neuronal damage and decreased reperfusion injuries in spinal cord ischemia (Cassada et al., .2002). However Adenosine A2A receptor inhibition was found to be neuroprotective during ischemia via inhibition of P38 MAPK (Melani et al., 2006).

Adenosine also plays a very important role in cerebral and cardiac ischemic preconditioning (PC). PC is a phenomenon in which an initial, brief non-lethal ischemic

event renders the tissue resistant to a subsequent more severe ischemic insult. PC is of interest as it reduces ischemic tissue damage and enhances tolerance to ischemia. Inactivation of the A1 receptor using a selective antagonist DPCPX (1,3 –dipropyl-8-cyclpentylxanthine) significantly reversed protective effect of PC in hippocampal neurons (Hiraide et al., 2001).

Several studies have strongly supported the protective role of adenosine against ROS. Adenosine receptor activation can counteract with the deleterious cellular consequences of ROS (Nie et al., 1998). Adenosine formed by breakdown of ATP during ischemic and oxidative stress attenuates the cellular damage caused by reactive oxygen species via A (1) receptor activation thus boosting neuronal recovery in rat hippocampus (Almeida et al., 2003). Studies using pharmacological manipulations of A(1) receptor using the A(1) receptor agonist CCPA or A(2A) receptor blockade elicited neuroprotection and cardioprotection against H_2O_2 induced oxidative stress which otherwise leads to both apoptotic and necrotic death (Fatokun, 2007, Karmazyn and Cook, 1992, P. Narayan, 2001). A recent study has shown that Adenosine A1 receptor stimulation leads to inhibition of presynaptic neurotransmission via activation of P38 mitogen activated protein kinase (MAPK) (Brust et al., 2006). Other investigations reported that purinergic stimulation induced activation of the prosurvival ERK and PI3-K pathways which lead to cell differentiation in glioma cell line (Jacques-Silva et al., 2004) and mediated ischemic preconditioning in heart (Germack et al., 2004). Adenosine mediated suppression of pro-death p38 and Bad was reported in astocytes exposed to oxygen glucose deprivation (D'Alimonte et al., 2007; Ciccarelli et al., 2007). These studies indicate that the complex series of intracellular signaling pathways mediates the

protective effects of adenosine. This range of interactions of adenosine with the AKT pathway/MAPKs family members and the Bcl-2 family of proteins confirms their central role in regulation of apoptosis.

Apoptosis

Apoptosis is a tightly regulated process in which a cell in response to an injury activates a programmed sequence of signaling events causing a loss of cellular integrity which finally leads to self destruction. To date three different mechanisms of apoptosis are described in the literature. One that is regulated at the mitochondrial level and triggered by signals generated intrinsically by the cell is generally referred to as the intrinsic pathway of apoptosis (Luo et al., 1998). Another mechanism is by death activators that bind externally to receptors on the cell surface namely TNF-α and Fas ligand and trigger the activation of the death receptor pathway, also described as the extrinsic pathway of apoptosis (Dhein et al., 1995). Third mechanism is via the release of apoptosis inducing factor (AIF) triggered by reactive oxygen species released from the intermembrane space of the mitochondria and is a caspase independent mechanism (Tsujimoto, 2003). The most widely observed mechanism leading to cell death during ischemia and oxidative stress is the mitochondrial/intrinsic pathway which is regulated by the Bcl-2 family of proteins.

Role of the Bcl-2 family of proteins and their interactions with MAPK'S

The Bcl-2 family of proteins tightly regulates the process of apoptosis. In the cell there exists a delicate balance between pro-apoptotic and anti-apoptotic proteins. A shift in the levels of expression of these proteins will control the overall cellular response to an apoptotic signal (Green and Reed, 1998). The mitochondrial pathway of apoptosis

process is regulated by the coordinated action of Bcl-2 family members which are classified based on the presence of BH (Bcl2-homology) domains (Adams and Cory, 2001). The groups of proteins that contain all four domains are classified as the anti-apoptotic proteins and include Bcl-2, Bcl-xL. Some members of the pro-apoptotic group that includes Bax and Bak consist of BH1-3 domains whereas others including Bad, Bid consist of the BH-3 domain only (Gross et al., 1999). Bax and Bid the pro-apoptotic members of the Bcl-2 family of proteins cause the opening of the intracellular membrane to the entry of ions and proteins as well as forming protein-conducting pores in the outer mitochondrial membrane, whereas the anti-apoptotic members Bcl-2 and Bcl-XL have the opposite effect on membrane permeability (Harris and Thompson, 2000). Bcl-2 acts as a major component that blocks apoptosis signals downstream of a complex signaling cascade and it plays a major role in suppression of apoptosis (Reed, 1994, Strasser et al., 1996). Bcl-2 is located on the outer membrane of mitochondria and maintains membrane potential as well as mitochondrial homeostasis (Wolter et al., 1997). On receiving an apoptotic signal, the pro-apoptotic proteins are translocated to the mitochondria where they alter the mitochondrial metabolism by interfering with the oxidative phosphorylation. This leads to the collapse of the mitochondrial membrane potential, which triggers the opening of the mitochondrial permeability transition pore (Shimizu et al., 1999, Sharpe et al., 2003, Tsujimoto, 2003). The opening of the mitochondrial permeability transition pore leads to the release of apoptogenic proteins such as cytochrome C into the cytoplasm which contributes to the activation of the caspase cascade (Shimizu et al., 1999, Lucken-Ardjomande and Martinou, 2005).

Recent studies investigating PC as a protective strategy against ischemia have suggested a neuroprotective role of Bcl-2. Hypoxic preconditioning renders the brain tissue more resistant to subsequent anoxic stress by increasing Bcl-2 expression which stabilizes the Mitochondrial Membrane Potential (MMP) and thus prevents the apoptotic cascade (Wu et al., 2005). Cerebral ischemic/hypoxic preconditioning prevents neuronal apoptosis by increasing expression of Bcl-2 and decreasing expression of pro-apoptotic Bax (Gao et al., 2006). Bcl-2 overexpression following experimental stroke has been shown to inhibit both caspase activation and the release of apoptogenic proteins such as cytochrome c and thus prevents neuronal loss within the ischemic area (Zhao et al., 2003). The results from several other ischemic model systems indicate that apoptosis can be inhibited by manipulation of Bcl-2 family members by viral-vector mediated gene transfer, transgenic mouse techniques and antisense oligonucleotides (Shimazaki, 2000, Hata, 1999, Chen et al., 2000, Shimuzu et al., 2001). Additionally Bcl-2 protein has was shown to play a major role in suppressing cell death during apoptotic and oxidative stress cell injury by enhancing the levels of anti-oxidants as well as suppressing generation of free radicals (Hockenbery, 1993; Kane et al., 1993; Ellerby et al., 1996; Lee et al., 1998, 2001, Saitoh et al., 2003). However the underlying mechanisms which initiate and transmit cell death signals or which allow anti-apoptotic signals to rescue the cells, still need to be elucidated. Thus the cumulative evidence suggests that by potentially targeting these critical elements an effective clinical strategy may potentially be developed for treatment of stroke.

Comparative anoxia-tolerant models

It can be difficult to identify protective mechanisms in the mammalian brain, however, because hypoxia or ischemia induces both physiological and pathological responses simultaneously (Dirnagl and Meisel, 2008). As mentioned earlier, the mammalian brain activates a series of systemic responses (physiological adaptations) within few seconds of oxygen deprivation including increasing cerebral blood flow, increasing anaerobic glycolysis, increasing ventilation, decreasing energy-consuming processes which helps to conserve its the high energy demand (Yuan et al.,1990; LaManna et al., 2004). Several tissue responses are observed within minutes to hours of hypoxia which promotes improved vascularization and enhancement of oxygen supply to the brain. All these take place rapidly at the onset of anoxia using the proteins present in the tissue. However a rapid upregulation of several HIF-1 mediated protective genes including EPO (erythropoietin), glycolytic enzymes, glut-1, VEGF (Vascular endothelial growth factor) to enhance angiogenesis, and inducible NO synthase that increases vasodilation are necessary for long term survival (Semenza, 2000; LaManna, 2007). These protective physiological and molecular events are masked by the simultaneous occurrence of pathological events which are more apparent during hypoxia/ischemia. The pathological responses include lactate acidosis due to anaerobic glycolysis, massive excitatoxicity due to glutamate, alteration in calcium homeostasis and activation of complex signal transducers which culminates by triggering the mitochondrial pathway of apoptosis (Berger et al., 2002; Lutz et al., 2003). Reoxygenation also leads to cell death, mainly through reactive oxygen species production and inflammatory responses (Michiels, 2004).

Exploring and analyzing intracellular signaling events is thus very complicated in mammalian neurons due to their exquisite sensitivity to stressors such as hypoxia, or ischemia/reperfusion. Such events can be dissected and analyzed in the anoxia tolerant fresh water turtle, which can inherently survive the conditions of oxygen deprivation and post-anoxic reoxygenation without brain damage. It is likely in such a model that modulation of a particular molecular signaling cascade is adaptive rather than pathological. Uncovering these molecular mechanisms in an alternate model such as the turtle might help to disentangle the complexities of vulnerable mammalian neurons and provide insights into studying the mechanisms of mammalian anoxic brain damage. These pathways can be clearly studied in the anoxia tolerant fresh water turtle as it serves as a promising tool for analyzing the cellular and molecular events during hypoxic /ischemic episodes. Exploration and elucidation of these protective pathways in an anoxia tolerant model may provide novel therapeutic strategies for stroke and other neurodegenerative disorders.

Anoxia tolerant brain

The extraordinary capacity to tolerate total oxygen deprivation is not common to all lower vertebrates. The only well recorded species of this phenomenon are the crucian carp (Carassius carassius), the goldfish (C. auratus) and two freshwater turtle species, Chrysemys picta and Trachemys scripta (formerly Pseudemys scripta) (Nilsson, 1990; Lutz and Nilsson, 1994). This scenario is not a consequence of ectothermy exhibited by most reptiles as other species in this class are extremely sensitive to oxygen deprivation. This indicates that turtles have evolved specific physiological and molecular mechanisms that prepare the turtle to face conditions of oxygen deprivation and recover back without

activation of pathological processes. The freshwater turtle Trachemys scripta elegans has been studied extensively for more then 2 decades in terms of physiological adaptations that permit neuronal survival in the absence of oxygen; however a few recent investigations have analyzed the molecular changes and events in realm of reoxygenation. This species displays a unique ability to withstand oxygen deprivation for up to 48 hrs at room temperature and for weeks at 3^{o} Celsius (Jackson, 2000). This unique ability is the result of application of at least three distinct mechanisms uncommon in mammals. First, these animals have large amounts of fermentable fuel in the form of glycogen which predisposes them to face anoxia. Secondly, they display a high tolerance of lactate-generated acidosis resulting from anaerobic glycolysis due to inherent natural buffering system. Lastly, they are able to lower energy consumption to match the anaerobic energy production levels thus avoiding a drastic drop in ATP levels, which would otherwise result in catastrophic cell death (Clark & Miller, 1973; Lutz et al., 2003; Lutz and Milton, 2004; Milton and Prentice, 2007). The anoxia and recovery episode consists of the initial transition phase, the maintenance phase and the recovery phase which are accomplished by application of range of physiological and molecular adaptations (Milton and Prentice, 2007)

Physiological adaptations

The initial hour of anoxia is really a time of increasing hypoxia while external and internal oxygen levels decline. An immediate response to this event is a drastic decrease in metabolic activities which decrease the energy consumption by the brain tissue. This hypometabolic state is unlike mammals and the tissue energy requirements are maintained by anaerobic glycolysis which maintains the basal metabolic activities (Lutz,

2003). To further decrease neuronal energy requirements the turtle decreases ion flux through a process termed channel arrest (Perez-Pinzon et al., 1992; Pek-Scott and Lutz, 1998). Though ATP levels are maintained at a steady state in anoxia in the turtles (Lutz et al., 1984) the turtle brain experiences an initial steep drop in ATP levels which releases the purine adenosine in the extracellular space. In turtles, it has been demonstrated that AD is critical at the physiological level to anoxic neuronal survival, acting as a "retaliatory metabolite" to balance energy supply and demand (Nilsson and Lutz, 1992; Lutz and Nilsson, 1997). AD levels increase as much as 12-fold over the initial 1-2 hr anoxia (Nilsson and Lutz, 1992) accompanied by an increase in A_1 receptor sensitivity (Lutz and Manuel, 1999). Adenosine receptor (ADR) activation has several protective effects like increased glycolysis, decreased extracellular levels of excitotoxins, increased blood flow and is a key element of channel arrest (Nilsson and Lutz, 1992; Perez-Pinzon et al., 1993; Hylland et al., 1994; Pek and Lutz, 1997, Milton et al., 2002; Milton and Lutz, 2005). The net effect of these events is prevention of anoxic depolarization. Other physiological events include marked decrease in the conductance of K^+ ions at the end of 2 h of anoxia (Pek and Lutz, 1998). The opening of the K^+ dependent ATP channels (K_{ATP} channels) and adenosine receptor stimulation further prevents anoxic depolarization (Pek and Lutz, 1997; Pek and Lutz, 1998). In addition to this, the calcium homeostasis is maintained by regulation of the NMDA receptor (Buck et al., 1998). The release and uptake mechanisms of excitatory amino acids like glutamate and dopamine are maintained in turtles during prolonged anoxic episodes (Milton et al., 2002; Milton et al., 2005). Further more, there is a significant decrease in the neuronal electrical activity (Fernandes et al., 1997), which is reversed quickly upon reoxygenation.

Overall, far less is known about adaptations for reoxygenation than for the induction and maintenance of the anoxic hypo-metabolic state. A critical phase following anoxic stress is exposure to elevated levels of reactive oxygen species (ROS) upon reoxygenation. A few minutes of anoxia is sufficient to reduce the activity of electron carriers of the mitochondrial respiratory chain to such an extent that ROS could be generated in toxic amounts once oxygen supply is restored (Lutz et al., 2003). Upon return to normoxic conditions, however, tissues become quickly reoxygenated, with arterial oxygen levels returning from 0 to 30 torr within 5 minutes (Milton, 1994). Elevated levels of ROS also result in activation of stress pathways including SAPKs, which have the potential to lead to neuronal death. In mammalian neurons ROS defense mechanisms include several antioxidant enzymes including superoxide dismutase, Catalase and Glutathione peroxidase. The turtle either has mechanisms to prevent the formation of ROS, and/or has mechanisms to protect against ROS damage (Lutz et al., 2003). Recent *in vivo* and *in vitro* work in our laboratory (Milton et al., 2007) has demonstrated the unique ability of the turtles in suppressing ROS formation upon reoxygenation (Milton et al., 2007). Real time measurements of ROS production did not increase in relation to the normoxic levels unlike mammals. Suppression of the adenosine receptors using selective A1 receptor antagonist resulted in increased adenosine receptors increased survival and decreased ROS production (Milton et al., 2007). Additionally, turtle neurons were found to be extremely vulnerable to extrinsic oxidative stress, indicating that anoxia and reoxygenation survival is mainly due to activation of several physiological and molecular adaptations (Milton et al., 2007).

Molecular adaptations

Recent studies have indicated increased upregulation of several protective genes in response to prolonged anoxia. Studies in our lab have shown high constitutive levels of heat shock proteins Hsp72 and Hsp73 in normoxia, which are induced to a greater extent on exposure to 4h anoxia (Prentice et al., 2004). Another investigation (Milton et al., 2008) from our laboratory has shown activation of neuroprotective signaling cascades AKT/PI3k and ERK whereas suppression of the presumed pro-death p38 cascade during the initial 1h hour of anoxia. The activation of these neuroprotective signaling cascades were modulated by adenosine as the employment of the general adenosine receptor antagonist, aminophylline resulted in total suppression of ERK and AKT and increased levels of p38. These results suggested that the upstream metabolite adenosine released during anoxia plays a major role in regulating the diverse MAPK's and AKT pathway (Milton et al., 2008). The activation of the ERK and AKT suggested that these pathways are the core molecular strategies used by the turtle in defense against anoxia. Similar observations were made in a recent in-vitro study; however an activation of p38 and JNK was also seen. The protective effects of AKT and ERK were increased significantly by A1R agonist 2-Chloro-N-cyclopentyladenosine (CCPA, 0.6 nmol/L) and were abrogated by employing a adenosine receptor antagonist 8-Cyclopentyl-1,3-dihydropylxanthine (DPCPX, 1 nmol/L) (Nayak et al., in prep).

Similar observations are reported by Haddad (2007) in some in-vivo studies using anoxia tolerant model Chrysemys picta but in slightly different conditions. Haddad (2007) reported that JNK expression remain unaltered after weeks of hypoxic submergence, although a significant increase in ERK was observed after 5 h hypoxia, a

slight increase in Bcl-2 compared to Bax levels was also seen. In anoxic submerged turtles organ specific elevation in the levels of JNK and ERK was reported (Greenway and Storey, 2000). However hypoxic and anoxic submergence can trigger different responses in this model which can further activate a different set of signaling cascades compared to hypoxia and anoxia exposures (Greenway and Storey, 1999, 2000; Prentice et al., 2004). The overall observations clearly indicate a link between the physiological adaptations and molecular regulations in this anoxia tolerant model. The combined application of a suite of physiological and molecular events such as release of adenosine, opening of K_{ATP} channels, suppression of the NMDA receptor, maintenance of calcium homeostasis, induction of protective genes, activation of the neuroprotective cascades and high levels of antioxidative enzymes help to preserve the neuronal integrity during oxygen crisis and recovery in the anoxia tolerant turtle. The occurrence of these protective events also indicates that the turtle brain may be constitutively preconditioned. Many of these protections are also observed in mammalian preconditioning.

Ischemic Preconditioning and neuroprotectants

A brief exposure to "sublethal" ischemia can make the brain resistant to future ischemic episodes by activating a concerted series of endogenous protective events which include induction of several protective genes (Webster et al., 1995; Dirnagl et al., 2003). This phenomenon is referred to as ischemic preconditioning/ischemic tolerance (IP / IT) and it offers robust protection via several molecular adaptations and increases cell survival by 50% (Malaughlin et al., 2003). There are two phases of (IP/IT) in the brain IP: early or rapid phase in which the sensor activates and induces protection within minutes and late phase which is induced over hour and days and provides opportunity for

transcription and translation of critical prosurvival protein thus preventing cell death (Schurr et al., 1986; Dirnagl et al., 2003; Yellon and Downey, 2003). IP consists of involvement and activation of an orderly arranged set of factors such as sensors which respond to the stimulus, transducers/mediators which processes the sensor signals and the effectors which generates the response (Dirnagl et al., 2003; Baker 2004). The major sensors or triggers include NMDA receptor (Dirnagl et al., 2003), HIF (Semenza, 2007), adenosine and adenosine receptor agonists (Dirnagl et al., 2003; Ciccarelli et al., 2007) and K(ATP) channels (Watanabe, 2008). Studies have demonstrated some of these major candidate factors which play a critical role as transducers at the different levels of PC include Nitric oxide (Dirnagl et al., 2003; Scorziello et al., 2007), MAPK's (Dirnagl et al., 2003) Phosphokinase C or PKC (Van Kolen et al., 2008) and PI3K/AKT (Hillion et al., 2006). ROS are generated during hypoxia/ischemia also acts as major transducers for the IP in the brain (Zhang et al., 2004) by activating PKC (Perez-Pinzon et al., 2005). Similarly ROS acts as major transducers of myocardial PC by activating PKC epsilon, ERK and PI3K/AKT pathway (Liu et al., 2008). The induction of the effector proteins determines the degree of cell survival during IP and these include heat shock proteins (Baker et al., 2004), GABA, GABA receptor upregulation, VEGF (Dirnagl et al., 2003), and Bcl-2 family proteins which are considered to be the major regulators of cell survival/death (Dirnagl et al., 2003; Rami et al., 2008).

As the endogenous neuroprotective mechanisms can be effectively turned on in the mammalian brain, it suggests clearly indicates that these factors may reside naturally in all vertebrates. Similarly, there exists endogenous neuroprotectants which have a remarkable potential to sustain respiration under compromised oxygen conditions and

offer protection upon oxygen restoration. One other potential pro-survival molecule is the recently discovered neural heme protein neuroglobin (Ngb), which has been proposed to have a variety of neuroprotective functions. A likely role for Ngb is as a "neuronal myoglobin" transferring oxygen to the mitochondrial respiratory chain in the brain and retina (Burmester and Hankeln, 2004). Other potential roles include acting as a terminal oxidase that regenerates NAD+ under anaerobic conditions, a role in the detoxification of reactive oxygen species (ROS), or as a sensor to detect cellular oxygen concentrations (Weber and Vinogradov, 2001; Burmester and Hankeln, 2004). Burmester et al., first reported Ngb in the mouse brain in the year 2000, and since then it has been identified in all vertebrates (Burmester and Hankeln, 2004) including reptiles (Milton et al., 2006) using *in vivo* models (Sun et al., 2001; 2003; Burmester and Hankeln, 2004) and *in vitro* analyses (Khan et al., 2006; 2007). Several investigations showing an induction of Ngb during oxygen-deprived conditions points to a critical role of Ngb in the modulation of hypoxic/ischemic brain injury. Similarly recent studies suggest Ngb could act as a major stress sensor, and does activate signal transduction in the brain (Wakasugi et al., 2003, 2005; Kitasuji et al., 2007, Khan et al., 2008) as well as protect neurons against oxidative stress (Li et al., 2008).

These observations suggest a major role of Ngb as an endogenous neuroprotectant (Greenberg et al., 2008). A significant induction of Ngb during hypoxia and reoxygenation upon anoxia and a moderate upregulation in the anoxia tolerant fresh water turtle points to a critical role in ROS regulation (Milton et al., 2006). Ngb knockdown using antisense oligonucleotides exacerbated neuronal injury in several stroke models (Sun et al., 2003), and over expression of Ngb is reported to reduce stroke-induced cell

death (Khan et al., 2006; Sun et al., 2001; Sun et al., 2003). *In vitro* studies have shown that knocking down Ngb using siRNA increased levels of ROS in the human SH-SY5Y neuroblastoma model system (Fordel et al., 2006), while Li et al., (2008) report that siRNA against Ngb increased ROS accumulation and oxidative damage in PC12 cells. To date majority of studies on Ngb have been carried out on mammalian species. However, a comparative approach can offer a promising tool for the identification of conserved Ngb features and thus for the further understanding of Ngb function. The advantage of the turtle model is that *in vivo* experiments can be performed on severely hypoxic and anoxic animals, as they are able to survive and recover from long-term anoxia (Lutz et al., 2004). Studying the expression pattern of this gene in the anoxia tolerant turtle will provide the unique opportunity to examine and understand the unique neuroprotective responses to oxygen deprivation in the absence of pathological processes associated with hypoxic responses in mammalian neurons.

Hypothesis

Physiological pathways that protect the anoxia tolerant turtle during anoxia are widely studied (Milton and Lutz, 2002). These physiological adaptations are certainly linked with several molecular aspects which protect the turtle during oxygen deprivation. Survival pathways upregulated by anoxia in the anoxia tolerant fresh water turtle Trachemys scripta also provide protection against reoxygenation stress. By investigating these pathways in an alternative animal model and uncovering how these underlying mechanisms help protect these anoxia tolerant creatures under conditions of oxidative stress we can obtain an insight into potential therapeutic targets for a variety of

pathologies attributed to ROS stress. This may eventually help clinicians design new therapies based on naturally tolerant organisms. Briefly, the specific aims are:

1. To investigate the neuroprotective role of adenosine in anoxia survival and ROS defense in turtle neuronal cultures.

2. To investigate the role of Mitogen activated protein kinase (MAPK) and AKT signaling pathways in neuroprotection and ROS tolerance.

3. To determine whether altered expression of Bcl-2 family members may underlie the protection against anoxia and oxidative stress in anoxia tolerant turtle.

4. To analyze the neuroprotective role of Ngb in anoxia and oxidative stress in the anoxia tolerant turtle brain and neuronally enriched cell cultures.

CHAPTER 2

METHODS AND MATERIALS

All experiments were approved by the Florida Atlantic University Institutional Animal Care and Use Committee.

Small Trachemys (juvenile, 4 to 6 inches) were obtained from a commercial supplier (Clive Longdon, Tallahassee, FL) and were used for all experiments. Turtles were maintained in freshwater aquaria on a 12 h light-dark cycle and fed 3x weekly. Animals are sacrificed for cell culture harvest by cervical separation in accordance with animal care standards established by the American Veterinary Medical Association for reptiles.

In vivo anoxic exposure and tissue preparation

To induce anoxia/hypoxia five experimental sets of N=5 included normoxic controls, anoxic animals exposed to 1 or 4 h anoxia, 4 h anoxia/4 h normoxic recovery, and 4 h hypoxia (5% O_2). For anoxic exposure, animals were individually placed in sealed 2 L plastic chambers at room temperature (22-23°C) under 99.99% N_2 (positive pressure flow-through, County Welding, Pompano Beach, FL). Normoxic controls were utilized directly from the aquaria. Hypoxic animals were placed in individual open holding boxes in a hypoxia chamber (Sheldon Manufacturing, Cornelius, OR) under 5% O_2 for 4 h. Animals subjected to reoxygenation were allowed normal normoxic recovery in room air at room temperature. Chamber PO_2 was determined hourly to ascertain O_2

levels (Cameron Instrument Company, Port Aransas, TX). Animals were sacrificed by cervical separation and the brains removed into liquid nitrogen in less than 2 min.

Turtle neuronally enriched Cell Cultures

Cell culture: Juvenile Trachemys scripta elegans (150-350g) were sacrificed by cervical separation and the brain quickly excised aseptically. The isolated cerebral hemispheres and hindbrain were finely minced and digested in a cocktail of MEM–L-glutamine and Earl's Salts (Mediatech, Inc., Herndon, VA) supplemented with 10% FBS (Gibco/Invitrogen, Grand Island, NY) containing 56U/ml penicillin and 56ug/ml streptomycin (Gibco), with a protease cocktail of 1300U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO), 25U/ml collagenase (Gibco) and 0.32U/ml dispase (Gibco). The homogenate was incubated for 4h at room temperature (23°C) with agitation. Neurons were separated utilizing differential concentrations of Optiprep (Sigma-Aldrich) and Minimum Essential Medium (MEM) dispensed as layers onto the surface of each preceding layer to create a density gradient (Brewer, 1997). The liquid portion of the brain homogenate was placed onto the surface of the gradient medium and centrifuged for 15 minutes at 750g (Beckman Coulter) at 30ºC. This creates distinct layers of neurons and glial cells; the neuronally enriched layer was pipetted out and cultured in 6 well TC-plates with MEM supplemented as above and incubated in a humidified chamber at 30°C in air/5% CO_2 (Kendro Laboratory Products, Asheville, NC). The medium was changed at 24 hrs and then weekly until cultures were utilized at 10-14 days (grown to confluence). Cultures were double-labelled with the neuronal markers NeuN and NCAM and the glial marker GFAP, which revealed that cultures are consistently >98% neuronal cells.

Immunostaining

Cells were fixed with 1-2 ml of freshly prepared 4% paraformaldehyde for 10 min. Cells were washed three times with PBS and further permeabilized with 0.1% Triton X-100 in PBS at room temperature. Cells were then washed with 0.1% Triton X-100 and non-specific binding was blocked for 15 min with blocking solution (0.5% Bovine Serum Albumin (BSA) in PBS. Cells were incubated overnight at 4°C with primary Alexa flour anti-mouse NeuN antibody (1:500, Chemicon, Billerica, MA) to confirm neuronal identity. Similarly primary anti-rabbit neurofilament antibody (1:500, Chemicon, Billerica, MA) and secondary alexa Fluor 568 goat anti-rabbit antibody (1:500, Chemicon, Billerica, MA) were also used to confirm the neuronal phenotype. After washing, cells were prepared with mounting medium and coverslipped. Cells were observed using a confocal Microscope equipped for epifluorescence.

Additionally double labeled immunostaining with primary anti-chicken Ngb polyclonal antibody (Biovendor, Candler, NC), anti-mouse NeuN antibody and anti-rabbit neurofilament antibody (Chemicon, Billerica, MA), anti-rabbit GFAP (1:100, Dako, Carpintera, CA) was carried out as described earlier. Alexa Fluor 568 goat anti-mouse/rabbit antibody and rabbit anti-chicken IgG, FITC conjugate secondary antibody (Chemicon, Billerica, MA) were employed as secondary antibodies (Invitrogen, Molecular probes, Eugene, OR). After washing, cells were prepared with mounting medium and coverslipped. Cells were observed using a confocal Microscope equipped for epifluorescence.

Anoxic and Hypoxic Cell Culture Experiments

All anoxia and hypoxia experiments were conducted in a Shel-Lab Bactron anaerobic chamber (Sheldon Manufacturing Inc Cornelius, OR) at 30°C under anoxic gas mixture (90% N_2, 5% He, 5% CO_2, AirGas, Miami, FL). Oxygen inside the chamber was continuously monitored with an OM-4 oxygen meter (Microelectrodes Inc, Bedford, NH).

***In vitro* Drug Exposure Protocol**

Adenosine receptor stimulation and inhibiton

For experimental treatments, existing medium was replaced with MEM containing the specific A_1 adenosine receptor blocker 8-Cyclopentyl-1,3-dihydro-pylxanthine (DPCPX, 1 nmol/L) or the adenosine recepetor agonist 2-Chloro-N-cyclo-pentyladenosine (CCCPA, 0.6 nmol/L) (2 wells + 2 wells + 2 wells control). Drugs were added either prior to the anoxic exposure (chronic stimulation/blockade, or only for the last hour of anoxia (acute exposure). Medium and drugs were degassed in the anoxia chamber prior to use to ensure continued anoxia. Propidium iodide (PI, Molecular Probes, Inc) staining to determine cell death was performed on identically treated cultures.

Blocking of the MAPK's and AKT pathway

Cell cultures were pre-exposed to specific MAPK's (UO126 for ERK pathway, SP00612 for JNK pathway, SB3082 for P38 pathway) and AKT (LY20027 for P13-K) inhibitors before subjecting those to experimental conditions. The concentrations used were determined by a dose dependent titration without significantly altering other MAPK pathways. The blockers were added prior to the experimental exposures. Medium and

drugs were degassed in the anoxia chamber prior to use to ensure continued anoxia. Propidium iodide (PI, Molecular Probes, Inc) staining to determine cell death and protein level analyses was carried out using western blot on identically treated cultures.

In vitro ROS measurements

Amplex Red assay

Cultures were utilized in three treatment groups: normoxic controls, 4 hr anoxia, and 4 hr anoxia-2 hr reoxygenation. All cell culture experiments were performed in a Bactron Environmental/Anoxia Chamber (Sheldon Manufacturing) at 30°C under anoxic gas mixture (90% N_2, 5% He, 5% CO_2, AirGas, Miami, FL). Normoxic controls were incubated in the incubation chamber in air/ 5% CO_2. H_2O_2 production was assessed by fluoresence following each treatment using Amplex Red® according to the manufacturer's protocol (Sun et al., 2005). 50 µl culture medium was removed from the cells and incubated at 30°C for 30 min with 100 uM Amplex Red and 0.2 U/ml HRP prepared in MEM. Fluorescence was measured on a fluorescent microplate reader at 550nm absorbance and compared to a standard curve.

Cell Death and Viability Assays

Propidium iodide staining

Cultures were utilized in three treatment groups: normoxic controls, 4 hr anoxia, and 4 hr anoxia-2 hr reoxygenation. All cell culture experiments were performed in a Bactron Environemental/Anoxia Chamber (Sheldon Manufacturing) at 30°C under anoxic gas mixture (90% N_2, 5% He, 5% CO_2, AirGas, Miami, FL). Normoxic controls were incubated in the incubation chamber in air/ 5% CO_2. Propidium iodide (PI, Molecular Probes, Inc) staining to determine cell death was performed on identically

treated cultures. After experimental exposures cell were stained with propidium iodide (5 μg ml^{-1} final concentration) for 30 min 1μg/ml PI for 20 minutes, washed two times with PBS, and analyzed by confocal microscopy. Cell viability was assessed on the basis of nuclear morphology and staining with propidium Iodide using a confocal microscope equipped with epifluorescence. Cell counts were performed manually.

Resistance to external oxidative stress

To determine whether anoxia increases cellular resistance to ROS stress, five experimental sets of cultured neurons (N=3-5 independent experiments per analysis) were subjected to normoxia, anoxia for 1hr, 4hr and 12 hr in the anoxic chamber followed by 2 hr of H_20_2, or 12 hr anoxia/2 hr reoxygenation and 2 hr of H_2O_2.

The effect of adenosine stimulation and blocking was determined by pretreating the cultures with DPCPX or CCCPA and exposing them to H_2O_2. Preliminary experiments determined the LD_{50}s of H_2O_2 for normoxia, 1, 4, and 12 hr anoxia, plus anoxia/reoxygenation; treatments were followed by PI staining as described above.

Measurement of mitochondrial membrane potential (ψ)

JC-1 ((Invitrogen, Molecular probes, Eugene, OR) was used to measure the membrane potential of the Bcl-2 knockdown and control treated neurons in the cell culture after they were subjected to anoxia and oxidative stress. JC-1, a fluorescent dye undergoes reversible change based on the membrane potential from red to green. The mitochondrial of normal viable cells are stained red. During stress, the mitochondrial potential collapses and the dye remains in the cytoplasm which fluoresces green. Confocal microscopy was carried out to analyze the green to red ratio of the cells.

Cultures were utilized in three treatment groups: normoxic controls, 4 hr anoxia, and 4 hr anoxia-4 hr reoxygenation for both Bcl-2 knockdown and control treated cells. All cell culture experiments were performed in a Bactron Environemental/Anoxia Chamber (Sheldon Manufacturing) at 30°C under anoxic gas mixture (90% N_2, 5% He, 5% CO_2, Air-Gas, Miami, FL). Normoxic controls were incubated in the incubation chamber in air/ 5% CO_2. JC-1 staining was performed on identically treated cultures. After experimental exposures cells were stained by adding 10 µL of 200 µM JC-1 (2 µM final concentration).The cells were incubated at 37°C, 5% CO_2, for 15 to 30 minutes, washed once with phosphate-buffered saline and observed under confocal microscope.

RNA extractions

Total RNA was extracted using the TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol and RNA was subjected to treatment with DNase I to eliminate DNA contamination.

RT-PCR

Complementary DNA was synthesized from total RNA using primers specific for Bcl-2 , Bax, Neuroglobin and actin, respectively. The PCR using Taq polymerase comprised denaturation for 7 min, 94^0C, PCR: 40 cycles (Bcl-2 and Bax) (1 min, 94^0C; 45 s, 55^0C; 1.0 min, 72^0C), (Ngb) (1.0 min, 94^0C; 45 s, 59^0C; 1.0 min, 72^0C) followed by elongation: 10 min, 72^0C or 30 cycles (actin) (1 min 94^0C; 45 s, 55^0C; 1.5 min 72^0C) followed by elongation: 10 min 72^0C. Primers specific to turtle Bcl-2, Bax and neuroglobin cDNA were designed from a partial cDNA sequence that was obtained previously by RT-PCR analysis of turtle brain mRNA using degenerate primers homologous to neuroglobin sequences from mouse and zebrafish. The primers employed for PCR were

the following: turtle brain specific: Bcl-2 primers 5'-GGTGCCACCTGT-GGTCCACCTG- 3' (forward) and 5'-CTTCACTTGTGGCCCAGATAGG-3'(reverse); Bax 5'-CCGGAATTCCGGAT GGACGGGTCCGGGGAGCAG- 3' (forward) and 5'TGCTCTAGAGCATCAGC CCATCTTCTTCCAG 3' (reverse). Neuroglobin primers: 5'-GTTGTTTGATCTGGACCCTGAC- 3' (forward) and 5'-TTGCCCAAGTTGGAGA GATATT-3' (reverse); actin primers: 5'-CAC CAACTGGGACGACATGG-3' (forward) and 5'-GTCGGCCAGCTCGTAGCTCT-3' (reverse). PCR products were separated by gel electrophoresis, visualized by ethidium bromide and photographed using a digital camera for quantification using National Institute of Health Image J 1.60 software. For semi-quantitative measurement of neuroglobin transcript levels, RT-PCR signal intensities were calculated as a ratio of levels of PCR products amplified from turtle actin cDNAs. Data are expressed as percent increase above normoxic control.

Protein Extractions

In vivo extraction analyses

Whole brain proteins were extracted from the frozen brains by homogenizing them in cell lysis buffer (5mM EDTA, pH 8.0, 0.15M NaCl,1% Triton X-100, 10Mm Tris-Cl, 2mM protease inhibitor,pH 7.4; with added 5 M DTT , 100 mM PMSF, 5 M mercaptoethanol diluted 1:1000) using a glass homogenizer at 4°C . The homogenate was then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant was removed and frozen for further analyses. Protein concentrations were determined using a standard BCA assay using the manufacturer's protocol (Pierce Biotechnology, Inc., Rockville, IL).

In vitro extraction

Existing media in the plates was disposed, and neurons were washed with ice cold Phosphate-Buffered Saline (PBS). Cells were further treated with 50 µl of RIPA lysis buffer (5mM EDTA, pH 8.0, 0.15M NaCl, 1% Triton X-100, 10mM Tris-Cl, 2mM protease inhibitor, pH 7.4; with added 5 M DTT , 100 mM PMSF, 5 M mercaptoethanol diluted 1:1000) and cells were then scraped. The buffer/cell mixture was then put into an Eppendorf tube (1 per individual) and kept on ice for 10 minutes. Liquid was then triturated and centrifuged for 10 minutes at 15000 RPM and 4°C. The supernatant was then frozen at –80ºC until use. The protein concentration was determined using BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

Western Blotting Protocol

Equal amounts of proteins from whole brain and cell lysates were separated electrophoretically by SDS-PAGE (12%) at 100 V for 2 hrs. Proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Piscataway, NJ). Nonspecific binding was inhibited by incubation in TBST [20mM Tris-buffered saline (pH 7.5) with 0.1% Tween 20] containing 5% nonfat dried milk for 1 h at room temperature. Membranes were further incubated with the appropriate primary antibody. Membranes were later washed in 3x TBST and incubated with anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Southern Biotech#4050, Birmingham, AL) for one hour at room temperature. The protein- secondary antibody complexes were identified using an electrochemiluminescent system (GE Healthcare, Piscataway, NJ) after washing. Densitometric analysis was conducted using National Institute of Health Image J 1.60 software, and band densities were expressed as a

percentage of the β-actin signal. Actin was used as a loading control and its expression remain unchanged in all experimental conditions. Earlier studies in our laboratory have also indicated that β-actin levels do not change with anoxia or reoxygenation in this model (Prentice et al., 2004; Milton et al., 2008). Data are expressed as percent change of the control group.

Primary antibodies

The unphosphorylated MAPK'S rabbit polyclonal antibodies and rabbit Bax polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These included ERK 1/ 2 (SC-93), JNK1 (SC-571) p38MAPK (SC-535) and BAX (SC-526). Rabbit polyclonal Ab to Akt (#9272) and phosphorylated antibodies phospho-Akt (#9271), phospho-p38MAPK (#4631), phospho-JNK (#4671), phospho-ERK1/2 (#4376), all from Cell Signaling Technology (Danvers, MA). Anti rabbit Bcl-2 anti-body was from Chemicon (Temecula, CA) and anti chicken Ngb polyclonal antibody (1 μg/ml) was from Biovendor (Candler, NC) diluted in TBST containing 5% milk. As a loading control, the membranes were blotted with antibody against β-actin (Sigma Aldrich, St. Louis, MO).

Secondary antibodies

HRP-conjugated anti-rabbit secondary antibody (Southern Biotech #4050, Birmingham, AL) was used as secondary antibody for detecting unphosphorylated and phosphorylated MAPK's and AKT immunoreactive protein bands. It was also used to determine Bcl-2 and BAX protein bands. The Ngb protein bands were detected with anti-chicken horseradish peroxidase (HRP)-conjugated secondary antibody (Chemicon, Billerica, MA).

siRNA design and transfection

Specific siRNA against turtle Ngb and Bcl-2 were designed using the siRNA design tool of Invitrogen/Molecular Probes (Eugene, OR) from the turtle specific sequence Ngb and Bcl-2 sequence respectively.

Turtle specific Ngb siRNA and Bcl-2 siRNA ((Invitrogen) designed from turtle specific Ngb sequence (ref: NM_021257.3) and Bcl-2 sequence (ref: NG_009361) were transfected using Lipofectamine 2000 in 6-well plates. Scrambled siRNA sequences from the same manufacturer were used as controls. Briefly Lipofectamine was diluted in MEM (5 µl/100 µl) and incubated for 5 min at room temperature. Turtle specific siRNA and control siRNA (250 pmol) were diluted in MEM and mixed with Lipofectamine. The incubation was continued for an additional 20 min before addition to the cultures. 48 h after transfection, the cell cultures were subjected to experimental conditions followed by protein extraction for Western blotting, cell viability testing and analysis of ROS levels. Transfections utilizing green fluorescent protein (GFP) demonstrated that this method has a 90% rate of transfection under these conditions.

Statistical Analysis

Statistically significant differences between treatments were evaluated using determined by analysis of variance ANOVA with post-hoc analysis using the SPSS statistical package. Differences were considered statistically significant at $p < 0.05$.

CHAPTER 3

RESULTS

I. Immunostaining of neuronally enriched cell cultures.

Neuronal fractions plated in 6 well plates were immunstained on day 10 after plating to determine the neuronal phenotype. Figure 3-1 shows the bright field morphology and immunostainings with several neuronal markers. The different staining patterns clearly demonstrate that cultures are consistently > 85% neuronal cells.

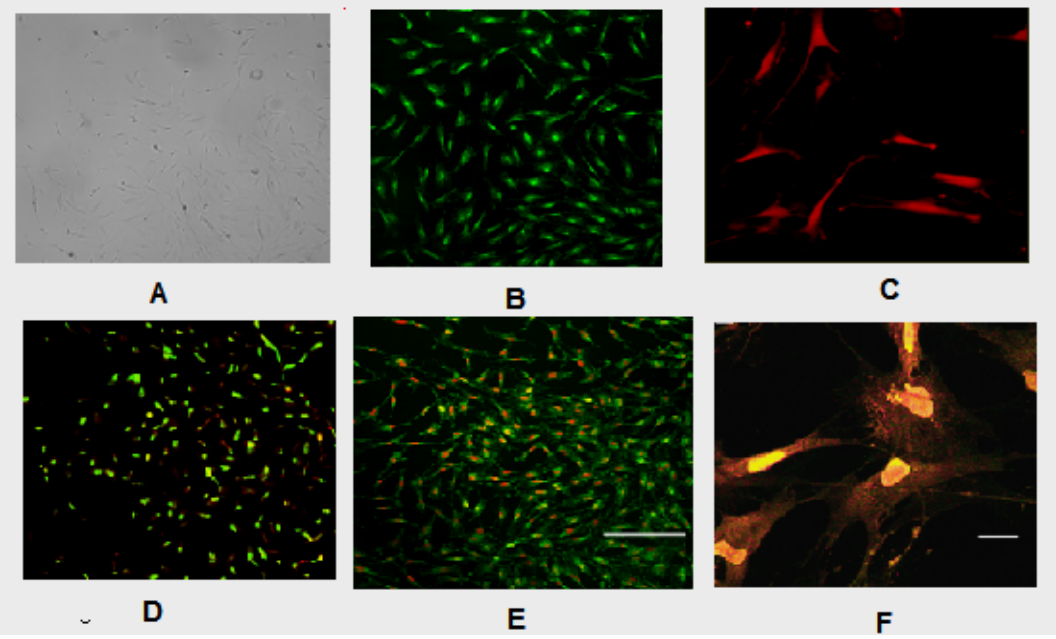


Figure 3-1: Representative views of neuronally enriched primary cultures prepared from juvenile Trachemys scripta forebrain, 10 day culture.

A. Bright field view, 10X magnification on Nikon Eclipse TE2000-S. **B**. Anti- NeuN antibody staining nuclei (20X). **C**. Anti-Neurofilament antibody staining neuronal filaments

(20X). **D.** Merged image (20X) of double-labelled NeuN (green)-and GFAP (red)-positive cells. **E.** Merged image (20 X) of double-labelled NeuN (red) and Ngb (green). **F.** Merged image (40X) of double-labelled NeuN- and Neurofilament. Cells were immunostained and viewed with a Rehga 1300 Inverted Fluorescent microscope.

II. Studies of adenosine receptor stimulation and receptor blocking on cell death and ROS production.

Neuronal resistance to anoxia and reoxygenation stress in culture with and without adenosine receptor stimulation: The anoxia tolerant fresh water turtle has a remarkable ability to withstand long-term anoxia, however anoxia does increase cell death in primary turtle neuronally enriched cell cultures (though there was no significant increase in cell death from 1 hr through 12 hr anoxia). A 2- to 3-fold increase in cell death (PI-positive cells) were observed in all anoxia treated cultures compared to their respective controls as determined by PI staining; the number of dead cells was highest in wells subjected to reoxygenation following 12 hr anoxia (Table 1). Studies have shown that adenosine acts as a key retaliatory metabolite that promotes neuronal survival during anoxia ((Nilsson and Lutz, 1992; Lutz and Nilsson, 1997). The upsurge in the extracellular levels of adenosine in anoxic conditions might provide protection against reoxygenation stress. Treatment with the specific A_1ADR antagonist DPCPX increased cell death significantly under almost every timeframe (with the exception of 1 hr normoxia); this increase was small (50%) though statistically significant in normoxic cultures. The percentage of PI-positive cells in anoxic DPCPX-treated cultures increased 2- to 3-fold compared to untreated anoxic controls, indicating the significance of A_1R-linked survival pathways for turtle neurons (Table 1). The anoxia-induced increase in cell death was ameliorated by

CCPA treatment; while CCPA had no effect on normoxic cultures, cell death was significantly reduced by 22% (reox) to 48% (12 hr anoxia) compared to untreated controls. There was no differences between percent cell death in chronic (4 hr) vs. acute (1 hr) drug exposures in anoxic cultures (data not shown), thus blocking A_1ADR during both the transition to the anoxic state (chronic exposure) or only after the transition to full anoxia has occurred (acute exposure) are both lethal to cell survival.

Table 1

Effect of specific A_1ADR agonist CCPA, the antagonist DPCPX on cell survival

	Control (no drug)	CCPA (0.6nmol/L)	DPCPX (1nmol/L)	DPCPX + CCPA
1h normoxia	7.1 ± 0.9	7.2 ± 1.3	8.6 ± 3.2	
4 h normox	8.3 ± 1.1	8.9 ± 1.2	12.8 ± 0.7*	9.8 ± 1.0
12 h normo	8.3 ± 1.1	8.9 ± 1.1	12.8 ± 0.9*	
1 h anoxia	15.8 ± 3.7†	8.5 ± 0.9*	38.9 ± 3.2*†	11.5 ± 5.4
4 h anoxia	21.8 ± 1.3†	13.9 ± 2.6*†	39.6 ± 6.8*†	13.2 ± 3.3*
12 h anoxia	15.3 ± 4.9†	7.9 ± 1.4*	53.1 ± 7.0*†	16.1 ± 3.1
12h anox/reox	25.2 ± 0.4†	19.8 ± 1.6*	52.6 ± 2.9*†	19.4 ± 2.2*

Table 1: Effects of chronic exposure to the specific A_1ADR agonist CCPA, the antagonist DPCPX, and agonist/antagonist administered simultaneously on cell survival in turtle primary neuronal cultures. Data are mean cell death (% PI-positive cells) ± SEM, N=5 individual cultures/group. * = significantly different from untreated controls, † = significantly different from normoxic value within same treatment group, $p < 0.05$.

***In vitro* ROS production:**

The turtle brain survives reoxygenation stress not solely by increasing defense mechanisms, but by suppressing ROS production upon reoxygenation both *in vivo* and in neuronal cultures. Figure 3-2 shows that ROS formation is decreased to near zero by 4 hr anoxia in the turtle neuronal cultures and reoxygenation also increases ROS production only to normoxic levels. Peroxide levels decreased dramatically by 4 hr anoxia in CCPA treated cells, though the blockade of AD receptors during this phase resulted in increased H_2O_2 in the medium. This observation is consistent with our hypothesis that the adaptations exhibited by the turtle at the physiological and molecular level to survive anoxia also prevent excess ROS formation upon reoxygenation. The protective role of ADR stimulation is evident in the lower levels of H_2O_2 release in CCPA treated cells (mean 49 ± 8 % of reoxygenated controls). As with the effect on cell death (above), treatment with the A_1R antagonist DPCPX significantly increased ROS production upon reoxygenation, to a mean of 314 ± 41 μM.

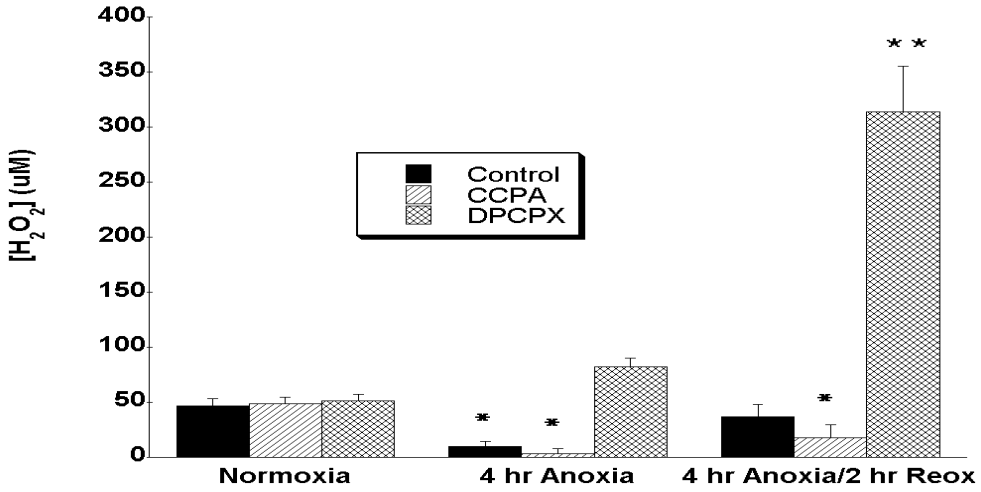


Figure 3-2: H_2O_2 concentration in the medium of neuronally enriched primary cell cultures treated with either the adenosine agonist CCPA or antagonist DPCPX.

4 hr anoxia significantly decreased ROS production except in DPCPX treated cells. Reoxygenation increases ROS production only to normoxic levels in controls, while CCPA reduces and DPCPX increases ROS production. * = significantly different from normoxic cells, $p < 0.05$, ** $p<0.01$. Data are mean ± SEM, N = 3 independent experiments/group.

Resistance to external oxidative stress (H_2O_2): As the turtle neurons both *in vivo* and *in vitro* are apparently able to significantly suppress ROS increases upon anoxia-reoxygenation, we further investigated the vulnerability of turtle neurons to ROS stress by exposure of cultures to externally imposed oxidative stress (H_2O_2). Initial titration experiments were conducted with concentrations ranging from 10 to 100 μM identified the concentration required to kill approximately 50% of the cells in normoxia (LC_{50}) and after 1h, 4h, and 12h anoxia, and after 12 hr anoxia/4h reoxygenation, to determine if the physiological and molecular changes induced by anoxia also increased resistance to oxidative stress. Contrary to our expectations, the LC_{50} for cells in anoxia decreased with increasing time in anoxia (Figure 3-3). The LC_{50} determined for each time point was then utilized for all other experiments at that timepoint.

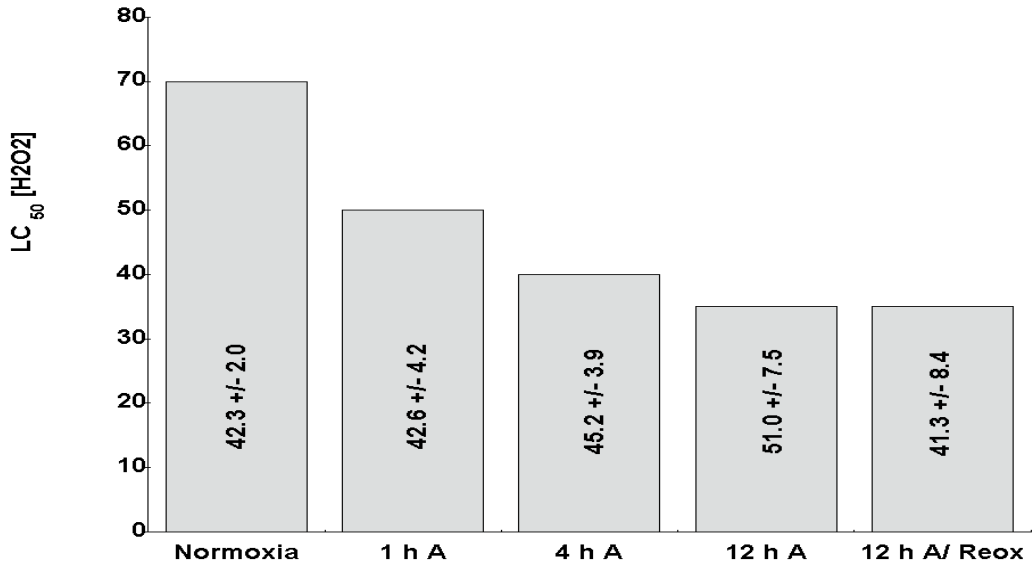


Figure 3-3: Concentration of hydrogen peroxide (µM) necessary to kill approximately 50% of the cells in a primary neuronal enriched culture (identified by PI staining).

Percent cell death (± SEM) at each concentration is included on the respective bars. N = 5 independent experiments/ timepoint. The LC_{50} decreases with increasing time in anoxia.

Resistance to externally imposed oxidative stress was then compared in cells treated with AD agonist and antagonist. As was seen with intrinsic anoxic/reoxidative stress, cell death due to externally imposed oxidative stress was increased by DPCPX, though in the case of treatment with hydrogen peroxide cell death was also generally ameliorated by CCPA compared to non-drug controls, though this was not statistically significant except after 12 h anoxia or anoxia/reox (Figure 3-4).

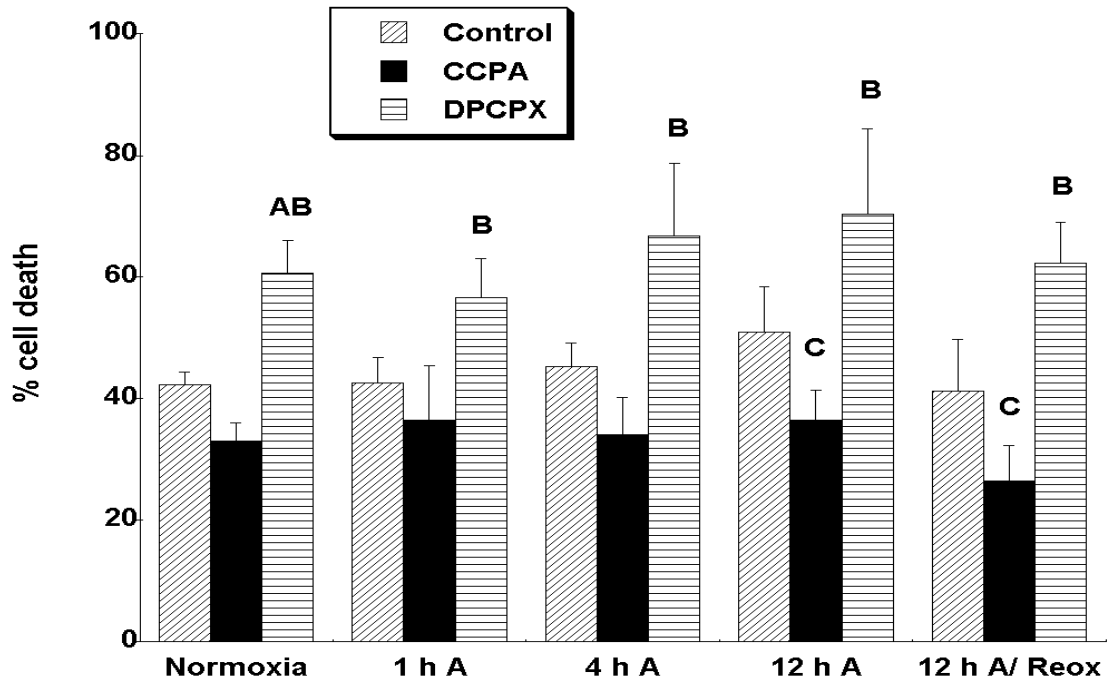


Figure 3-4: Blockade of adenosine receptors (DPCPX) increases cell death (% PI positive cells) during imposed oxidative stress, while increased receptor stimulation (CCPA) increases cell survival slightly (significant differences only after 12 h anoxia (12 h A) or 12 anoxia/reoxygenation). (N = 5 individuals /timepoint. A = sig. diff. from controls and CCPA, $p<0.05$, B = sig. diff. from CCPA, $p<0.05$, C = sig. diff. from controls and DPCPX, $p<0.05$).

1II. Effect of Adenosine receptors stimulation and suppression on the Mitogen activated protein kinase pathways (MAPK's) /AKT pathway and regulators of apoptosis (Bcl-2 and Bax)

Adenosine receptor activation or suppression may modulate the down stream signaling of the MAPKS and P13/AKT which could then lead to cell injury/death. Fewer studies have focused on the immediate effects of adenosine on preventing neuronal cell

death following ischemia (Gervitz et al., 2002; Higashi et al., 2002). We have previously shown that the initial increases in p-ERK and p-AKT and the suppression of p-p38 in the first hour of anoxia in the turtle brain are in fact linked to AD, and could be blocked with the general AD receptor antagonist aminophylline (Milton et al., 2008). We exposed turtle neurons to AD receptor agonist CCPA (cyclopentyladenosine) and antagonist DPCPX (1,3 –dipropyl-8-cyclpentylxanthine) respectively before subjecting them to respective experimental conditions. Phosphorylated ERK 1/2 (p-ERK) and AKT (p-AKT) as well as Bcl-2 are upregulated in anoxic neuronally enriched primary cultures from turtle brain; this native upregulation is further increased by the selective A1R activation using the specific agonist CCPA while AD1R suppression using specific antagonist DPCPX has opposite effects, suggesting that there is indeed a direct link between adenosine signaling and the MAPKs (Figure 3-5, Figure 3-6, Figure 3-7, Figure 3-8). The levels of actin remained unchanged in all experimental conditions, indicating that the increases in protein levels were not a generalized stress effect on the cells (Figure 3-9).

A)

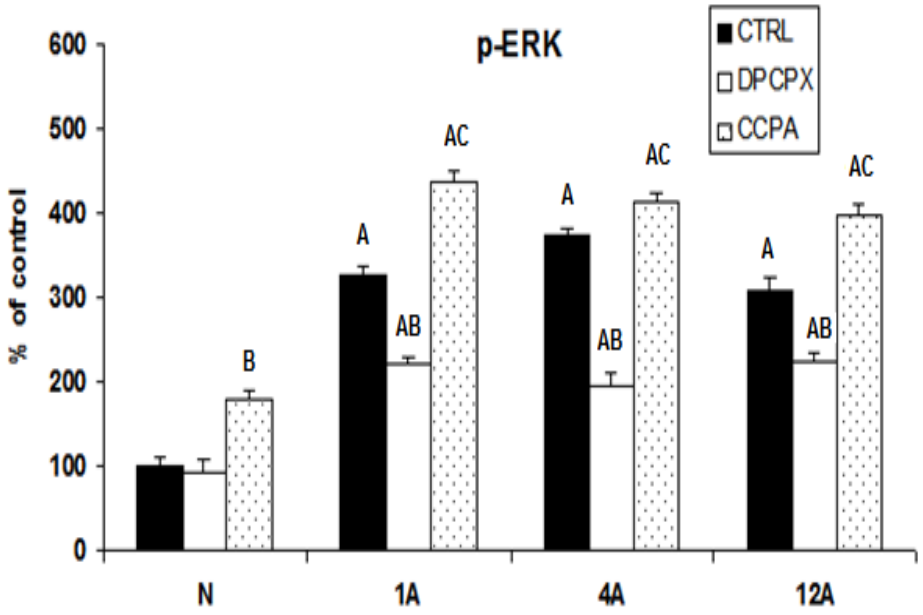
p-ERK

p-AKT

C DP CP C DP CP C DP CP C DP CP

Norm 1hr Anox 4hr Anox 12 h Anox

B)



C)

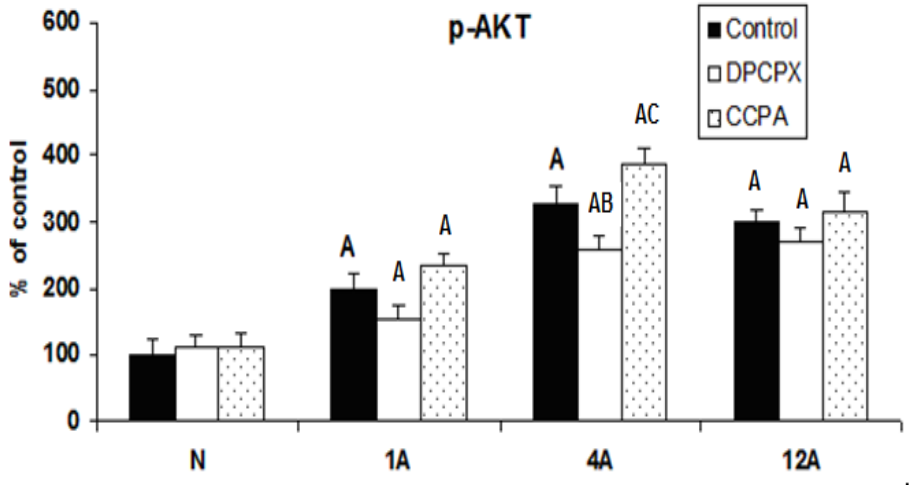
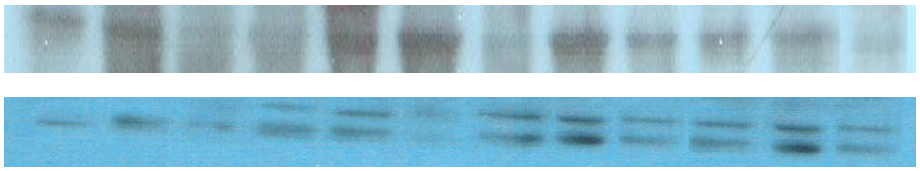


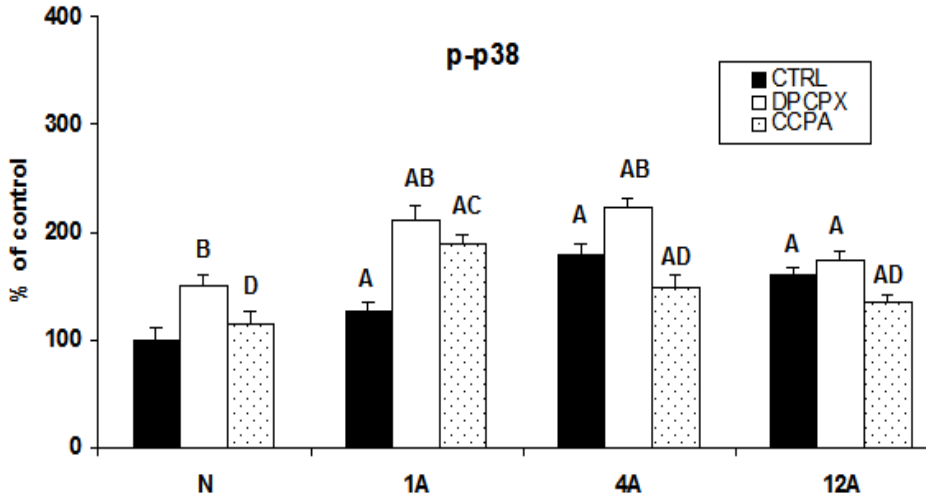
Figure 3-5: Representative western blot of phospho-ERK and phospho-AKT (A) and densitometric analyses (B) of pERK and (C) p-AKT levels under control and anoxic experimental conditions (treated with ADR agonist and antagonist) in turtle

neuronally enriched cell cultures. C = controls; DP = DPCPX treated; CP = CCPA treated. Data are mean ± SEM, N = 5 independent experiments/group. A = sig. diff. from normoxia control for the same treatment, $p<0.05$, B = sig. diff. from controls at the same time point, C = sig. diff. from controls at that point and DPCPX, $p<0.05$.

A)



B)



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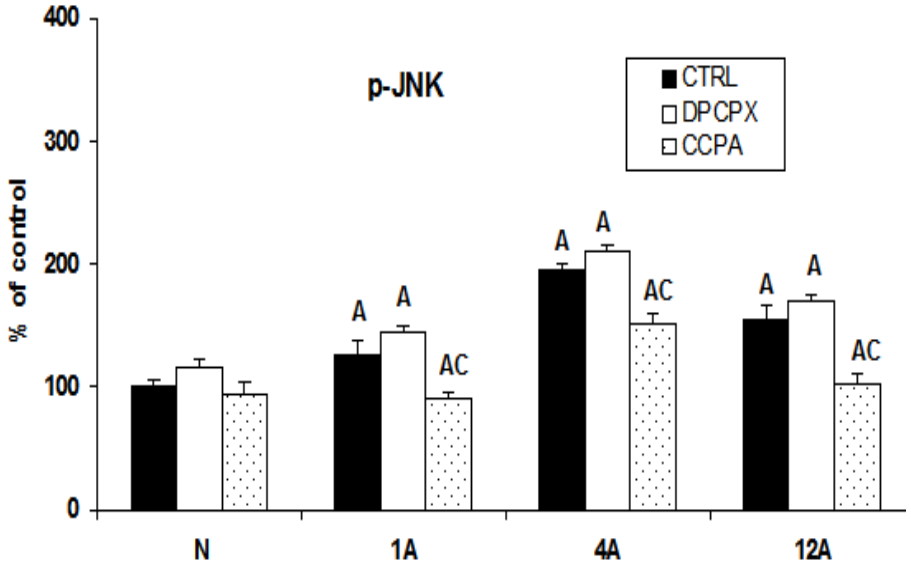
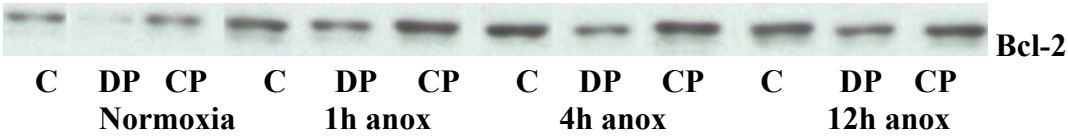


Figure 3-6: Representative western blot of phospho-P38 and phospho-JNK (A) and densitometric analyses (B) of p-P38 and (C) p-JNK levels under control and anoxic experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. C = controls; DP = DPCPX treated; CP = CCPA treated. Data are mean ± SEM, N = 5 independent experiments/group. A = sig. diff. from normoxia control for the same treatment, $p<0.05$, B = sig. diff. from controls at the same time point, C = sig. diff. from controls at that point and DPCPX, $p<0.05$.

A)



B)

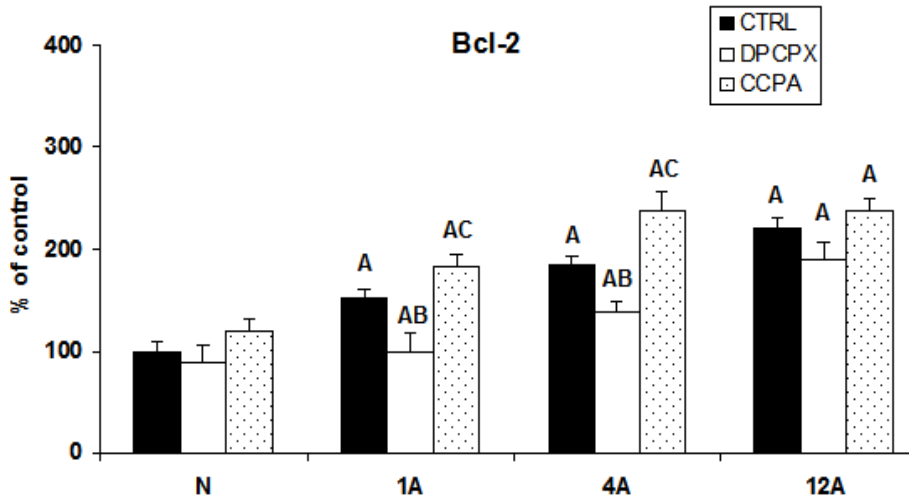


Figure 3-7: Representative Western blots of Bcl-2 (A) and densitometric analyses (B) of Bcl-2 levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. C = controls; DP = DPCPX treated; CP = CCPA treated. Data are mean ± SEM, N = 5 independent experiments/group. A = sig. diff. from normoxia control for the same treatment, $p<0.05$, B = sig. diff. from controls at the same time point, C = sig. diff. from controls at that point and DPCPX, $p<0.05$.

A)

C DP CP C DP CP C DP CP C DP CP

Normoxia 1h anox 4h anox 12h anox

B)

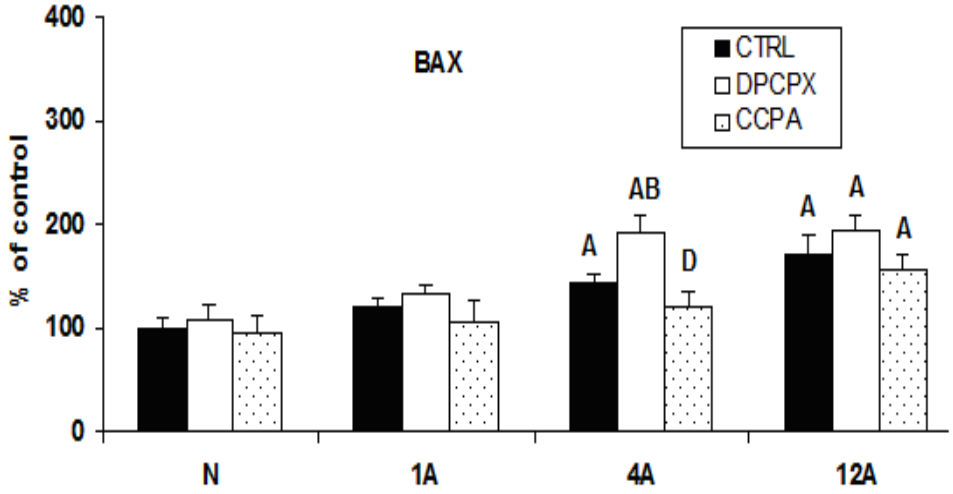


Figure 3-8: Representative Western blot of Bax (A) and densitometric analyses (B) of Bax levels under control and experimental conditions (treated with ADR agonist and antagonist) in turtle neuronally enriched cell cultures. C = controls; DP = DPCPX treated; CP = CCPA treated. Data are mean ± SEM, N = 5 independent experiments/group. A = sig. diff. from normoxia control for the same treatment, $p<0.05$, B = sig. diff. from controls at the same time point, C = sig. diff. from controls at that point and DPCPX, $p<0.05$, D = sig. diff. from DPCPX, $p<0.05$.

Actin

C DP CP C DP CP C DP CP C DP CP

Norm **1hr Anox** **4hr Anox** **12 h Anox**

Figure 3-9: Representative Western blot showing expression of ß-actin in turtle neuronally enriched cell cultures. Actin expression is unchanged in all experimental conditions. C = controls; DP = DPCPX treated; CP = CCPA treated.

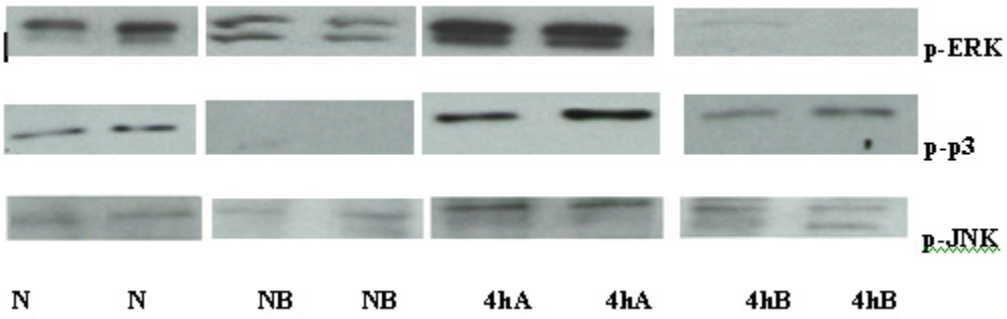
IV. Analysis of MAPK's activation in response to stress and effect of respective MAPK's blockers on its activation.

Shaped by millions of years of evolution, it is likely that pathways in the turtle brain that are strongly upregulated during anoxia promote cell survival, whereas those that are strongly suppressed would otherwise promote cell death, or may potentially deplete limited physiological resources such as anaerobically produced ATP (e.g. protein synthesis (Fraser et al., 2004). Recent work on the Trachemys whole brain has shown a number of changes at the molecular level in signaling pathways that are thought to promote cell survival and prevent apoptosis, including the upregulation of ERK (but not JNK) in the hypoxic cortex (Haddad, 2007a; 2007b), with increases in both Bcl-2 and Bax (Haddad, 2007b), and the activation of ERK and Akt with a concomitant suppression of p38MAPK activation in the whole brain at 1 hr anoxia (Milton et al., 2008). While the upregulation of Erk and Akt and suppression of p38MAPK are presumed to be protective in the turtle brain, as they are under many (but not all) conditions in the mammalian brain (Martindale and Holbrook, 2002), the development of neuronal cultures allows us to manipulate these pathways and determine if they are critical to neuronal survival in this anoxia tolerant model.

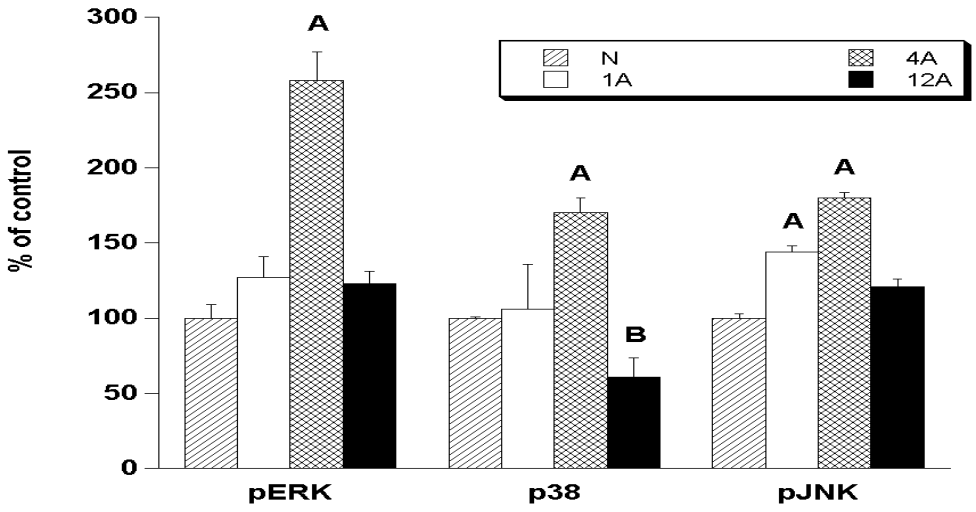
We analyzed the levels of MAPK's activation after exposing neuronally enriched cell cultures to different duration of anoxia. All three MAPK pathways were activated over 4 h anoxia, followed by a subsequent decrease as anoxia continued; p-ERK levels increased to a greater degree than p-JNK or p-p38MAPK (Figure 3-5). Further we preexposed the cell cultures to specific MAPK's blocker to abrogate the activation of the MAPK's. We used pathway-specific pharmacological blockers for p38MAPK, ERK, and JNK

activation (SB203580, U0126, and SP600125, respectively in DMSO). Activation of all the MAPK's was significantly suppressed by employment of the respective blockers (Figure 3-10).

A)



B)



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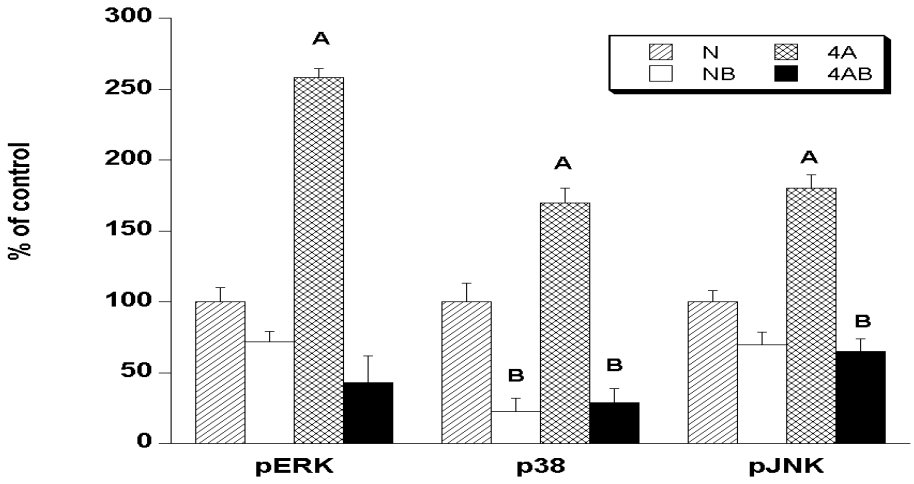


Figure 3-10: Anoxia-induced increases in phosphorylated MAPKs are abrogated by specific pathway blockers. (A) Representative immunoblots: anoxia-induced MAPK activation is abrogated by specific pathway blockers **(B)** Densitometric analysis of changes in phosphorylated MAPKs in cultures of primary turtle neurons at different anoxic timepoints. N = normoxic controls, 1A etc. = 1, 4, and 12 h anoxia. A = sig. higher than normoxia, B = sig. lower than normoxia, $p<0.05$. **(C)** MAPK activation is reduced after treatment with specific pathway blockers (UO126 for ERK pathway, SP00612 for JNK pathway, SB3082 for P38 pathway) N = normoxia, NB = normoxic controls with blocker, 4hA = untreated 4h anoxic controls, 4AB = 4 h anoxic cultures treated with blocker. N = 5 individuals/treatment. A = s.d. from normoxia, B = s.d. from untreated controls, $p<0.05$. Data are normalized to actin signal.

V. Analysis of AKT activation in response to stress and suppression of its activation using specific blocker

Upregulation of the Akt pathway is generally accepted to be anti-apoptotic in mammalian systems (Kilic et al., 2006); Akt and ERK activation are hallmarks of the preconditioning response in the brain and heart (Hauesenloy and Yellon, 2007), and are thought to regulate components of the apoptotic pathway (Noshita et al., 2001; Li et al., 2003) including Bcl-2 and BclxL (Kaushal et al., 2004). We analyzed the activation of AKT in turtle neurons when exposed to different durations of anoxia. p-AKT levels increased significantly in during 1h and 12 h anoxia (Figure 3-11). Similarly preexposure to specific AKT blocker LY294002 abrogated the activation of AKT significantly (Figure 3-12).

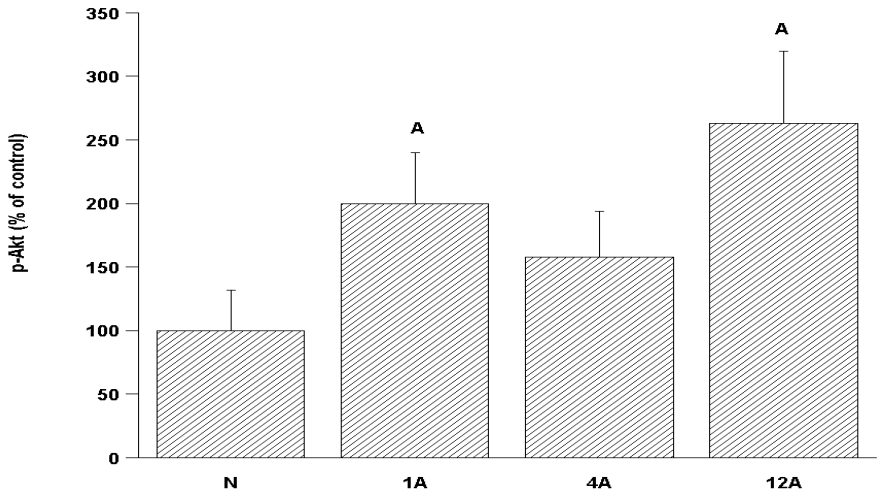
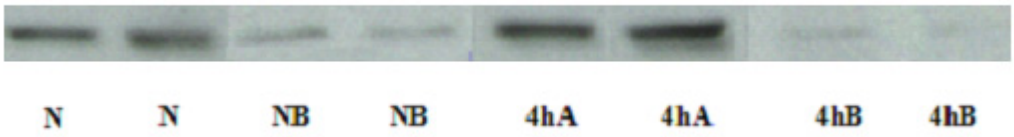


Figure 3.11: **Densitometry analysis of changes in phosphorylated Akt in anoxic cultures of primary turtle neurons**. * = significantly different from normoxic controls, $p<0.05$. Data are normalized to actin signal. N = normoxic controls, A = 1, 4, and 12 h anoxia.

A)



B)

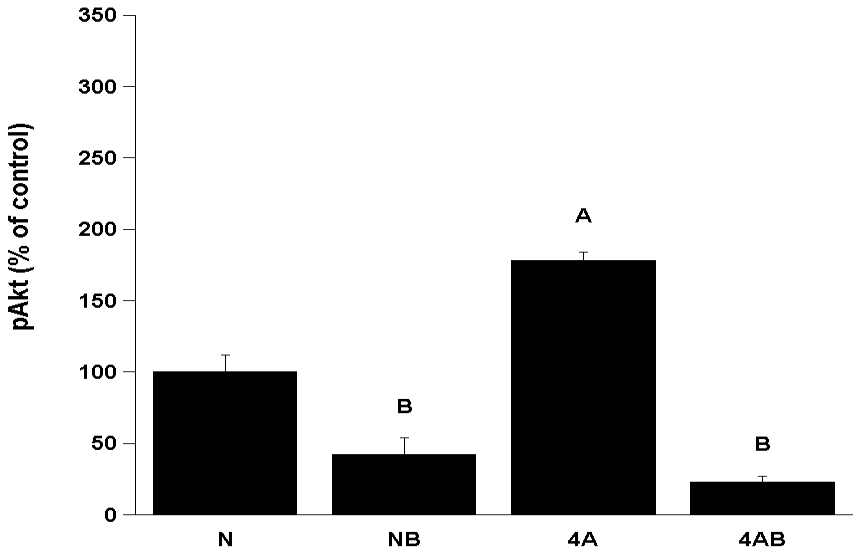


Figure 3-12: Activation of AKT (p-AKT) in untreated controls and in cell cultures treated with the specific AKT-blocker LY294002. (A) Representative western blot and (B) densitometric analysis. N = normoxia, NB = normoxic controls with blocker, 4hA = untreated 4h anoxic controls, 4AB = 4 h anoxic cultures treated with blocker. N = 5 individuals/treatment. A = significantly different from normoxic untreated control, $p<0.05$. B = significantly different from control at that time point, $p<0.05$.

Impact of MAPK's and AKT blockers on cell death

We used pathway-specific pharmacological blockers for AKT, p38MAPK, ERK, and JNK activation (LY294002, SB203580, U0126, and SP600125, respectively in DMSO) to determine the impact of each pathway on cell survival (Table 2). At low concentrations of individual drugs, blockade of ERK activation significantly increased cell death, while blockade of p38MAPK and JNK phosphorylation pathways increased cell survival (Table 1). DMSO alone did not alter cell survival compared to controls. The results are consistent with mammalian systems indicating that activation of ERK may act to increase cell survival or apoptosis (Martindale and Holbrook, 2002), while p38MAPK and JNK activation are pro-apoptotic (Horstmann et al., 1998; Irving et al., 2000; Sugino et al., 2000; Gu et al., 2001; Guan et al., 2006).

Table 2

Effect of low doses of specific MAPK blockers on neuronal cell survival

	Percent cell death (Mean ±SEM)	
	Control (no drug)	**MAPK Inhibitor**
Akt blocker (LY294002, 40 μM)		
Normoxia	7 ± 2	15 ± 1
4 hr anoxia	20 ± 1	56 ± 4
ERK blocker (U0126, 5.0 μM)		
Normoxia	12 ± 2	16 ± 2
4 hr anoxia	18 ± 3	40 ±1[1,2]
JNK blocker (SP600125, 5.0 μM)		
Normoxia	7 ± 2	12 ± 4
4 hr anoxia	23 ± 2[1]	13 ± 1[2]
P38 blocker (SB203580, 1.0μM)		
Normoxia	7 ± 2	10 ± 3
4 hr anoxia	23 ± 3[1]	14 ± 1[1,2]

Table 2: Effect of low doses of specific MAPK blockers on cell survival in turtle primary neuronal cultures on turtle neuronal cell death. Low doses of specific

MAPK blockers significantly altered cell death, with ERK blockade increasing cell death and JNK and p38MAPK blockade decreasing cell death. Superscripts: [1]= significantly different from normoxic control, [2] = significantly different from anoxic controls, $p<0.05$, N = 3-4 individuals per group.

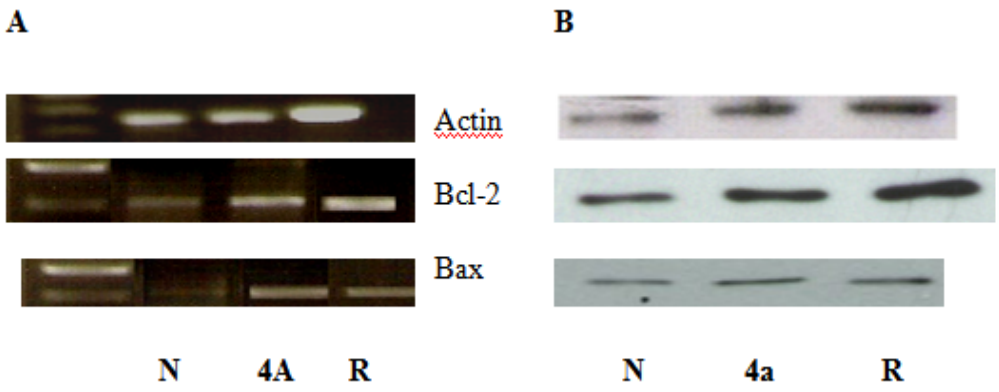
V1. Role of the Bcl-2 and Bax in neuroprotection

The Bcl-2 family of proteins tightly regulates the process of apoptosis. In the cell there exists a delicate balance between pro-apoptotic and anti-apoptotic proteins. A shift in the levels of expression of these proteins will control the overall cellular response to an apoptotic signal (Green and Reed 1998). Analyzing the expression of these genes in the anoxia tolerant turtle brain as well as in turtle neuronal cultures may provide a useful insight into some unique mechanisms of mammalian anoxia/ischemic brain damage.

In turtle neuronal cell cultures both Bcl-2 and Bax transcription and protein levels increase in 4h anoxic cells and upon anoxia/reoxygenation (4h/4h), but the increases in Bcl-2 transcription and translation were relatively greater (Figure 3-13 A,B,C), leading to increased Bcl-2:Bax ratios. These high Bcl-2:Bax ratios might offer neuroprotection during experimental conditions. Further we employed turtle specific Bcl-2 siRNA to knock-down expression of Bcl-2 in turtle neuronal cultures using lipofectamine transfection. SiRNA transfection resulted in a dose-dependent knockdown of Bcl-2 ex-pression when exposed to 4 hr anoxia, at the highest dose decreasing Bcl-2 to a mean 38 ± 7% of control (Figure 3-13E) and increasing cell death significantly ($p<0.01$) from 11± 2% to 31 ± 1% in anoxic cultures and from 19 ± 2% in anoxia/reoxygenation cells treated with control (scrambled) siRNA oligomer to 46 ± 3% in cells with siRNA against Bcl-2 (Figure 3-13D). SiRNA treatment itself did not result in increased cell death; cell death in cells

treated with scrambled siRNA was not significantly different under any condition compared to levels of cell death in untreated cultures.

The increase in cell death resulting from Bcl-2 knockdown may have resulted from increases in Bax expression, resulting in decreased Bcl-2: Bax ratios compared to control cells and shifting cells away from survival towards apoptosis. The mean 62% decrease in Bcl-2 levels was correlated with an increase to 238 ± 2% of basal ($p<0.01$) in Bax levels (vs. 113 ± 11% in cells transfected with scrambled siRNA), and detectable levels of activated caspase-3 (not detectable in scrambled siRNA treated cultures). Caspase-3 levels do not increase in anoxia in untreated cell cultures, nor in whole brain even over 24h anoxia (Kesaraju et al., 2009), showing the extraordinary resistance of turtle neurons to apoptosis even in extended anoxia.



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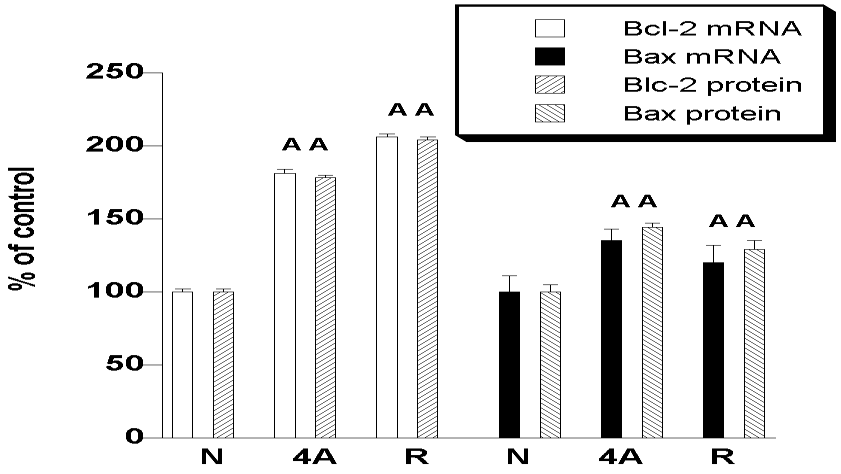
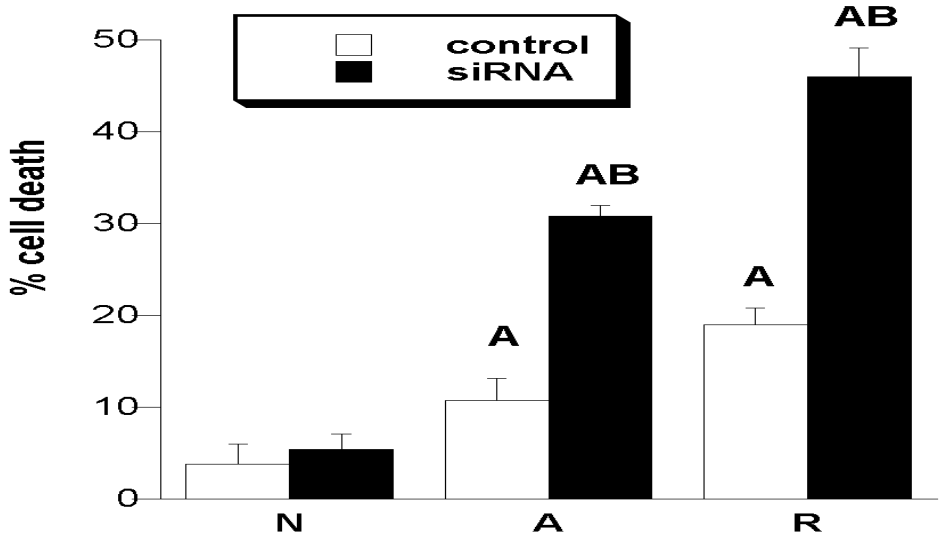

Bcl-2 mRNA
Bax mRNA
Blc-2 protein
Bax protein
% of control
250
200
150
100
50
0
A A
A A
A A
A A
N
4A
R
N
4A
R

D


control
siRNA
% cell death
50
40
30
20
10
0
AB
AB
A
A
N
A
R

E

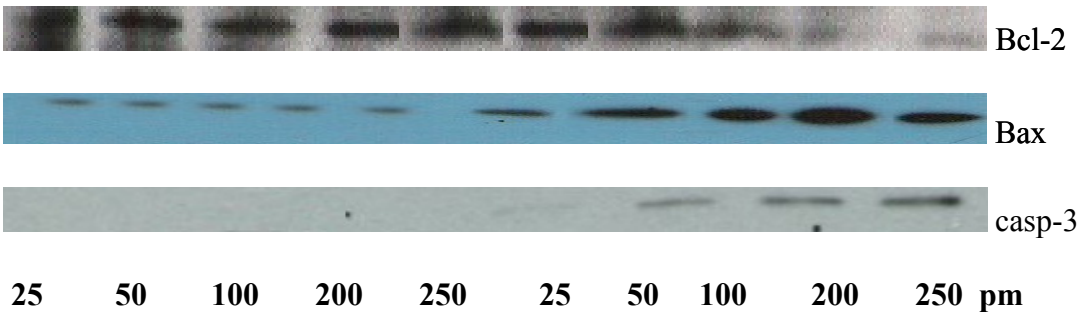


Figure 3-13: **Bcl-2 transcription in neuronally enriched cell cultures increases significantly in anoxia and anoxia/reoxygenation compared to the pro-apoptotic Bax; protein levels also increase significantly (A)** Representative gels from N= 5 semi-quantitative RT-PCR. **(B)** Representative western blots from N=5. N = normoxia, 4hA = 4 h anoxia, R = 4 h anoxia/4 h reoxygenation. Actin levels are not significantly altered by anoxia or reoxygenation in this model. **(C)** Densitometric analysis of Bcl-2 and Bax mRNA and protein levels. A = s.d. from respective control **(D)** Bcl-2 knockdown resulted in increased cell death. A = s.d. from normoxia, B = s.d. from control within treatment group **(E)** Representative western blots (of N=5/treatment) showing Bcl-2 Stealth™ siRNA decreases anoxia-induced induction of Bcl-2 in a dose dependent manner. Scrambled siRNA does not affect Bcl-2 expression. Bcl-2 decreases were correlated with increases in Bax and caspase-3.

Effect of Bcl-2 knockdown on ROS release

Several studies have indicated a strong link between Bcl-2 and its role in oxidative damage. Localization of Bcl-2 in the mitochondria enhances its anti-oxidant capacity by suppressing reactive oxygen species thus protecting cells from oxidants such as hydrogen

peroxide (Hockenbery 1993). Bcl-2 protein has shown to play a major role in suppressing cell death during apoptotic and oxidative stress cell injury by enhancing the levels of anti-oxidants as well as suppressing generation of free radicals (Hockenbery 1993; Kane et al., 1993; Ellerby et al., 1996; Lee et al., 1998, 2001, Saitoh et al., 2003).

Bcl-2 knockdown by specific siRNA in turtle neuronal cultures resulted in elevated ROS release in normoxic, anoxic, and reoxygenated cells as determined by Amplex Red staining, with H_2O_2 release increasing more than 2-fold in cells exposed to anoxia followed by 4h reoxygenation (Figure 3-14). ROS levels in untreated cells, by contrast, return only to normoxic levels in untreated turtle neuronal cultures upon reoxygenation following 4h anoxia, without the excess release characteristic of mammalian cells subjected to ischemia/reperfusion (Milton et al., 2007; Pamenter et al., 2007). High ROS levels in anoxic cells results from increased release during the initial hour of anoxia, as cells undergo increasing hypoxia and physiological stress (Milton et al., 2007). SiRNA treatment alone (scrambled controls) does not affect ROS release, as H_2O_2 levels followed the same pattern as is observed in untreated cells with a significant decrease in anoxia followed by a return only to basal levels upon reoxygenation (Figure 3-14).

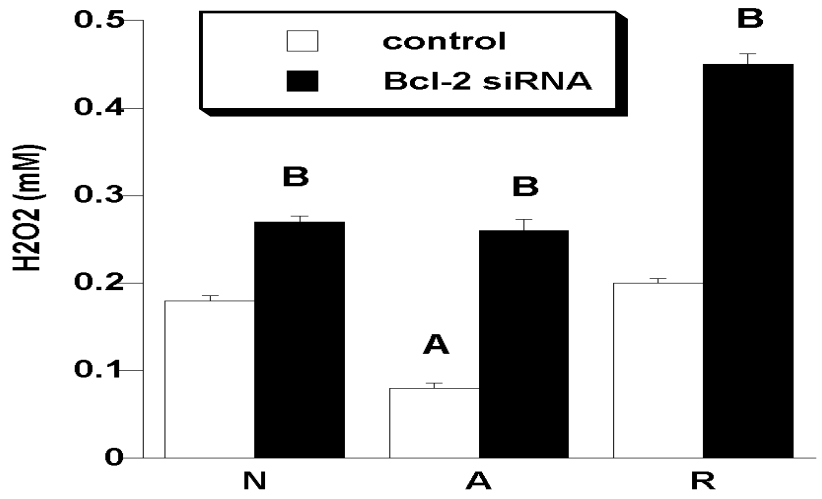


Figure 3-14: Bcl-2 knockdown using specific Bcl-2 Stealth™ RNA in turtle primary neuronal cell cultures increases the release of H202 significantly ($p<0.01$) relative to the respective controls treated with scrambled siRNA.

Effect of Bcl-2 knockdown on mitochondrial membrane potential (ψ)

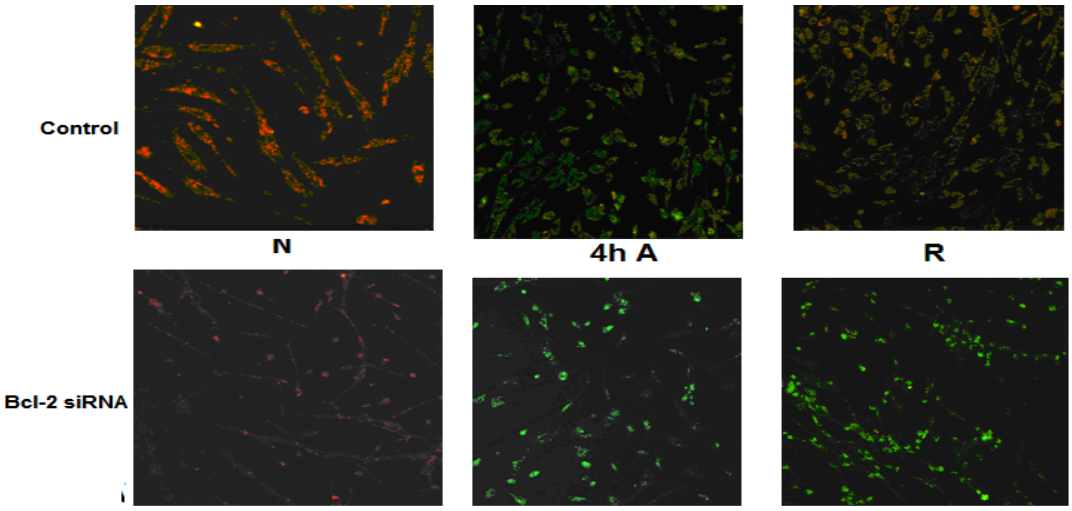


Figure 3-15: Effect of Bcl-2 knockdown on mitochondrial membrane potential (ψ). Bcl-2 knockdown in turtle neuronal cultures leads to the complete loss of the mitochondrial membrane potential (ψ) in all experimental conditions compared to its

respective controls. In controls, the cells losses it potential to a greater extent but this is regained on 4 h anoxia/ 4 h reoxygenation. N= Normoxia, 4h A = 4 h anoxia, R = 4 h anoxia/ 4 h reoxygenation

V11. *In vivo* and *In vitro* analysis of Ngb gene transcription and translation and its role in ROS regulation and cell survival

Ngb is the recently discovered neural heme protein that has a remarkable potential to sustain respiration under compromised oxygen conditions and offer protection upon oxygen restoration (Burmester 2000). Several investigations have predicted several prosurvival functions of Ngb during oxygen crisis. To date, however, it is unclear if Ngb is regulated under varying O_2 levels, with some studies reporting increased expression of Ngb mRNA and protein in cerebral tissue culture after 24 h anoxia-reperfusion (Sun et al., 2001), while other investigations (Mammen et al., 2002) did not find increased expression in hypoxic (10% O_2) mice *in vivo*. As mammalian studies are complicated by pathological responses to stress the brain of the anoxia- tolerant turtle offers as a unique model to identify the expression of Ngb in varying oxygen levels. *In vivo* and *in vitro* transcription and translation of Ngb increased significantly in response to hypoxia with further increases on reoxygenation indicating its oxygen dependent activation (Figure 3-16, 3-17, 3-18).

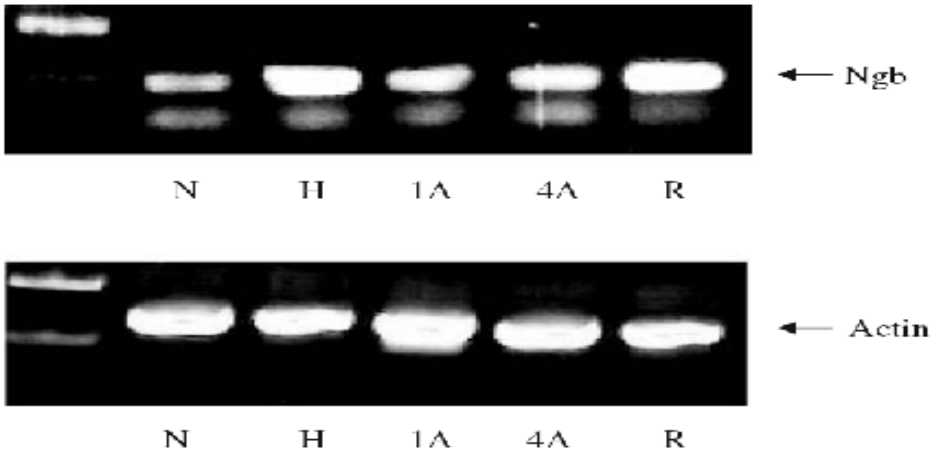


Figure 3-16: Representative gels showing increased neuroglobin transcription relative to actin controls in the T. scripta brain. Gels were visualized with ethidium bromide and digitally photographed for analysis. N = normoxia, H = 4 h hypoxia, 1A = 1 h anoxia, 4A = 4 h anoxia, R = 4 h anoxia/4 h reoxygenation.

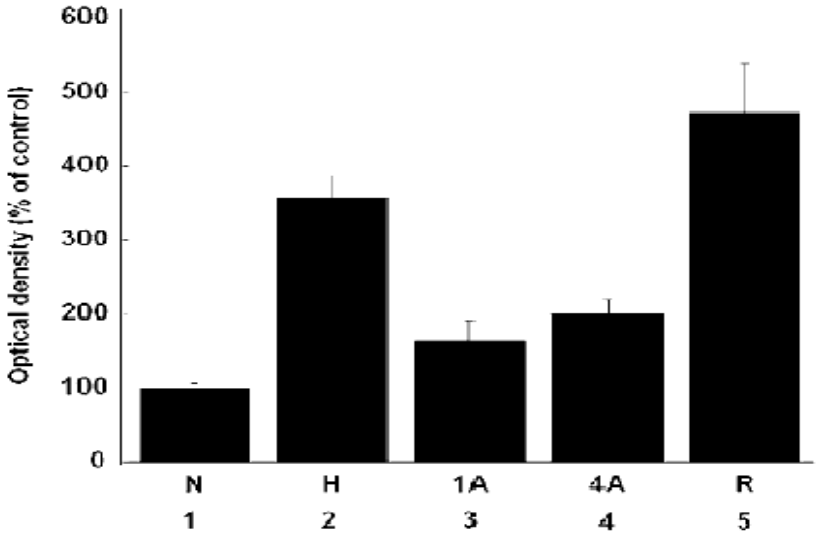


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Figure 3-17: Changes in neuroglobin mRNA in the hypoxic and anoxic turtle brain, expressed as percent of normoxic control. Each experiment utilized five individuals per group. N = normoxia, H = 4 h hypoxia, 1A = 1 h anoxia, 4A = 4 h anoxia, R = 4 h anoxia/4 h reoxygenation. Columns are numbered 1–5. Column 1 (N) differs significantly

from column 2 (H), ($p<0.01$); column 1 (N) differs significantly from column 4 (4A), ($p<0.05$); column 4 (4A) differs significantly from column 5 (R), ($p<0.01$).

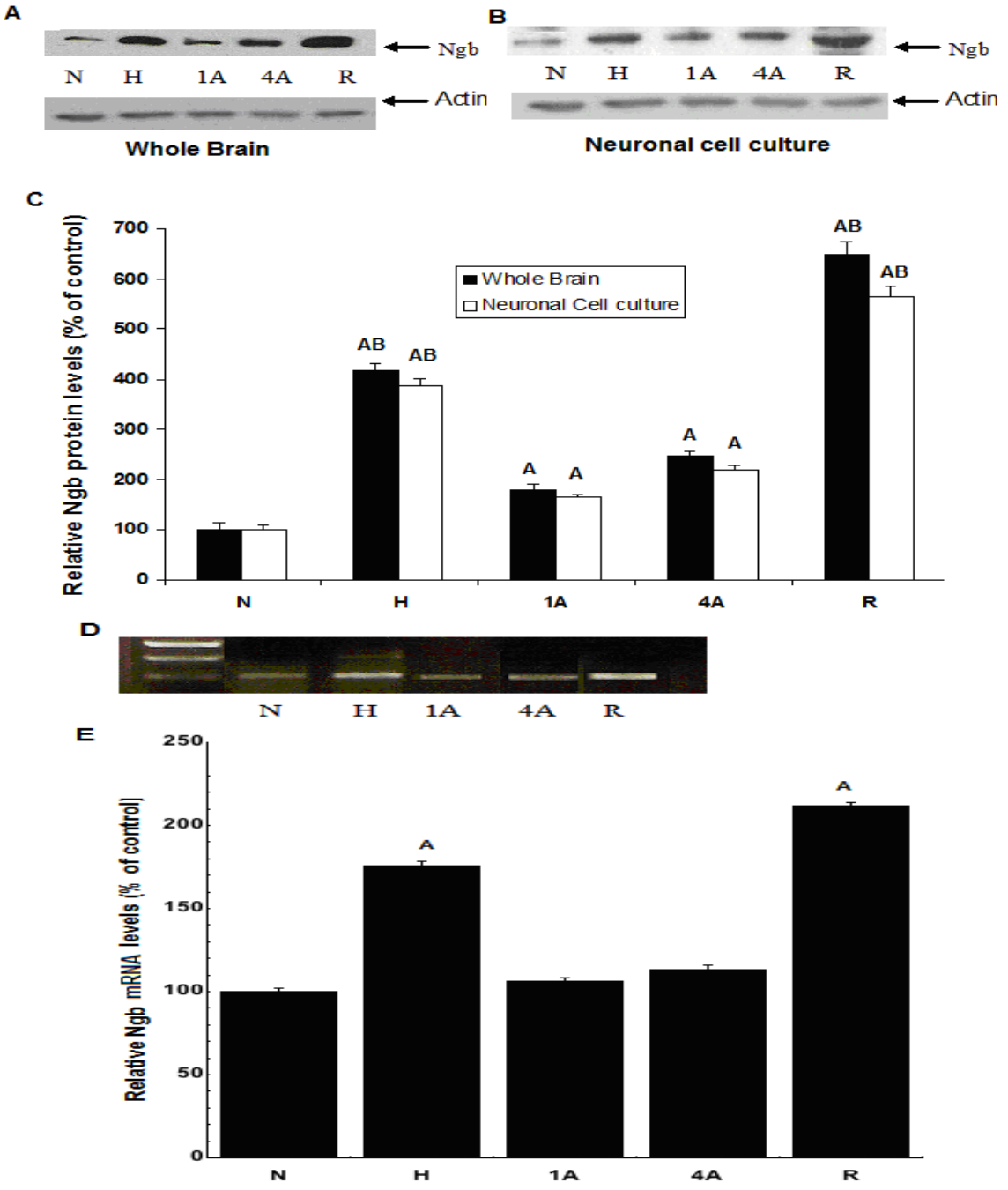

Figure 3-18: ***In vivo*** **Ngb protein levels and** ***in vitro*** **Ngb m-RNA and protein levels increased significantly during hypoxia and reoxygenation compared to normoxic**

controls. Representative Western blots (A, B) showing increased Ngb protein levels in the T. scripta whole brain and neuronally enriched cell cultures, respectively, relative to actin controls and (C) densitometric analyses of Ngb protein levels in the whole turtle brain and cell cultures. Representative gel (D) showing increased Ngb transcription in primary T. scripta neuronal cell culture visualized with ethidium bromide and digitally photographed for analysis and (E) densitometric analyses of Ngb mRNA levels in turtle primary neuronal cell cultures. (N = 5 individuals /timepoint. N = normoxia, H = 4 h hypoxia, 1A = 1 h anoxia, 4A = 4 h anoxia, R = 4 h anoxia/4 h reoxygenation. A = sig. diff. from the respective normoxic controls, $p < 0.01$, B = sig. diff. from the respective 1 h and 4h anoxia, $p < 0.01$).

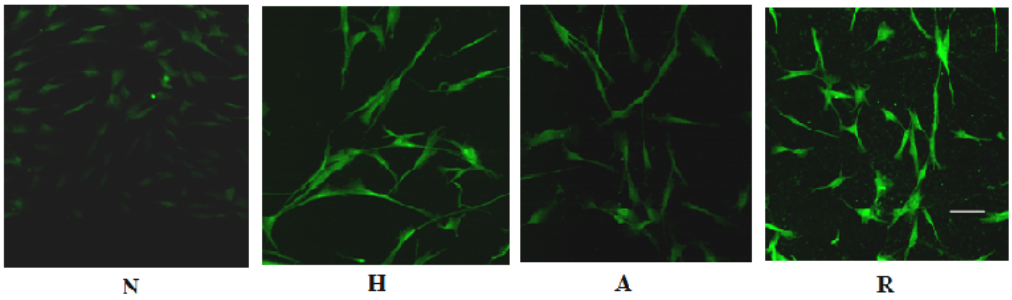

Figure 3-19: Upregulation of Ngb is clearly evident in representative immunostained cells subjected to hypoxia and reoxygenation compared to normoxia and anoxia. N= Normoxia, H= 4 h hypoxia, A= 4 h anoxia, R= 4 h anoxia/4 h reoxygenation. (N = 3 independent experiments/group, scale bar = 50µm, 40x).

Effect of Ngb knockdown on cell survival and ROS release

In mammalian ischemia/reperfusion models, knockdown of Ngb results in increased cell death (Sun et al., 2003), and increased Ngb levels are reported to reduce stroke-induced cell death (Khan et al., 2006; Sun et al., 2001; Sun et al., 2003). To further

elucidate the role of Ngb in neuroprotection, we analyzed the effects of Ngb knockdown in turtle neuronally enriched cell cultures exposed to 4 h hypoxia, 4 h anoxia, and anoxia/reoxygenation. Turtle specific Ngb siRNA transfection resulted in a dose-dependent decrease in anoxic Ngb protein expression in cell culture (Fig.3-20). A concentration of 250 pM resulted in more than 70% decrease in Ngb protein compared to untreated anoxic cultures. Scrambled siRNA was used as control; scrambled siRNA did not change Ngb levels compared to the controls. Additonally, the knockdown of Ngb by more than 70% resulted in increased ROS release into the medium by approximately 2-fold in all experimental conditions with the exception of normoxic controls (Figure 3-21), however no significant change in cell survival was observed (Figure 3-22).

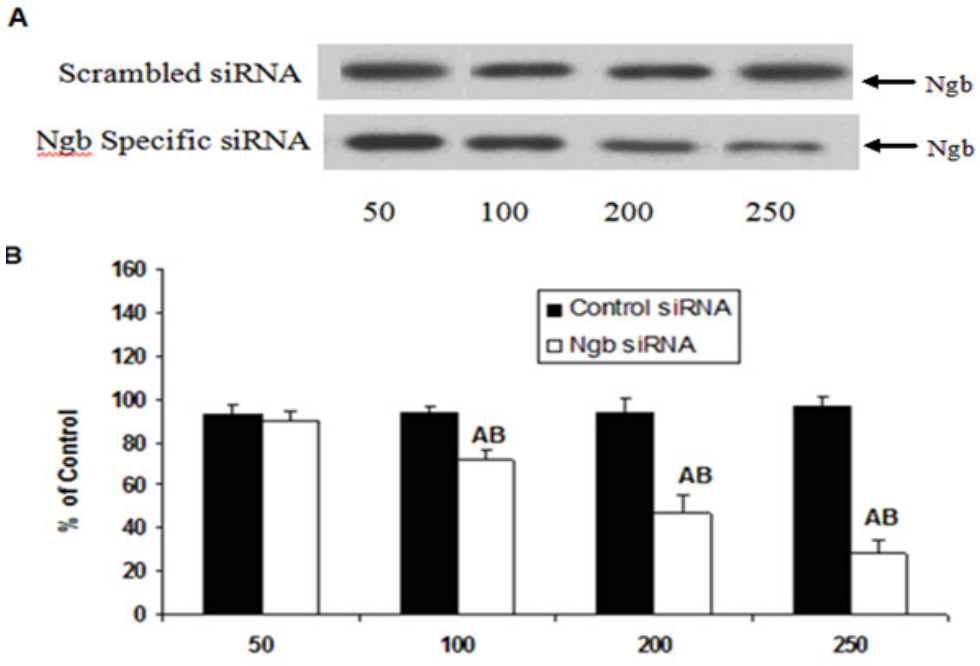

Figure 3-20: A. Representative Western blot showing reduced Ngb expression in Ngb specific siRNA transfected cells compared to their respective controls.

Concentrations ranging from 50 to 250 pM of Ngb siRNA and scrambled siRNA (control) were used and the transfected cells were subjected to 4 h anoxia. B. Densitometric analyses of Ngb protein expression in cell cultures treated with Control siRNA and Ngb siRNA, exposed to 4 h anoxia and expressed as % of untreated controls. A = sig. different from untreated 4h anoxia controls (100% expression level), B = sig. different from control siRNA treated cells in same treatment group. Data are mean ± SEM, N = 3 independent experiments/group.

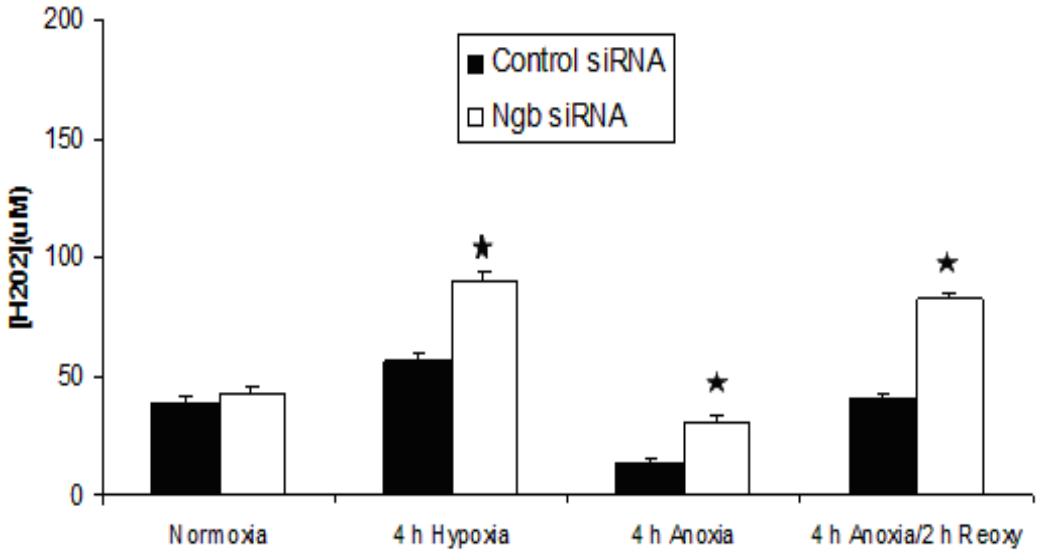

Figure 3-21: Knockdown of Ngb significantly increased the release of H_20_2 into the medium by approximately 2-fold during hypoxia and upon reoxygenation following 4 h anoxia. A significant increase of 2.5 -fold was observed during 4 h anoxia. H_20_2 concentration was calculated based on a standard curve. * = significantly different their respective control siRNA groups. Data are mean ± SEM, N = 3 independent experiments/ group.

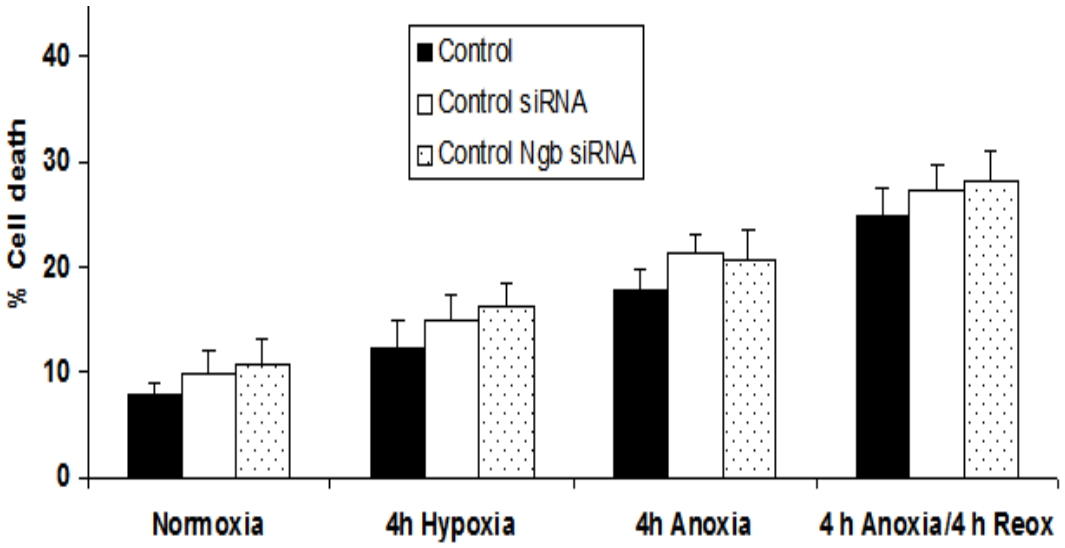

Figure 3-22: Ngb knockdown did not alter cell death in 4 h hypoxia, 4 h anoxia, or and upon reoxygenation following 4 h anoxia compared to their respective controls. Data are mean ± SEM, N = 3 independent experiments/groups.

Effect of external oxidative stress on Ngb knockdown cell cultures

Previous studies have shown that siRNA against Ngb increased susceptibility of PC12 cells to H_20_2 induced oxidative injury (Li et al., 2008; Fordel et al., 2006) investigation has demonstrated that cultured human neuroblastoma cells transfected with Ngb-expressing plasmid were resistant to H_20_2 induced oxidative injury. We observed that Ngb knockdown in turtle neuronally enriched cell cultures using turtle specific siRNA increased cell death under conditions of imposed oxidative stress using H_20_2 (Figure 3-23 B) while in control cultures Ngb protein expression was significantly induced by H_20_2, suggesting that external oxidative stress can act as a modulator of Ngb expression (Figure 3-23 A).

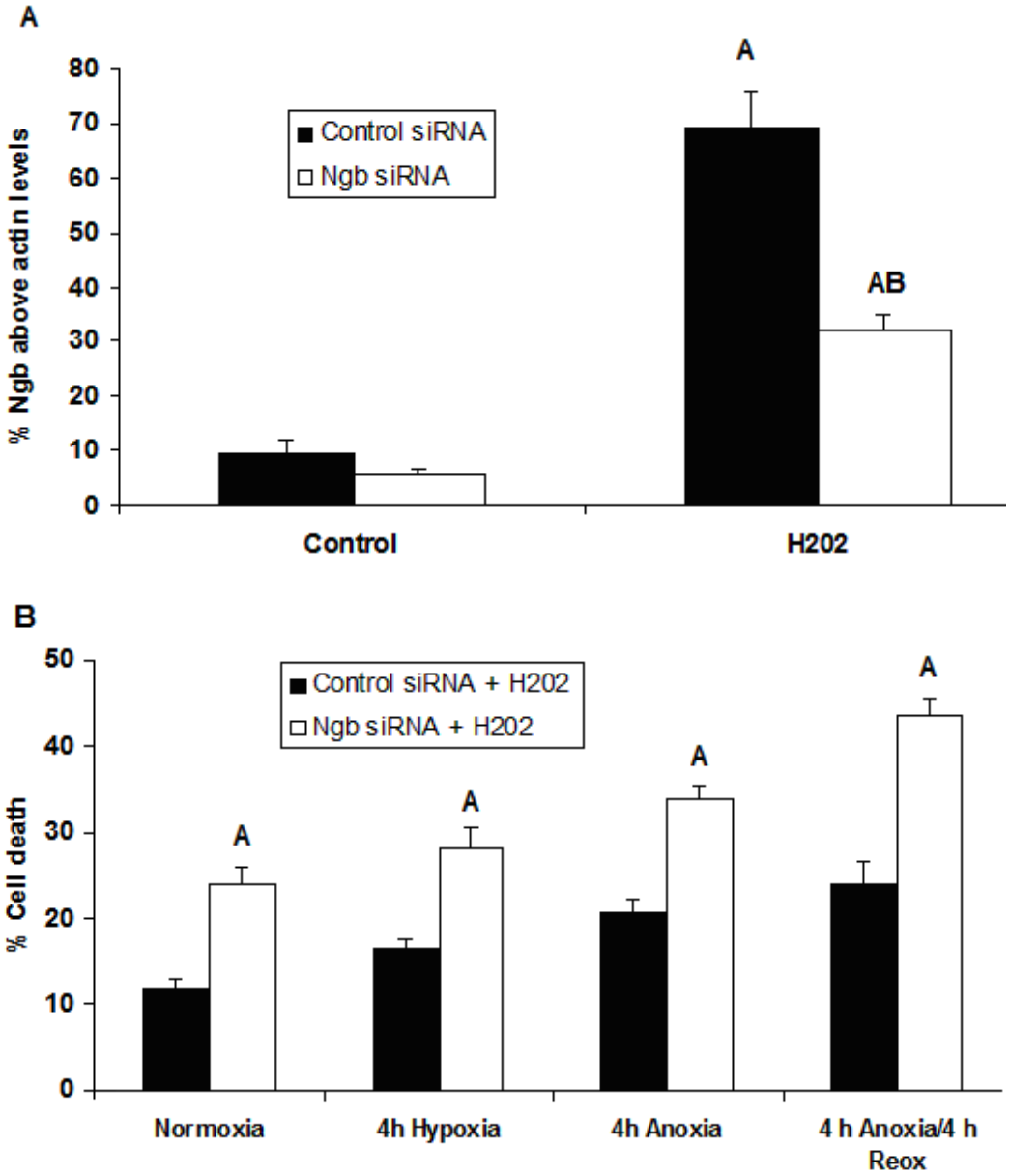

Figure 3-23: A: External oxidative stress (H_20_2) induced Ngb protein expression in control siRNA transfected cells. This induction was abrogated in cells transfected with Ngb siRNA and further exposed to H_20_2. Data are mean ± SEM, N = 3 independent experiments/group. A= significantly different from untreated (no H_20_2) control, B= significantly different from control siRNA treated with H_20_2.

B: Cell death increased significantly in all experimental conditions in Ngb knockdown cell cultures when exposed to external oxidative stress (H_20_2) compared to their respective controls. Concentrations ranged from 35 μmol/L to 70 μmol/L as previously published (Milton et al., 2007). N = 3 independent experiments/ timepoint. A= sig. different from controls in each treatment group.

Effect of Ngb knockdown on the prosurvival ERK pathway

It has been suggested that Ngb alters cellular levels of MAPK proteins (Zhu et al., 2002), specifically through the cross talk with the Extracellular Regulated Kinase (ERK) pathway. We examined the changes in activated ERK (p-ERK) expression in knockdown cultures subjected to differing of oxygen availability conditions. Ngb siRNA increased p-ERK levels in hypoxia, anoxia, and A/R (Figure 3-24) indicating that the high rate of cell survival that we found in Ngb knockdown cultures may be linked to activation of the pro-survival extracellular regulated kinase (ERK) signal transduction pathway in this model.

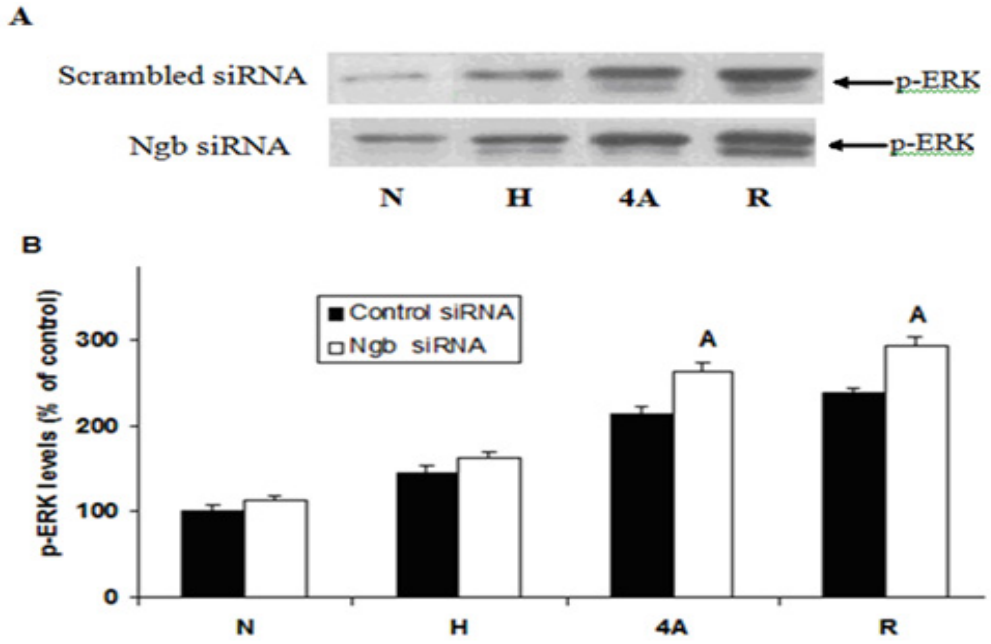

Figure 3-24: Ngb knockdown using siRNA increased p-ERK expression in Ngb knockdown cultures compared to the scrambled siRNA treated groups. Graphical representation showing densitometric analyses of p-ERK protein levels. N = normoxia, H = 4 h hypoxia, 1A = 1 h anoxia, 4A = 4 h anoxia, R = 4 h anoxia/4 h reoxygenation. A = sig. diff. from controls, $p < 0.01$. Data are mean ± SEM, N = 3 independent experiments/groups.

CHAPTER 4

DISCUSSION

Mammalian neurons exhibit rapid and irreversible degeneration when oxygen supply is curtailed (see reviews Lipton 1999, Mehta et al 2007; Rami et al., 2008). The restoration of oxygen supply after a brief ischemic event causes additional damage to the oxygen deprived tissue due to generation of reactive oxygen species (ROS) which further causes direct damage to the major cellular components and can lead to cell death. Both pathological and adaptive changes occur simultaneously during anoxia/ischemia in mammalian neurons, however, which hinders the accurate analysis of these overlapping events. The highly anoxia-tolerant freshwater turtle thus serves as an alternative model to analyze protective mechanisms in the absence of evident pathologies. As the gene responses to hypoxia are highly conserved, the brain of the anoxia- tolerant turtle offers a unique model to identify strategies to enhance neuronal survival during hypoxia/reoxygenation stress and hence can provide a useful insight into mechanisms behind mammalian failure and survival (Hochachka et al., 2001; Bickler et al., 2002). This may eventually help clinicians design new therapies based on mechanisms employed by naturally anoxia tolerant organisms. The current studies were carried out to analyze neuroprotective strategies including the role of AD, MAPK's/AKT, the Bcl-2 family of proteins and the putative free radical scavenger Ngb in neuronal survival during anoxia and in suppression of excess ROS formation upon reoxygenation by pharmacological and

molecular manipulations in turtle neuronal cultures. These studies may help to determine adaptations exhibited by the turtle at the physiological and molecular level to survive anoxia and also prevent excess ROS formation upon reoxygenation and how it differs in vulnerable mammalian neurons.

We are the first laboratory to successfully establish neuronal-enriched cultures from juvenile turtle brains. The protocol used (Milton et al., 2007) is a modification of Brewer's (1997) method of density gradient isolation of neurons. Several neuronal markers (NCAM, NeuN, Ngb, CSP, Neurofilament and GFAP) were used to confirm the consistency of the cells to be > 98% neuronal. The neuronal enriched cultures have several advantages such as it helps to isolate the protective events occurring specifically in the neurons, to manipulate one brain for several experimental analyses and testing several different variables on one brain tissue maintaining the internal controls effectively.

Despite numerous investigations using the mammalian ischemic model systems the mechanisms leading to cell death are not fully understood. Recently the focus of several studies has been on the signal transduction cascades as it is evident that the activation of these complex cascades act as crucial mediators of extracellular signals and their modulation can regulate the process of cell death/apoptosis during compromised oxygen conditions. The timing and degree of upregulation of the upregulation of the presumed prosurvival ERK and AKT pathway will promote anoxic survival. However the activation of the MAPK's play significant role in neuroprotection as well as pathogenesis of ischemia, hence its significance in ischemic neuroprotection is yet controversial. Studies have indicated that the overall outcome of an ischemic event via the activation of

these dynamic cascades depend upon the trigger, dynamics and duration of its activation, cellular conditions and model system as well the cell type (Martindale and Holbrook 2002; Chu et al., 2004). Additionally, studies have also shown that there exist several important points where therapeutic intervention may block the prodeath pathways thus inhibiting the death cascade. To study the important link of these pathways to cerebral ischemia several pharmacological agents have been used as specific inhibitors of the crucial pathways. Inhibition of the ERK pathway using a specific inhibitor has been seen to reverse its protective effects during cerebral ischemia (Park et al., 2004, Franceschini et al., 2006). While it has been suggested that JNK may play a protective role under some conditions (Dougherty et al., 2002), activation of JNK is generally associated with increased apoptosis and cell death, while blockade of JNK activation in mammalian neuronal cultures is protective (Kuan et al., 2003; Kanzawa et al., 2006). A recent study using a JNK knock-out model has clearly determined that JNK defiency protects the brain from glutamate -induced excitotoxicity and cerebral ischemia (Guan et al., 2005, 2006). Similar neuroprotective effects are seen after the inhibition of the p38 pathway during focal cerebral ischemia (Barone et al., 2001; Cao et al., 2005).

We employed pathway-specific pharmacological blockers for p38MAPK, ERK, and JNK activation (SB203580, U0126, and SP600125 respectively in DMSO) and PI3K/AKT (LY294002 in DMSO) to determine the role of these mediators in regulating cell death. We were successful in suppressing the MAPK's/AKT activation in turtle neurons using these blockers. After preexposing the neurons to the respective blockers and subjecting them to different durations of anoxia, the effects on cell survival were determined by propidium iodide staining. Our observations were consistent with the

mammalian ischemic studies showing that inhibition of ERK and AKT increased cell death whereas inhibition of the P38 and JNK pathway increased cell survival (Milton et al., in prep). DMSO alone did not alter cell death compared to controls. These inhibitors were found to be effective in turtle neuronal cultures, as in mammalian cells, lending support to the concept that the turtle and mammalian brain share similar molecular pathways; survival pathways, however, are clearly more robust in the turtle, as this research addresses.

Using these specific pathway blockers we aimed to determine whether the neuroprotection provided by some members of the MAPK family/AKT are activated by independent but overlapping signaling cascades. This activation may lead to the translocation of the protective terminal kinase to the nucleus which might ultimately lead to the transcription of the prosurvival genes. We found that blocking the protective ERK and AKT pathway lead to the upregulation of the presumed prodeath p-JNK and p-p38 levels which eventually lead to cell death. Similarly, blocking of the JNK and p38 pathway lead to the induction of the prosurvival p-ERK and p-AKT levels, which protected neuronal cell death (Milton et al., in prep). These results indicated that activation of the prosurvival ERK/AKT signal transduction cascades and suppression of the pro-death pathway can promote neuronal survival during anoxia in turtle neurons. Additionally, these observations also indicate the presence of an upstream sensor molecule that has significant effect on the intracellular mediators including theMAPK's/AKT which regulate the downstream molecular events. We attempted to find a link between the critical upstream signals that regulate and modulate the intracellular events.

One upstream signaling molecule thought to affect AKT and the MAPK pathways is adenosine (AD), a neuroprotectant rapidly formed during ischemia as a result of the intracellular breakdown of ATP (Braun et al., 1998; Dunwiddie and Masino 2001). AD also plays a role in preconditioning in the brain (Ciccarelli et al., 2007) and heart (Downey et al., 2007). Determining these links in this alternate model might help to disentangle the complexities of vulnerable mammalian neurons and provide insights into the mechanisms of mammalian anoxic brain damage.

We employed turtle neuronal cultures to analyze the protective role of adenosine during anoxia as well as its downstream effects on the signal transduction cascades in regulating cell survival. Cumulative evidence shows a protective role for adenosine during anoxia/ischemia in brain (Gervitz et al., 2002; D'Alimonte et al., 2007; Ciccarelli et al., 2007) and heart (Schulz et a.,l 2002; Germack et al., 2004). Stimulation of adenosine A1 receptors (A1R) reduces neuronal damage whereas blockade of A1 receptors exacerbates damage (Logan and Sweeney 1997; Safran et al., 2001; Hiraide et al., 2001). We have observed similar processes in turtle neuronal cell cultures after activation and blocking of A1R. In anoxic and reoxygenated cultures cell death was increased or decreased over untreated controls by administration of an A_1R antagonist or agonist, respectively (Milton et al., 2007).

Due to their ability to survive repeated anoxia-reoxygenation events, we had hypothesized that turtle neurons would prove to be highly resistance to oxidative stress. Indeed, when exposed to 12 hr complete anoxia/4 hr reoxygenation, turtle neurons demonstrated survival rates of nearly 75%. This high rate of survival, however,

apparently arises from low levels of ROS production (and presumably highly effective scavenging *in vivo*), rather than to an inherent resistance to ROS damage.

T. scripta has constitutively high levels of antioxidant enzymes including ascorbate, catalase, glutathione peroxidase (GPOX), superoxide dismutase (SOD), and alkyl hydroperoxide reductase (Rice et al., 1995; Willmore and Storey, 1997a; 1997b). Catalase and GPOX both convert H_2O_2 to water before hydroxyl radicals are formed, and are thus critical elements of the antioxidant defense system (Wang et al., 2003). As both hydroxyl radicals (*in vivo*) and hydrogen peroxide (in culture) were also readily detected under normoxic conditions, antioxidant defenses are not completely preventing the accumulation of ROS, and thus are not likely to fully explain the lack of ROS accumulation following anoxia-reoxygenation. Therefore the turtle brain is equipped with other intrinsic factors which can suppresses ROS formation during anoxia and upon reoxygenation. Blocking AD receptors increased ROS levels during both the hypoxic transition to anoxia and in particular upon reoxygenation and stimulation of AD receptors decreased ROS formation thus indicating a critical role of adenosine in modulation of ROS levels (Milton et al., 2007). While these would give the turtle a degree of "constitutive preconditioning" for reoxygenation stress (Lutz and Milton 2004), antioxidant defenses *in vitro*, at least, are clearly not sufficient to withstand extrinsic ROS stress.

We further investigated the vulnerability of turtle neurons to ROS stress by exposure of cultures to externally imposed oxidative stress (H_2O_2). Turtle neurons proved surprisingly susceptible to externally imposed ROS stress (H_2O_2). We propose that the suppression of ROS formation, coupled to high antioxidant levels, is necessary for

reoxygenation survival. This effect was then analyzed in cell cultures treated with AD agonist and antagonist. As was seen with intrinsic anoxic/reoxidative stress, cell death due to externally imposed oxidative stress was increased by DPCPX, though in the case of treatment with hydrogen peroxide cell death was also generally ameliorated by CCPA compared to non-drug controls, though this was not statistically significant except after 12 h anoxia or anoxia/reox. Our study has shown that the turtle neuronal cultures in fact proved to be no more resistant to imposed ROS stress than mammalian neurons, with similar concentrations proving lethal to cultured neurons (Jiang et al., 2001; Gum et al., 2004), nor did anoxia increase resistance to extrinsic ROS stress. Turtle neurons are even more vulnerable to ROS stress than some mammalian cell types, including cultured hepatocytes (Rosseland et al., 2005), and human microvascular endothelial cells (Rah et al., 2005) that can withstand 5-10 mM H_2O_2 exposure (30 mins.) with 70-90% cell survival. Our study has determined that the turtle brain survives reoxygenation stress not solely by increasing defense mechanisms, but by suppressing ROS production upon reoxygenation in neuronal cultures. This might be another strategy shaped by millions of years of evolution by the anoxia tolerant turtle to survive repeated bouts of anoxia/reoxygenation without apparent damage.

To understand further the mechanisms of adenosine-mediated protection, we wanted to analyze the interactions between adenosine and the signaling cascades. AD1R receptor stimulation reduces apoptosis by suppressing the activation of the pro-death JNK and p38 MAPK, simultaneously activating the prosurvival ERK and AKT pathway, and decreasing the levels of proapoptoic proteins including Bad (Ciccarelli et al., 2007). We have made similar observation in turtles, indicating that adenosine plays a critical role in

providing the turtle a unique ability to survive anoxia, and that blocking the AD1R receptor can hinder this ability and make the turtle succumb to anoxia. We have demonstrated through this study that extracellular signal regulated kinase (ERK 1/2) and PI3-K/AKT activation is dependent on A1R stimulation and these in turn modulate downstream effects that promote neuronal survival. Phosphorylated ERK 1/2 (p-ERK) and AKT (p-AKT) as well as Bcl-2 are upregulated in anoxic neuronally enriched primary cultures from turtle brain; this native upregulation is further increased by the selective A1R agonist CCPA, while the selective antagonist DPCPX decreased p-ERK and p-AKT expression. Conversely, low anoxic levels of phosphorylated JNK (p-JNK), p38 (p-p38), and Bax were elevated in the presence of the A1 antagonist (Nayak et al., in prep). Similarly in T. scripta whole brain, we observed an activation of the ERK and AKT pathway, a moderate activation of the JNK pathway, and a total suppression of the P38 pathway (Milton et al., 2008) supporting the *in vitro* observations and suggesting that the activation of ERK and AKT pathways might be protective in the turtle. These results indicate that the observations made in Milton et al., 2007 investigation which showed that AD1R activation increases cell survival and decreases ROS production during anoxia/reoxygenation might be linked to the activation of the presumed prosurvival ERK and AKT pathways and induction of the anti-apoptotic Bcl-2 protein. Pretreatment with CCPA could influence the protective effects of the presumed ERK and the AKT pathway. The activation of the ERK pathway in mammalian neurons leads to phosphorylation of wide range of cellular substrates and activate transcription of cAMP response element binding protein (CREB) which activates the prosurvival Bcl-2 and suppresses the apoptotic proteins such as Bad, Bim and caspases (Sgambato et al., 1998; Chan et al.,

2004; Hetman and Gozdz, 2004, Sawe et al., 2008) and prevents apoptosis. Similarly, the protective role of AKT is thought to be in part through its interactions with the Bcl-2 family of proteins. AKT targets CREB to activate prosurvival mechanisms (Shibata et al., 2002) and also phosphorylates BAD- a proapoptotic protein (Datta et al., 1997; Chan et al., 2004; Kamada et al., 2007) thus preventing translocation of Bad to the mitochondria and inhibiting the release of apoptogenic proteins including the cytochrome c and caspase activation (Wang et al., 2007). Recent studies have shown that the activation of the pro-apoptotic stress-activated protein kinase (SAPK) cascade is downregulated by the pro-survival phosphatidylinositol 3-kinase/AKT pathway in cerebral ischemia through inhibiting Bad (Kamada et al., 2007; Wang et al., 2007).

Adenosine thus acts as an efficient sensor or signal during cerebral anoxia/ischemia for activation of a diverse array of signaling cascades, which then further activate the downstream effectors including anti-apoptotic Bcl-2, resulting in neuroprotection (Dirnagl et al., 2003). Additionally, the Bcl-2 protein has been shown to play a major role in suppressing cell death during apoptotic and oxidative stress cell injury by enhancing the levels of anti-oxidants as well as suppressing generation of free radicals (Hockenbery 1993; Kane et al., 1993; Ellerby et al., 1996; Lee et al., 1998; 2001; Saitoh et al., 2003). As reported in Milton et al., 2007, the suppression of ROS and decreased cell death can be linked to the induction of anti-apoptotic protein Bcl-2 and inhibition of pro-apoptotic BAX in the adenosine stimulated neurons. Thus intracellular signaling pathways are apparently affected by adenosine (Gervitz et al., 2002; Schulte et al., 2004; Brust et al., 2006) and the protective effects can be duplicated and abrogated by appropriate pharmacological intervention through employing specific adenosine receptor

agonists and antagonists. The overall observations from previous investigations on turtle brain together with this current study clearly indicate a link between the physiological adaptations and molecular regulations in this anoxia tolerant model. These studies could contribute to stroke therapy as at least part of the brain tissue can be protected by pharmacological manipulations by targeting adenosine signaling. The results of these studies are consistent with our hypothesis that adenosine may have a neuroprotective role in turtle neurons which may help them survive repeated anoxia-reoxygenation events by interacting with the MAPKs/PI3-K/AKT pathway and adenosine may also play a key role in suppressing the generation of reactive oxygen species, thus avoiding apoptosis.

Apoptosis is a complex process that is tightly regulated by several upstream and downstream regulators which may be potential therapeutic targets in stroke (Mehta et al., 2007). The Bcl-2 family of proteins is a major downstream group of regulators and these include members that serve for both defenses such as Bcl-2 and cell execution such as Bax (Green and Reed 1998; Letai, 2005). There are several lines of evidence which show that the Bcl-2 family of proteins plays a major role in cerebral and myocardial ischemia as well as pre-conditioning (see reviews Graham et al., 2000, Ouyang and Gifford 2004, Mehta et al., 2007). Interestingly the elements that are known to be protective may be elevated in the fresh water turtle brain during anoxia (Haddad et al., 2007). We have made similar observations with both Bcl-2 and Bax transcription and protein levels increasing in anoxic cells and upon anoxia/reoxygenation, but the increases in Bcl-2 transcription and translation are relatively greater than those of Bax and result in increased Bcl-2: Bax ratios in anoxia and reoxygenation. To confirm the potential neuroprotective role of Bcl-2 we employed turtle specific Bcl-2 siRNA and our data

demonstrated a clear increase in cell death. Bcl-2 knockdown is most likely related to increases in Bax expression, resulting in decreased Bcl-2: Bax ratios compared to control cells and shifting cells away from survival towards apoptosis. Bcl-2 knockdown also resulted in elevated expression of activated caspase-3. In the absence of Bcl-2 siRNA, caspase-3 levels do not increase in anoxia in cell cultures, or in whole brain (Kesaraju et al., submitted), showing the extraordinary resistance of turtle neurons to apoptosis even in extended anoxia. Bcl-2 knockdown using siRNA also resulted in elevated ROS release in normoxic, anoxic, and reoxygenated cells as determined by Amplex Red staining thus pointing to the contribution of Bcl-2 in ROS regulation. In non-transfected anoxic/reoxygenated turtle neurons ROS production only to basal normoxic levels without the ROS overproduction typically observed in mammalian models suggesting that the anoxia tolerant turtle has an inherent ability to suppress ROS generation (Milton et al., 2007). High ROS levels in anoxic cells results from increased release during the initial hour of anoxia, as cells undergo increasing hypoxia and physiological stress (Milton et al., 2007). SiRNA treatment alone (scrambled controls) does not affect ROS release, as H_2O_2 levels followed the same pattern as is observed in untreated cells with a significant decrease in anoxia followed by a return only to basal levels upon reoxygenation. Analyzing the regulation of these key downstream molecular events which prevents the triggering of death cascade as well as lethal effects of oxidative stress in this alternate animal model may provide insights into the mechanisms of mammalian anoxic and ischemic/reperfusion brain damage. An overview of the current understanding from several ischemic model systems including the anoxia tolerant species will help to

design paradigm that will incorporate many endogenous neuroprotective mechanisms thus highlighting future treatment strategies for stroke.

In addition to the above mentioned strategies we have also investigated the role of a recently discovered neuroprotective heme protein Ngb, an intracellular protein that helps sustain cellular respiration and would aid cellular survival under hypoxic conditions. The regulation and function of the recently described neuroglobin protein are as yet a matter of debate. In response to the growing interest in understanding the role of Ngb in cerebral ischemia and stroke protection, we analyzed the regulation of Ngb in hypoxia (5% O_2), acute (1h) and long-term (4h) anoxia and upon anoxia-reoxygenation in whole turtle brains and turtle neuronal cell cultures. Hypoxia resulted in a greater induction of Ngb mRNA and protein in the turtle brain than anoxia. A further greater induction of Ngb mRNA and protein was observed upon reoxygenation following anoxia. Acute (1 hr) and chronic (4 hr) anoxia, however, resulted in a much smaller degree of induction relative to normoxia, implying that the role or regulation of Ngb in the turtle brain is hypoxia specific. Ngb mRNA and protein expression in the neuronally enriched turtle cultures follows a pattern similar to the whole brain. The upregulation of neuroglobin transcripts during anoxia and during hypoxia may reflect its potential role as a hypoxia sensor, or may relate to an alternative function such as an intracellular oxygen carrier or a component in a detoxification pathway (Herold et al., 2004, Sowa et al., 1998, Kriegl et al., 2004). The greater induction of Ngb by hypoxia over anoxia, and higher additional increases upon reoxygenation, indicate a more likely hypoxia specific role in the turtle brain, perhaps as an ROS scavenger, rather than as a means to increase oxygen delivery. The induction of Ngb by hypoxia in turtles suggests the specific regulation of

Ngb through hypoxia signaling pathways (HIF-1ά), although alternative regulatory pathways have also been suggested (Wystub et al., 2004). The alternate hypoxic induction of Ngb could occur through MAPK signal transduction pathways (Zhu et al., 2002).

We further examined the neuroprotective effects of Ngb by knocking down Ngb with turtle specific siRNA using lipofectamine transfection. Ngb knockdown in neuronally enriched cell cultures resulted in significant increases in H_20_2 release compared to controls but no change in cell death. Cell survival may be linked to activation of other protective responses such as the extracellular regulated kinase (ERK) transduction pathway, as phosphorylated ERK levels in anoxia were significantly higher in Ngb knockdown cultures compared to controls (Nayak et al., submitted).

We also analyzed the effect of imposed oxidative stress on Ngb knockdown cell cultures. Ngb was significantly induced when exposed to H_20_2. Cell death also increased significantly in Ngb knockdown cell cultures when exposed to external oxidative stress. The greater expression of Ngb when ROS are likely to be high, and the increased susceptibility of neurons to H_20_2 release and external oxidative stress in knockdown cultures, suggests a role for Ngb in reducing ROS production or in detoxification, though it does not appear to be critical to survival in the anoxia tolerant turtle in the presence of compensatory survival mechanisms which include a range of physiological and molecular adaptations. As an evolutionarily selected adaptation, the ability to suppress ROS formation could prove an interesting path to investigate new therapeutic targets in mammals.

In conclusion, by investigating these pathways in an alternative animal model and uncovering how these underlying mechanisms help protect these anoxia tolerant creatures under conditions of oxidative stress, we can obtain an insight into potential therapeutic targets for a variety of pathologies attributed to ROS stress. It is likely in such a model that the up- and down-regulation of particular molecular pathways is adaptive rather than pathological. Uncovering these molecular mechanisms in such an alternate model might help to disentangle the complexities of vulnerable mammalian neurons and provide insights into studying the mechanisms of mammalian anoxic brain damage. In our studies we have shown that by modulating the critical upstream neuromodulator, adenosine during ischemia we can successfully regulate the key events leading to apoptosis. Pharmacological stimulation of the A1 adenosine receptor using specific agonist can turn on the presumed protective signaling cascades like ERK and AKT while suppressing the prodeath pathways like JNK and P38. Similarly these protective events are seen to be abolished by use of a specific A1 adenosine receptor antagonist. The anoxia tolerant turtle shares many of the same molecular pathways as mammals and thus can be manipulated by pharmacological intervention. The pathways we have characterized are significant for the neuroprotection in this model and might be helpful in mammalian neuroprotection. This study has established the protective role of the anti-apoptotic protein Bcl-2 in this model. The study has shown that turtle maintains a high Bcl2: Bax ratio during anoxic stress which is critical for neuronal survival and a reversal of this ratio can lead to neuronal cell death. This study has for the first time provided a novel link between physiological and molecular adaptations in maintaining functional integrity of the neuronal network in long term anoxia and oxidative stress. This major link of the

key physiological and molecular events can guide in designing a paradigm which will eventually help in developing a clinical therapeutic strategy for stroke.

This study has also opened new avenues in analyzing the effect of several inherent neuroprotectants like Ngb which can offer neuroprotection by sustaining respiration during compromised oxygen conditions and oxidative stress. This study also clearly emphasizes the importance of the down stream regulators in regulating the process of apoptosis. These events activated in response to stress are linked to the excitatory events that occur during oxygen deprivation and oxidative stress and thus activate the intrinsic mitochondrial pathway of apoptosis. Other *in vivo* and *in vitro* studies in our laboratory have shown that caspase levels during anoxic stress as well as reoxygenation are unaltered in the anoxia tolerant fresh water turtle.

Future directions to the project will be are analyzing the possible role of caspase independent pathway, inhibitor of apoptotic proteins as well as protective events that prevent the mitochondrial MPTP opening. Studies from our laboratory have indicated that the anoxia tolerant turtle has a remarkable ability to suppress ROS formation upon reoxygenation (Milton et al., 2007). Hsp-72 and Bcl-2 knockdown however lead to permanent loss of the mitochondrial membrane potential unlike untreated controls and resulted in increased cell death in turtle neuronal cultures exposed to anoxia (Milton et al., in prep). Mammalian studies have shown that apoptotic signal leads to the translocation of the pro-apoptotic proteins to the mitochondria which alters the membrane potential and lead to the opening of MPTP (Shimizu et al., 1999; Sharpe et al., 2003, Tsujimoto 2003). Cyclophilin D (Cyp D), a prolyl isomerase enzyme guards the mitochondrial membrane opening and hence can be a critical target for analyses in the

turtle neurons. Another promising study will be analyzing the role of cyclosporine A in preventing opening of mitochondrial MPTP opening. Mammalian studies have shown a significant role of cyclosporine in protecting mitochondrial during hypoxia/reperfusion injury (Godvadze and Richter 1993; Uchino et al., 1998; Oka et al., 2008). A recent Mayanagi et al (2007) investigation has emphasized the importance of mito K_{ATP} channels in preventing neuronal apoptosis. The study has shown that mito K_{ATP} channel opener, BMS191095 prevented ROS generation while this effect was reversed by mito K_{ATP} blocker 5- Hydroxydecanoate (5HD). The pharmacological manipulations of the mitochondrial ATP can provide effective strategy for neuronal protection during oxygen deprivation and oxidative stress.

A recent study has shown that the inhibitor of apoptosis proteins (IAPs) can potentially prevent apoptosis by inactivating caspases during cerebral ischemia/reperfusion injury (Deveraux and Reed, 1999; Nakka et al., 2008). It will be interesting to analyze the role of these proteins in the stress-resistant turtle neurons. Additionally the role of calpains cannot be ruled out as these calcium dependent cysteine proteases also play a major role in neuronal damage during ischemia (Rami 2003). An interesting aspect would be to analyze the regulation of calpains, and to use calpain inhibitors in this model to analyze effect on neuronal survival.

It will be interesting to analyze the role of Ngb and its interaction with other signal transduction pathways. It might help to clear the missing links, which will help to reveal the neuroprotective role of Ngb. Since adensosine A1 receptor activation is crucial in the freah water turtle for ROS regulation (Milton et al., 2007), determining the role of adensosine A1 receptor in regulating Ngb expression will also be a critical study. It will

help to determine the upstream regulator of Ngb expression during compromised oxygen availability.

Most importantly with this study we emphasize the role of comparative study models in successfully understanding the key events during ischemia and determining future targets and neuroprotective cascades which can help in mammalian neuronal survival by application of gene therapy or efficient pharmacological intervention.

REFERENCES

Adams, J. M. and Cory, S. (2001) Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci, **6,** 61-66.

Allen, N. J., Karadottir, R. and Attwell, D. (2005) A preferential role for glycolysis in preventing the anoxic depolarization of rat hippocampal area CA1 pyramidal cells. J Neurosci, **25,** 848-859.

Almeida, C. G., A. de Mendonca, et al. (2003) Adenosine promotes neuronal recovery from reactive oxygen species induced lesion in rat hippocampal slices. Neurosci Lett **339,** 127-30.

Amir, G., Ramamoorthy, C., Riemer R. K., Reddy V. M.**,** and F. L. Hanley Neonatal Brain Protection and Deep Hypothermic Circulatory Arrest: Pathophysiology of Ischemic Neuronal Injury and Protective Strategies. Ann. Thorac. Surg, **80,** 1955 – 1964.

Baker A (2004) Ischemic preconditioning in the brain. Can J Anaesth **51**, 201-205.

Barone, F. C., E. A. Irving, et al. (2001) SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. J Pharmacol Exp Ther **296,** 312-321

Beguin PC, Belaidi E, Godin-Ribuot D, Levy P, Ribuot C (2007) Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk1/2. J Mol Cell Cardiol **42,** 343-351.

Berger, R., Y. Garnier, et al. (2002). Perinatal brain damage: underlying mechanisms and neuroprotective strategies. J Soc Gynecol Investig **9,** 319-328.

Braun, N., Y. Zhu, et al. (1998). Upregulation of the enzyme chain hydrolyzing extracellular ATP after transient forebrain ischemia in the rat. J Neurosci **18,** 4891-4900.

Brewer, G. J. (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods **71,** 143-155.

Brust, T. B., F. S. Cayabyab, et al. (2006). p38 mitogen-activated protein kinase contributes to adenosine A1 receptor-mediated synaptic depression in area CA1 of the rat hippocampus. J Neurosci **26,**12427-12438.

Buck, L. T. and Bickler, P. E. (1998). Adenosine and anoxia reduce Nmethyl-D-aspartate receptor open probability in turtle cerebrocortex. J. Exp.Biol **210**, 289-297.

Burmester, T. and Hankeln, T. (2004) Neuroglobin: A respiratory protein of the nervous system. News Physiol. Sci **19,** 110–113.

Canals, M., E. Angulo, et al. (2005). Molecular mechanisms involved in the adenosine A and A receptor-induced neuronal differentiation in neuroblastoma cells and striatal primary cultures. J Neurochem **92,** 337-348.

Cao, W., Q. F. Collins, et al. (2005). p38 Mitogen-activated protein kinase plays a stimulatory role in hepatic gluconeogenesis. J Biol Chem **280**, 42731-42737.

Cassada, D. C., C. G. Tribble, et al. (2002). Adenosine A2A analogue improves neurologic outcome after spinal cord trauma in the rabbit. J Trauma **53,** 225-229; discussion 229-231.

Chan PH (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab **21,** 2-14.

Chan (2004) Future Targets and Cascades for Neuroprotective Strategies.Stroke **35,** 2748-2750.

Chen, J., R. P. Simon, et al. (2000). Suppression of endogenous Bcl-2 expression by antisense treatment exacerbates ischemic neuronal death. J Cereb Blood Flow Metab **20**,1033-1039.

Chih, C. P. and Roberts Jr, E. L. (2003) Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron lactate shuttle hypothesis. J Cereb Blood Flow Metab **23,** 1263-1281.

Choi, B. H. (1993). Oxygen, antioxidants and brain dysfunction. Yonsei Med J **34,** 1-10.

Chu CT, Levinthal DJ, Kulich SM, Chalovich EM, DeFranco DB (2004) Oxidative neuronal injury. The dark side of ERK1/2. Eur J Biochem **271**, 2060-2066.

Ciccarelli R, D'Alimonte I, Ballerini P, D'Auro M, Nargi E, Buccella S, Di Iorio P, Bruno V, Nicoletti F, Caciagli F (2007) Molecular signalling mediating the protective effect of A1 adenosine and mGlu3 metabotropic glutamate receptor activation against apoptosis by oxygen/glucose deprivation in cultured astrocytes. Mol Pharmacol **71**, 1369-1380.

Clerk, A., S. J. Fuller, et al. (1998). Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J Biol Chem **273,** 7228-7234.

Collier, T. J., Steece-Collier, K., McGuire, S. and Sortwell, C. E. (2003) Cellular models to study dopaminergic injury responses. Ann N Y Acad Sci **991,** 140-151.

Cooke, H. J., Y. Wang, et al. (1999). Activation of neuronal adenosine A1 receptors suppresses secretory reflexes in the guinea pig colon. Am J Physiol **276,** G451-62.

D'Alimonte I, Ballerini P, Nargi E, Buccella S, Giuliani P, Di Iorio P, Caciagli F, Ciccarelli R (2007) Staurosporine-induced apoptosis in astrocytes is prevented by A1 adenosine receptor activation. Neurosci Lett **418,** 66-71.

Das, D. K., and Maulik, N. (2003): Preconditioning potentiates redox signaling and converts death signal into survival signal. Arch. Biochem. Biophys **420,** 305-311.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91,** 231-41.

Deveraux, Q. L. and Reed, J. C. (1999) IAP family proteins--suppressors of apoptosis. Genes Dev **13,** 239-252.

Dhein J., Walczak H., Baumler C., Debatin K. M., Krammer P. H. (1995) Autocine T-cell suicide mediated by APO-1/(Fas/CD95). Nature **6513**, 438-441.

Dirnagl, U., Simon, R. P. and Hallenbeck, J. M. (2003) Ischemic tolerance and endogenous neuroprotection. Trends Neurosci **26,** 248-254.

Dirnagl, U. and Meisel, A. (2008) Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? Neuropharmacology **55,** 334-344.

Dougherty, C. J., Kubasiak, L., Prentice, H., Andreka, P., Bishopric, N. H. and Webster, K. A. (2002). Activation of c-Jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress. Biochem. J. **362**,561 -571

Droge, W. (2002) Free radicals in the physiological control of cell function. Physiol Rev **82,** 47-95.

Dunwiddie, T. V. and S. A. Masino (2001). The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci **24,** 31-55.

Ellerby L., Ellerby H., Park S., Hlleran A., Murphy a Fiskum G., Kane D., Testa M., Kayalar C. and Bredesen D. 1996. Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. J. Neurochem **67,** 1259–1266.

Engelbrecht, A. M., C. Niesler, et al. (2004). p38 and JNK have distinct regulatory functions on the development of apoptosis during simulated ischaemia and reperfusion in neonatal cardiomyocytes. Basic Res Cardiol **99,** 338-50.

Fatokun, A. A., T. W. Stone, et al. (2007). Cell death in rat cerebellar granule neurons induced by hydrogen peroxide *in vitro*: mechanisms and protection by adenosine receptor ligands. Brain Res **1132,** 193-202.

Fernandes J. A., Lutz P. L., Tannenbaum A., Todorov A. T., Liebovitch L., and Vertes R. (1997) Electroencephalogram activity in the anoxic turtle brain. Am. J. Physiol **273,** R911 -R919.

Floyd R.A., and Carney J.M. (1992) Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. Lab. Invest **47,** 412-426.

Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Van Bockstaele, D., Moens, L. and Dewilde, S. (2006) Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. Neurosci Lett **410,** 146-51.

Gao, X., C. Chang, et al. (2006). Effect of hypoxic preconditioning on neural cell apoptosis and expression of Bcl-2 and Bax in cerebral ischemia-reperfusion in rats. J Huazhong Univ Sci Technolog Med Sci **26,** 17-20.

Germack, R. and J. M. Dickenson (2005). Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. J Mol Cell Cardiol **39,** 429-442.

Gervitz, L. M., D. Nalbant, et al. (2002). Adenosine-mediated activation of Akt/protein kinase B in the rat hippocampus *in vitro* and *in vivo*. Neurosci Lett **328,** 175-179.

Gogvadze, V. and Richter, C. (1993) Cyclosporine A protects mitochondria in an *in vitro* model of hypoxia/reperfusion injury. FEBS Lett **333,** 334-338.

Graham, S. H., J. Chen, et al. (2000). Bcl-2 family gene products in cerebral ischemia and traumatic brain injury. J Neurotrauma **17,** 831-41.

Green, D. R. and J. C. Reed (1998). Mitochondria and apoptosis. Science **281**(5381): 1309-12.

Greenberg DA, Jin K, Khan AA (2008) Neuroglobin: an endogenous neuroprotectant. Curr Opin Pharmacol **8,** 20-4.

Greenway, S. C. and K. B. Storey (2000). Mitogen-activated protein kinases and anoxia tolerance in turtles. J Exp Zool **287,** 477-84.

Greenway, S.C., Storey, K.B., 2000. Seasonal change and prolonged anoxia affect the kinetic properties of phosphofructokinase and pyruvate kinase in oysters. J. Comp. Physiol. B-Biochem. Syst. Environ. Physiol **170**, 285– 293.

Gross, A., McDonnell, J. M. and Korsmeyer, S. J. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev **13,** 1899-1911.

Guan, Q. H., D. S. Pei, et al. (2005) The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. Brain Res **1035,** 51-59.

Guan, Q. H., Pei, D. S., Liu, X. M., Wang, X. T., Xu, T. L. and Zhang, G. Y. (2006) Neuroprotection against ischemic brain injury by SP600125 via suppressing the extrinsic and intrinsic pathways of apoptosis. Brain Res **1092,** 36-46.

Gupta Y, Sinha K, Chaudhary G, and Jagannathan N. (2002) Protective effect of adenosine against neuronal injury induced by middle cerebral artery occlusion in rats as evidenced by diffusion-weighted imaging. Pharmacol. Biochem. Behav **72,** 569-576.

Guyton KZ, Gorospe M, Kensler TW, Holbrook NJ (1996a) Mitogen-activated protein kinase (MAPK) activation by butylated hydroxytoluene hydroperoxide: implications for cellular survival and tumor promotion. Cancer Res **56,** 3480-3485.

Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ (1996b) Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. J Biol Chem **271,** 4138-4142.

Haas, H. L. and O. Selbach (2000). Functions of neuronal adenosine receptors. Naunyn Schmiedebergs Arch Pharmacol **362,** 375-381.

Haberg, A., Qu, H. and Sonnewald, U. (2006) Glutamate and GABA metabolism in transient and permanent middle cerebral artery occlusion in rat: importance of astrocytes for neuronal survival. Neurochem Int **48,** 531-540

Haddad JJ (2007a) The role of Bax/Bcl-2 and pro-caspase peptides in hypoxia/reperfusion-dependent regulation of MAPK(ERK): discordant proteomic effect of MAPK(p38). Protein Pept Lett **14,** 361-371.

Haddad JJ (2007b) Discordant tissue-specific expression of SAPK/MAPK(JNK)-related cofactors in hypoxia and hypoxia/reoxygenation in a model of anoxia-tolerance. Protein Pept Lett **14,** 373-380.

Han BH and Holtzman DM (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury *in vivo* via the ERK pathway. J Neurosci **20**, 5775-5781.

Harris, M. H. and C. B. Thompson (2000). The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ **7,** 1182-1191.

Hashimoto T., Yonetani M., and Nakamura H. (2003) Selective brain hypothermia protects against hypoxic-ischemic injury in newborn rats by reducing hydroxyl radical production. Kobe J. Med. Sci **49,** 83-91.

Hata R, Gillardon F, Michaelidis TM, Hossmann KA (1999) Targeted disruption of the Bcl-2 gene in mice exacerbates focal ischemic brain injury. Metab Brain Dis **14,** 117-124.

Hata, R., Maeda, K., Hermann, D., Mies, G. and Hossmann, K. A. (2000a) Dynamics of regional brain metabolism and gene expression after middle cerebral artery occlusion in mice. J Cereb Blood Flow Metab **20,** 306-315.

Hata, R., Maeda, K., Hermann, D., Mies, G. and Hossmann, K. A. (2000b) Evolution of brain infarction after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab **20,** 937-946.

Hetman M, Gozdz A (2004) Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. Eur J Biochem **271**, 2050-2055.

Hillion, J. A., Li, Y., Maric, D., Takanohashi, A., Klimanis, D., Barker, J. L. and Hallenbeck, J. M. (2006) Involvement of Akt in preconditioning-induced tolerance to ischemia in PC12 cells. J Cereb Blood Flow Metab **26,** 1323-1331.

Hirai, K., T. Hayashi, et al. (2004). PI3K inhibition in neonatal rat brain slices during and after hypoxia reduces phospho-Akt and increases cytosolic cytochrome c and apoptosis. Brain Res Mol Brain Res **124,** 51-61.

Hiraide, T., K. Katsura, et al. (2001). Adenosine receptor antagonists cancelled the ischemic tolerance phenomenon in gerbil. Brain Res **910,** 94-98.

Hockenbery, D. M., Z. N. Oltvai, et al. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell **75,** 241-251.

Hylland, P., Nilsson, G. E. and Lutz, P. L. (1994) Time course of anoxia-induced increase in cerebral blood flow rate in turtles: evidence for a role of adenosine. J Cereb Blood Flow Metab **14,** 877-881.

Irving, E. A., F. C. Barone, et al. (2000). Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. Brain Res Mol Brain Res **77,** 65-75.

Jackson DC (2000) Living without oxygen: Lessons from the freshwater turtle. Comp biochem Physiol A Mol Integr Physiol **125,** 299-315.

Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. Nat Rev Drug Discov **5,** 247-264.

Jacques-Silva MC, Bernardi A, Rodnight R, Lenz G (2004) ERK, PKC and PI3K/Akt pathways mediate extracellular ATP and adenosine-induced proliferation of U138-MG human glioma cell line. Oncology **67,** 450-459.

Jin G, Omori N, Li F, Sato K, Nagano I, Manabe Y, Shoji M, Abe K (2002) Activation of cell-survival signal Akt by GDNF in normal rat brain. Brain Res **958**, 429-433.

Kahlert, S., Zundorf, G. and Reiser, G. (2005) Glutamate-mediated influx of extracellular Ca2+ is coupled with reactive oxygen species generation in cultured hippocampal neurons but not in astrocytes. J Neurosci Res **79,** 262-271.

Kamada, H., C. Nito, et al. (2007). Bad as a converging signaling molecule between survival PI3-K/Akt and death JNK in neurons after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab **27,** 521-533.

Kane D., Sarafian T., Anton R., Hahn H., Gralla E., Valentine J., Ord T. and Bredesen D. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science **262,** 1274–1277.

Karmazyn, M. and M. A. Cook (1992). Adenosine A1 receptor activation attenuates cardiac injury produced by hydrogen peroxide. Circ Res **71,** 1101-1110.

Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K. et al., (2006) Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. Proc. Natl Acad. Sci. U S A **103,** 17944-17948.

Khan, A. A., Mao, X. O., Banwait, S., DerMardirossian, C. M., Bokoch, G. M., Jin, K. and Greenberg, D. A. (2008) Regulation of hypoxic neuronal death signaling by neuroglobin. FASEB J **22,** 1737-1747.

Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM (2006) The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. FASEB J **20,** 1185-1187.

Kitatsuji, C., Kurogochi, M., Nishimura, S., Ishimori, K. and Wakasugi, K. (2007) Molecular basis of guanine nucleotide dissociation inhibitor activity of human neuroglobin by chemical cross-linking and mass spectrometry. J Mol Biol **368,** 150-160.

Kreisman, N. R., Lamanna, J. C., Rosenthal, M. and Sick, T. J. (1981) Oxidative metabolic responses with recurrent seizures in rat cerebral cortex: role of systemic factors. Brain Res **218,** 175-188.

Kulinsky V.I., Minakina L.N., and Usov L.A. (2001) Role of adenosine receptors in neuroprotective effect during global cerebral ischemia. Bull. Exp. Biol. Med **131,** 454-456.

LaManna, J. C., Light, A. I., Peretsman, S. J. and Rosenthal, M. (1984) Oxygen insufficiency during hypoxic hypoxia in rat brain cortex. Brain Res **293,** 313-318.

LaManna JC (2007) Hypoxia in the central nervous system. Essays Biochem **43,** 139-151.

Laderoute, K. R. and K. A. Webster (1997). Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes. Circ Res **80,** 336-44.

Lee L, Irani K, Finkel T. 1998. Bcl-2 regulates nonapoptotic signal transduction: inhibition of c-Jun N-terminal kinase (JNK) activation by IL-1 beta and hydrogen peroxide. Mol Genet Metab **64,** 19–24.

Lee M., Hun D., Marshall K., Ellerby L., Bredesen D., Jenner P. and Halliwell B. 2001. Effect of overexpression of Bcl-2 on cellular oxidative damage, nitric oxide production, antioxidant defenses, and the proteasome. Free Rad. Biol. Med **31**, 1550–1558.

Lennmyr, F., S. Karlsson, et al. (2002). Activation of mitogen-activated protein kinases in experimental cerebral ischemia. Acta Neurol Scand **106**, 333-340.

Leutner, S., Eckert, A. and Muller, W. E. (2001) ROS generation, lipid peroxidation and antioxidant enzyme activities in the aging brain. J Neural Transm **108,** 955-967.

Lewen A, Matz P, Chan PH (2000) Free radical pathways in CNS injury. J Neurotrauma **17,** 871-890.

Li F, Omori N, Sato K, Jin G, Nagano I, Manabe Y, Shoji M, Abe K (2002) Coordinate expression of survival p-ERK and proapoptotic cytochrome c signals in rat brain neurons after transient MCAO. Brain Res **958**, 83-88.

Li, R. C., M. W. Morris, Lee, S., Pouranfar, F., Wang, Y. and Gozal, D. (2008) Neuroglobin protects PC12 cells against oxidative stress. Brain Res **1190**, 159-166.

Lipton, P. (1999) Ischemic cell death in brain neurons. Physiol Rev **79,** 1431-1568.

Liu, Y., Yang, X. M., Iliodromitis, E. K., Kremastinos, D. T., Dost, T., Cohen, M. V. and Downey, J. M. (2008) Redox signaling at reperfusion is required for protection from ischemic preconditioning but not from a direct PKC activator. Basic Res Cardiol **103,** 54-59.

Logan, M. and M. I. Sweeney (1997). Adenosine A1 receptor activation preferentially protects cultured cerebellar neurons versus astrocytes against hypoxia-induced death. Mol Chem Neuropathol **31,** 119-133.

Lucken-Ardjomande, S. and J. C. Martinou (2005). Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane. C R Biol **328,** 616-631.

Luo, X., I. Budihardjo, et al. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell **94,** 481-490.

Lutz, P. L., McMahon, P., Rosenthal, M. and Sick, T. J. (1984) Relationships between aerobic and anaerobic energy production in turtle brain in situ. Am J Physiol **247,** R740-744.

Lutz, P. L. and G. E. Nilsson (1997). Contrasting strategies for anoxic brain survival--glycolysis up or down. J Exp Biol **200,** 411-419.

Lutz P. L., Nilsson G. E., Prentice H. (2003) The Brain without Oxygen: Causes of Failure Molecular and Physiological Mechanisms for Survival. Third edition. Dordrecht: Kluwers Press.

Lutz P.L., Milton S.L. (2004) Negotiating brain anoxia survival in the turtle. J. Exp. Biol **207,** 3141-3147.

Martindale J.L, Holbrook N.J. (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol **192,** 1-15.

Mayanagi, K., Gaspar, T., Katakam, P. V. and Busija, D. W. (2007) Systemic administration of diazoxide induces delayed preconditioning against transient focal cerebral ischemia in rats. Brain Res **1168,** 106-111.

McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E (2003) Caspase 3 activation is essential for neuroprotection in preconditioning. Proc Natl Acad Sci U S A **100,**715-720.

Mehta S.L., Manhas N., Raghubir R. (2007) Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res. Rev **54,** 34-66.

Melani, A., M. Gianfriddo, et al. (2006). The selective A2A receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. Brain Res **1073-1074**: 470-480.

Mielke K, Brecht S, Dorst A, Herdegen T (1999) Activity and expression of JNK1, p38 and ERK kinases, c-Jun N-terminal phosphorylation, and c-jun promoter binding in the adult rat brain following kainate-induced seizures. Neuroscience **91,** 471-483.

Milton, S.L. 1994. The physiology of hypoxia and anoxic tolerance in three species of turtle: The loggerhead sea turtles (Caretta caretta), green sea turtle (Chelonia mydas) and freshwater Trachemys scripta. Ph.D Thesis, University of Miami, 196pp.

Milton SL, Thompson JW, Lutz PL (2002) Mechanisms for maintaining extracellular glutamate levels in the anoxic turtle striatum. Am J Physiol Regul Integr Comp Physiol **282,** R1317-R1323.

Milton SL, Lutz PL (2005) Adenosine and ATP-sensitive potassium channels modulate dopamine release in the anoxic turtle (Trachemys scripta) striatum. Am J Physiol Regul Integr Comp Physiol **289,** R77-83.

Milton, S. L., Nayak, G., Lutz, P. L. and Prentice, H. M. (2006) Gene transcription of neuroglobin is upregulated by hypoxia and anoxia in the brain of the anoxia-tolerant turtle Trachemys scripta. J Biomed Sci **13,** 509-514.

Milton S. L., Prentice H.M. (2007) Beyond anoxia: the physiology of metabolic downregulation and recovery in the anoxia-tolerant turtle. Comp. Biochem. Physiol. A Mol. Integr. Physiol **147**, 277-290.

Milton, S. L., Nayak, G., Kesaraju, S., Kara, L. and Prentice, H. M. (2007). Suppression of reactive oxygen species production enhances neuronal survival *in vitro* and *in vivo* in the anoxia-tolerant turtle Trachemys scripta. J Neurochem **101,** 993-1001.

Milton SL, Dirk LJ, Kara LF, Prentice HM (2008) Adenosine modulates ERK1/2, PI3K/Akt, and p38MAPK activation in the brain of the anoxia-tolerant turtle Trachemys scripta. J Cereb Blood Flow Metab **28,** 1469-1477.

Moro, M. A., A. Almeida, et al. (2005). Mitochondrial respiratory chain and free radical generation in stroke. Free Radic Biol Med **39,** 1291-1304.

Nakka, V. P., Gusain, A., Mehta, S. L. and Raghubir, R. (2008) Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. Mol Neurobiol **37,** 7-38.

Narayan, P., R. M. Mentzer, Jr., et al. (2001). Adenosine A1 receptor activation reduces reactive oxygen species and attenuates stunning in ventricular myocytes. J Mol Cell Cardiol **33,** 121-129.

Nedergaard, M. and Dirnagl, U. (2005) Role of glial cells in cerebral ischemia. Glia **50,** 281-286.

Nie, Z., Y. Mei, et al. (1998). Oxidative stress increases A1 adenosine receptor expression by activating nuclear factor kappa B. Mol Pharmacol **53,** 663-669.

Nilsson, G. E. (1990) Long-term anoxia in crucian carp: changes in the levels of amino acid and monoamine neurotransmitters in the brain, catecholamines in chromaffin tissue, and liver glycogen. J Exp Biol **150,** 295-320.

Nilsson G.E., Lutz P.L. (1992) Adenosine release in the turtle brain; a possible mechanism for anoxic survival. J. Exp.Biol **162,** 345-351.

Nilsson, G. E. and Lutz, P. L. (2004) Anoxia tolerant brains. J Cereb Blood Flow Metab **24,** 475-486.

Nita DA, Nita V, Spulber S, Moldovan M, Popa DP, Zagrean AM, Zagrean L (2001) Oxidative damage following cerebral ischemia depends on reperfusion - a biochemical study in rat. J Cell Mol Med **5,** 163-170.

Oechmichen, M., Meissner, C. (2006) Cerebral Hypoxia and Ischemia: The forensic point of view; A review. J.Forensic.Res **51**, 880-887.

Oka, N., Wang, L., Mi, W., Zhu, W., Honjo, O. and Caldarone, C. A. (2008) Cyclosporine A prevents apoptosis-related mitochondrial dysfunction after neonatal cardioplegic arrest. J Thorac Cardiovasc Surg **135,** 123-130.

Ouyang, Y. B. and Giffard, R. G. (2004) Cellular neuroprotective mechanisms in cerebral ischemia: Bcl-2 family proteins and protection of mitochondrial function. Cell Calcium **36,** 303-311.

Park, E. M., T. H. Joh, et al. (2004). A neuroprotective role of extracellular signal-regulated kinase in N-acetyl-O-methyldopamine-treated hippocampal neurons after exposure to *in vitro* and *in vivo* ischemia. Neuroscience **123,** 147-154.

Pearson, G., J. M. English, et al. (2001). ERK5 and ERK2 cooperate to regulate NF-kappaB and cell transformation. J Biol Chem **276,** 7927-7931.

Pek M., and Lutz P.L. (1997) Role for adenosine in "channel arrest" in the anoxic turtle brain. J. Exp. Biol **200,** 1913 -1917.

Pek, M. and Lutz, P. L. (1998) K+ATP channel activation provides transient protection in anoxic turtle brain. Am.J. Physiol **44,** R2023-R2027.

Perez-Pinzon, M. A., Rosenthal, M., Sick, T. J., Lutz, P. L., Pablo, J. and Mash, D. (1992) Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain. Am J Physiol **262,** R712-R715.

Perez-Pinzon MA, Dave KR, Raval AP (2005) Role of reactive oxygen species and protein kinase C in ischemic tolerance in the brain. Antioxid Redox Signal **7,** 1150-7.

Perlman, J. M. (2006). Intervention strategies for neonatal hypoxic-ischemic cerebral injury. Clin Ther **28,** 1353-65.

Prentice HM, Milton SL, Scheurle D, Lutz PL (2004) The upregulation of cognate and inducible heat shock proteins in the anoxic turtle brain. J Cereb Blood Flow Metab **24,** 826-828.

Rami, A. (2003) Ischemic neuronal death in the rat hippocampus: the calpain-calpastatin-caspase hypothesis. Neurobiol Dis **13,** 75-88.

Rami, A., Bechmann, I. and Stehle, J. H. (2008) Exploiting endogenous anti-apoptotic proteins for novel therapeutic strategies in cerebral ischemia. Prog Neurobiol **85,** 273-296.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. J Cell Biol **124,** 1-6.

Ribeiro, J. A. (2005). What can adenosine neuromodulation do for neuroprotection? Curr Drug Targets CNS Neurol Disord **4,** 325-329.

Rudolphi, K. A., Schubert, P., Parkinson, F. E. and Fredholm, B. B. (1992) Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci **13,** 439-445.

Safran, N., V. Shneyvays, et al. (2001). Cardioprotective effects of adenosine A1 and A3 receptor activation during hypoxia in isolated rat cardiac myocytes. Mol Cell Biochem **217,** 143-152.

Saitoh, Y., R. Ouchida, et al. (2003). Bcl-2 prevents hypoxia/reoxygenation-induced cell death through suppressed generation of reactive oxygen species and upregulation of Bcl-2 proteins. J Cell Biochem **90,** 914-924.

Sawe, N., Steinberg, G. and Zhao, H. (2008) Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke. J Neurosci Res **86,** 1659-1669.

Scheid MP, Woodgett JR (2003) Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Lett **546,** 108-112.

Schubert, P., T. Ogata, et al. (1997). Protective mechanisms of adenosine in neurons and glial cells. Ann N Y Acad Sci **825,** 1-10.

Schulte, G., H. Sommerschild, et al. (2004). Adenosine A receptors are necessary for protection of the murine heart by remote, delayed adaptation to ischaemia. Acta Physiol Scand **182,** 133-143.

Schurr A, Reid KH, Tseng MT, West C, Rigor BM (1986) Adaptation of adult brain tissue to anoxia and hypoxia *in vitro*. Brain Res **374,** 244-248.

Sciotti, V. M., F. M. Roche, et al. (1992). Adenosine receptor blockade augments interstitial fluid levels of excitatory amino acids during cerebral ischemia. J Cereb Blood Flow Metab **12,** 646-655.

Scorziello A, Santillo M, Adornetto A, Dell'aversano C, Sirabella R, Damiano S, Canzoniero LM, Renzo GF, Annunziato L (2007) NO-induced neuroprotection in ischemic preconditioning stimulates mitochondrial Mn-SOD activity and expression via Ras/ERK1/2 pathway. J Neurochem **103,** 1472-1480.

Semenza GL (2000) Oxygen-regulated transcription factors and their role in pulmonary disease. Respir Res **1,** 159-162.

Semenza, G. L. (2007) Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE, **2007,** cm8.

Sgambato V, Pages C, Rogard M, Besson MJ, Caboche J (1998) Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J Neurosci **18,** 8814-8825.

Sharikabad M.N., Ostbye K.M., and Brors O., (2004) Effect of hydrogen peroxide on reoxygenation-induced Ca2+ accumulation in rat cardiomyocytes. Free Rad. Biol. Med **37**, 531-8.

Sharpe JC,Arnoult D,Youle RJ. (2004) Control of mitochondrial permeability by Bcl-2 family members: Biochim Biophys Acta **1644,** 107-113.

Sheng H., Enghild J.J., Bowler R., Patel M., Batinic-Haberle I., Calvi C.L., Day B.J., Pearlstein R.D., Crapo J.D., and Warner D.S. (2002) Effects of metalloporphyrin

catalytic antioxidants in experimental brain ischemia. Free Rad. Biol. Med **33,** 947-961.

Shimazaki, K., M. Urabe, et al. (2000). Adeno-associated virus vector-mediated Bcl-2 gene transfer into post-ischemic gerbil brain *in vivo*: prospects for gene therapy of ischemia-induced neuronal death. Gene Ther **7,** 1244-1249.

Shimizu, S., M. Narita, et al. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399,** 483-487.

Siesjo, B. K., Bendek, G., Koide, T., Westerberg, E. and Wieloch, T. (1985) Influence of acidosis on lipid peroxidation in brain tissues *in vitro*. J Cereb Blood Flow Metab **5,** 253-258.

Simon, R. P., Niro, M. and Gwinn, R. (1993) Brain acidosis induced by hypercarbic ventilation attenuates focal ischemic injury. J Pharmacol Exp Ther **267,** 1428-1431.

Siesjo B (1993) [Future treatment of stroke. Brain injury can be reduced with drugs]. Nord Med **108,** 316-318, 329.

Strasser, A., L. O'Connor, et al. (1996). Lessons from Bcl-2 transgenic mice for immunology, cancer biology and cell death research. Behring Inst Mitt **97,** 101-117.

Sun, Y., Jin, K., Mao, X. O., Zhu, Y. and Greenberg, D. A. (2001) Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc. Natl Acad. Sci. U S A **98,** 15306-15311.

Sun, Y., Jin, K., Peel, A., Mao, X. O., Xie, L. and Greenberg, D. A. (2003) Neuroglobin protects the brain from experimental stroke *in vivo*. Proc. Natl Acad. Sci. U S A **100,** 3497-3500.

Tsujimoto, Y. (2003). Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol **195,** 158-167.

Uchino, H., Elmer, E., Uchino, K., Lindvall, O. and Siesjo, B. K. (1995) Cyclosporin A dramatically ameliorates CA1 hippocampal damage following transient forebrain ischaemia in the rat. Acta Physiol Scand **155,** 469-471.

Van Kolen, K., Pullan, S., Neefs, J. M. and Dautzenberg, F. M. (2008) Nociceptive and behavioural sensitisation by protein kinase Cepsilon signalling in the CNS. J Neurochem **104,** 1-13.

Vornov JJ, Thomas AG, Jo D (1996) Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. J Neurochem **67,** 2379-2389.

Wakasugi, K., Nakano, T. and Morishima, I. (2003) Oxidized human neuroglobin acts as a heterotrimeric Galpha protein guanine nucleotide dissociation inhibitor. J Biol Chem **278,** 36505-36512.

Wakasugi, K. and Morishima, I. (2005) Identification of residues in human neuroglobin crucial for Guanine nucleotide dissociation inhibitor activity. Biochemistry **44,** 2943-2948.

Wang H., Cheng E., Brooke S., Chang P. and Sapolsky R. (2003) Over-expression of antioxidant enzymes protects cultured hippocampal and cortical neurons from necrotic insults. J. Neurochem **87,** 1527-1534.

Wang X, Zhu C, Qiu L, Hagberg H, Sandberg M, Blomgren K (2003) Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia. J Neurochem **86,** 351-362.

Wang ZQ, Wu DC, Huang FP, Yang GY (2004) Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia. Brain Res **996,** 55-66.

Wardas, J. (2002) Neuroprotective role of adenosine in the CNS. Pol J Pharmacol, **54,** 313-326.

Watanabe M, Katsura K, Ohsawa I, Mizukoshi G, Takahashi K, Asoh S, Ohta S, Katayama Y (2008) Involvement of mitoKATP channel in protective mechanisms of cerebral ischemic tolerance. Brain Res **1238,** 199-207.

Weber, R. E. and Vinogradov, S. N. (2001) Nonvertebrate hemoglobins: functions and molecular adaptations. Physiol Rev **81,** 569-628.

Webster, K. A., Discher, D. J. and Bishopric, N. H. (1995) Cardioprotection in an *in vitro* model of hypoxic preconditioning. J Mol Cell Cardiol **27,** 453-458.

Wolter, K. G., Y. T. Hsu, et al. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol **139,** 1281-1292.

Xia Z, Dickens M, Raingeaud J, Davis R J, Greenberg M E (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270,** 1326-1331.

Yamaguchi A, Tamatani M, Matsuzaki H, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M (2001) Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. J Biol Chem **276,** 5256-5264.

Yellon, D. M. and Downey, J. M. (2003) Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev **83,** 1113-1151.

Ying, W., Han, S. K., Miller, J. W. and Swanson, R. A. (1999) Acidosis potentiates oxidative neuronal death by multiple mechanisms. J Neurochem **73,** 1549-1556.

Zablocka, B., J. Dluzniewska, et al. (2003). Opposite reaction of ERK and JNK in ischemia vulnerable and resistant regions of hippocampus: involvement of mitochondria. Brain Res Mol Brain Res **110,** 245-252.

Zhang X, Xiong L, Hu W, Zheng Y, Zhu Z, Liu Y, Chen S, Wang X (2004) Preconditioning with prolonged oxygen exposure induces ischemic tolerance in the brain via oxygen free radical formation. Can J Anaesth **51,** 258-263.

Zhao, H., M. A. Yenari, et al. (2003). "Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. J Neurochem **85,** 1026-1036.

Zhuang, S. and Schnellmann, R. G. (2006) A death-promoting role for extracellular signal-regulated kinase. J Pharmacol Exp Ther **319,** 991-997.

Zhu, P. J. and K. Krnjevic (1993). "Adenosine release is a major cause of failure of synaptic transmission during hypoglycaemia in rat hippocampal slices. Neurosci Lett **155,** 128-131.