AKT: IT'S ROLE IN NEURONAL VIABILITY AND PROTECTION AGAINST ISCHEMIA IN THE RAT HIPPOCAMPUS

by

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TABLE OF CONTENTS

ABSTRACT

According to the American Stroke Association, about 700,000 people suffer a new or recurrent stroke each year in the United States. Of these people, approximately 163,000 die, making stroke the third leading cause of death in the U.S., only behind heart disease and cancer. Depending on the area of the brain affected by the stroke, functions such as motor activity, speech, behavior, and/or memory can be hampered. The hippocampus is a bilateral structure that is highly susceptible to hypoxic and/or ischemic insult.

One of the early responses to ischemia is the transient and reversible inhibition of synaptic activity mediated by endogenous adenosine acting on neuronal A_1 receptors. Increase in adenosine during ischemia is thought to play a key prosurvival role by attenuating excitotoxic damage through inhibiting glutamate release and activating Akt.

Akt is activated by PI3K-dependent and PI3K-independent mechanisms. Akt, also known as PKB, has been shown to be both necessary and sufficient to promote cell survival by growth factors *in vitro*. Akt directly phosphorylates multiple proteins resulting in the inhibition of apoptotic and/or necrotic cell death. Bcl-2 and Bcl-xL are two proteins that are disinhibited by the direct Akt phosphorylation of Bad. These two proteins function to maintain mitochondrial integrity during ischemia, thus inhibiting the release of cytochrome c which is a strong inducer of the apoptotic pathway.
This thesis explores the activation mediated by PI3K and the significance of this activation in neuronal survival mechanisms.

LIST OF FIGURES

CHAPTER I

INTRODUCTION

According to the American Stroke Association, about 700,000 people suffer a new or recurrent stroke each year in the United States. Of these people, approximately 163,000 die, making stroke the third leading cause of death in the U.S., only behind heart disease and cancer (American Heart Association 2004). The current treatment of acute ischemic strokes are carried out by early reperfusion and by interference of pathobiochemical cascades that lead to neuronal death (Heiss, Thiel et al. 1999). Reperfusion is achieved through thrombolytic actions that help in clearing obstructive vessel and is most effective when treated within 3 hours of the onset of ischemic symptoms. Neuroprotective strategies have focused on inhibiting the excitotoxic cascade in order to attenuate necrotic and apoptotic cell death (Choi 1998).

An early neuroprotective response to cerebral hypoxia and/or ischemia is a reversible inhibition of neuronal activity (Astrup, Siesjo et al. 1981). Further investigation has revealed that this reversible inhibition is attributed to the effects of endogenous adenosine acting on neuronal A_1 receptors (Fowler 1989; Wu and Saggau 1994; Latini, Bordoni et al. 1998; Gervitz, Lutherer et al. 2001). During this early stage of hypoxia/ischemia, endogenous adenosine levels increase (Fowler 1993; Pearson, Currie et al. 2003). This increase in adenosine is thought to play a protective role through attenuating glutamate release during ischemia (Fowler 1989; Katchman and Hershkowitz

1993; Zhu and Krnjevic 1993; Pearson and Frenguelli 2000) and also by stimulating the activation of Akt, a prosurvival and antiapoptotic enzyme (Gervitz, Nalbant et al. 2002).

Akt can regulate glucose metabolism through its effects on glucose uptake and glycogen synthesis (Kandel and Hay 1999). Akt induces upregulation of glucose transporters (GLUTs), namely GLUT1, GLUT3, and GLUT4, to the plasma membrane allowing greater glucose entry into cells throughout the body and brain (Kohn, Summers et al. 1996; Cong, Chen et al. 1997; Tanti, Grillo et al. 1997; Hajduch, Alessi et al. 1998; Barthel, Okino et al. 1999; Bondy and Cheng 2004; McEwen and Reagan 2004). Akt also phosphorylates glycogen synthase kinase 3β (GSK3β) causing inactivation of GSK3β. The inactivation of GSK3β relieves its inhibitory phosphorylating activity on glycogen synthase, thus promoting glycogen synthesis (Bondy and Cheng 2004).

Akt has been shown to play a critical role in neuronal cell survival (Dudek, Datta et al. 1997). The activation of Akt is induced by a variety of stimuli with the majority being PI3K-dependent (Datta, Brunet et al. 1999). Activated Akt phosphorylates multiple proteins that regulate apoptosis. For instance, the phosphorylation of Bad, caspase 9, and the Forkhead transcription factor FKHRL1 by Akt elicits an inhibition of the apoptotic cascade. Bcl-2 and Bcl-X_L, both cell survival promoting proteins, are also downstream targets of Akt, which have been shown to inhibit both necrotic (Kane, Ord et al. 1995) and apoptotic (Martinou, Dubois-Dauphin et al. 1994; Kluck, Bossy-Wetzel et al. 1997) pathways. Due to the vast number of regulatory processes that Akt performs, we chose to investigate the role of Akt in maintenance of neuronal cell viability under normal physiological conditions and its importance in survival against ischemic insult.

In summary, the conducted experiments attempted to probe the significance of the PI3K/Akt pathway in neuroprotection. The following experiments were designed to examine the activation of Akt mediated by PI3K and the importance of this activation in neuronal survival to ischemic-like conditions.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Stroke and the Relevance of Hippocampal Studies

Stroke is defined as an acute neurological deficit resulting from impaired cerebral blood supply. Strokes occur when the delivery of oxygen and nutrients to the brain is impeded by an occlusion (ischemic stroke) or rupture (hemorrhagic stroke) of a blood vessel. Ischemic strokes make up 88% of all strokes and 15-30% of those afflicted die within the first 30 days (Stedman 2000; American-Heart-Association 2004). Among the stroke survivors, there exists a broad range of debilitated functions. Depending on the area of the brain affected by the stroke, functions such as motor activity, speech, senses, behavior, and/or memory can be hampered. Memory formation has been shown to be impaired by injuries to the hippocampus brought on by ischemic insult (Zola-Morgan, Squire et al. 1992; Blumenfeld 2002).

It has been shown that the hippocampus has a selective vulnerability to ischemic insults (Kirino and Sano 1984; Schmidt-Kastner and Freund 1991). During an ischemic event, the hippocampus is one of the first regions to become electrically silent (Sugar 1938). Due to this high susceptibility to ischemia and its important role in memory formation, the hippocampus has been extensively studied and much understanding of the cytoarchitecture has been revealed.

The Hippocampus: Structure and Function

The hippocampal formation is an archeocortical gyrus that folds inward upon itself. It is located in the medial temporal lobe and consists of the dentate gyrus, subiculum, and hippocampus. Information enters the hippocampus from the projections of the pyramidal cells of the entorhinal cortex and subiculum via the perforant pathway and the alvear pathway. Through the perforant pathway, axons from the entorhinal cortex synapse on the granule cell layer of the dentate gyrus and the CA4 (CA for cornu Ammonis) region of the hippocampus. The axons of these granule cells, also called mossy fibers, synapse on the dendrites of CA3 pyramidal cells. Axons of the CA3 pyramidal cells exit the hippocampal formation through the fornix and send information to the hypothalamus and mammillary bodies. Other axons of the CA3 pyramidal cells form the Schaffer collaterals which synapse on apical dendrites of the CA1 pyramidal cells. The CA1 pyramidal cells send axons that exit the hippocampus through the alveus to send information to areas such as hypothalamus, mammillary bodies, and back to the subiculum and entorhinal cortex, which relay it back to the sensory cortex (Blumenfeld 2002). This relatively simple cytoarchitecture allows for detailed electrophysiological examination of synaptic transmission and hippocampal function.

The initial investigations into the functional role of the hippocampus were performed in the early 19th century. These investigations involved the examination of psychiatric patients suffering from different forms of temporal lobe epilepsy. It was not until the early 1950's that the role of the hippocampus was associated with memory formation.

In 1953, a 27-year-old male with the initials H.M. underwent a bilateral resection of his medial temporal lobes, including the hippocampal formation and parahippocampal gyri, in order to control his epileptic seizures. The surgery improved his seizures but left him with severe memory problems. His memories of events prior to the surgery and during childhood were still intact. However, he was unable to learn new facts or recall experiences that occurred after the surgery (Blumenfeld 2002). Studies of patient H.M., along with numerous other studies, have implicated the hippocampus as having a major role in short-term memory.

Homeostasis

The neuron, like any other cell, functions in a narrow range of physiological parameters. Under these normal physiological conditions, the cell maintains a steady state termed homeostasis. It is further able to withstand changes in these conditions by altering this steady state to maintain viability and function of the cell. However, this adaptive response has a limited range of effectiveness. When this range is surpassed, cell injury or death can occur. Many factors contribute to maintaining this homeostatic state in the neuron. Some trophic factors such as insulin-like growth factor 1 (IGF-1) stimulate mechanisms that are essential for cell viability and survival (Zheng and Quirion 2004). IGF-1 is a polypeptide that shares structural identity and amino acid composition with insulin and is highly expressed in the brain (Bondy and Cheng 2004). IGF-1 acts in an autocrine and/or paracrine manner to promote glucose utilization (Cheng, Reinhardt et al. 2000). This glucose utilization is mediated by the phosphatidylinositol 3 kinase

(PI3K)/Akt pathway (Bondy and Cheng 2004). The translocation of GLUT4 to the plasma membrane of both neurons and astrocytes is thought to facilitate this increase in glucose utilization (Cheng, Reinhardt et al. 2000; Bondy and Cheng 2004; McEwen and Reagan 2004). Also during IGF-1 stimulation, GSK3β is inactivated through direct phosphorylation by Akt. This inactivation relieves inhibition of glycogen synthase, thus promoting glycogen synthesis (Alessi and Cohen 1998; Cheng, Reinhardt et al. 2000; Bondy and Cheng 2004). Insulin, which is predominantly secreted by the pancreas, acts in much of the same manner as IGF-1 showing much cross-reactivity with IGF-1 receptors (Bondy and Cheng 2004). However, little circulating insulin crosses the blood-brain barrier and very little is synthesized in the brain; therefore, more of the trophic effects in the brain are due to IGF-1 than to insulin (Cheng, Reinhardt et al. 2000).

Necrosis

When homeostatic conditions are compromised by an injurious agent or stress, cell injury occurs. If the injurious stimulus is great enough or persists long enough, the injury can lead to irreversible damage eventually ending in cell death. Necrotic cell death is characterized by cellular and mitochondrial swelling, dilatation of the endoplasmic reticulum, chromatin clumping into poorly defined masses, and extensive vacuolization of the cytoplasm and is an ATP independent process (Yamashima 2000; Syntichaki and Tavernarakis 2003). Excessive increases in cytosolic Ca^{2+}, which can occur in acute ischemia, have been proposed to be a trigger for necrotic cell death (Yamashima 2000; Syntichaki and Tavernarakis 2003; Yuan, Lipinski et al. 2003). Recently suggested

mechanisms are thought to be mediated through the activation of calpains and aspartyl proteases by elevated intracellular Ca^{2+} (Yuan, Lipinski et al. 2003). Calpain can further cause membrane instability and leakage of lysosomal enzymes. These cells swell and eventually lyse, releasing their cellular contents into the extracellular space. This release of cellular contents often causes damage to neighboring cells and induces inflammation (Majno and Joris 1995; Yamashima 2000).

Apoptotic Cascade

Apoptosis, first introduced in 1972 (Kerr, Wyllie et al. 1972), is an inherent ATP dependent program for cell death that can be stimulated both intrinsically and extrinsically. A cell undergoing apoptosis can be characterized by membrane blebbing, aggregation of highly condensed chromatin at the nuclear membrane, shrinkage of the cytoplasm, and prelytic DNA fragmentation, activation of the Fas death-inducing ligand/receptor system, release of cytochrome c from mitochodria, and caspase 3 activation (Snider, Gottron et al. 1999). Apoptotic cells gradually form smaller fragments and are phagocytosed by neighboring cells or macrophages without releasing their contents into their surroundings, unlike necrosis.

The apoptotic pathway can be initiated by various factors that result in the activation of effector caspases which are involved in protein degradation. One such factor is cytochrome c, which is a key protein in the electron transport chain. Leakage of cytochrome c from the mitochondria occurs through the permeability transition pore complex (PTPC). This pore has been speculated to be composed of inner and outer

mitochondrial membrane proteins, adenine nucleotide transporter (ANT) and the voltage-dependent anion channel (VDAC) respectively (Green and Reed 1998). Cytosolic cytochrome c forms a complex with the apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 (Li, Nijhawan et al. 1997). This complex triggers the activation of procaspase-9 to caspase-9, which is an initiator caspase that activates the caspase cascade.

Bcl-2 is an anti-apoptotic protein found on the surface of organelles such as the endoplasmic reticulum, nucleus, and mitochondria. This protein has been found to inhibit the formation of the PTPC by maintaining the mitochondrial membrane potential ($\Delta\psi$) (Shimizu, Eguchi et al. 1998). During apoptotic signaling, there is an upregulation of Bcl-associated dimer (Bad) and Bcl-associated X protein (Bax). These pro-apoptotic proteins function by binding and thus inactivating bcl-2 and bcl-x_L.

Apoptosis and Necrosis in Cerebrovascular Injury

Stroke or cerebrovascular accident occurs with disturbances of the cerebral blood supply. An extensive decrease in blood supply to a region can produce an ischemic environment that can lead to neuronal death. Neurons may undergo both apoptosis and necrosis after such an ischemic insult. More severe insults favor the necrotic pathway while the less severe insults favor the apoptotic pathway.

The "core" ischemic region is defined by blood flow that is reduced to less than 15% of normal (Tamura, Graham et al. 1981; Nedergaard, Gjedde et al. 1986). Neurons in this area rapidly lose electrical activity , ATP falls to less than 25% of basal levels, and

undergo an ischemic depolarization within minutes of the insult (Folbergrova, Memezawa et al. 1992; Sun, Zhang et al. 1995). This ischemic core mainly undergoes necrotic cell death due to the enormous intracellular calcium overload and Na^+/K^+ - pump inactivation caused by ATP depletion (reviewed in (Lipton 1999). This core death can be observed in neurons by the early mitochondrial swelling and compromise of the cytoplasmic membrane with preservation of the nuclear membranes which are classical features of necrotic cell death.

The surrounding tissue that has a reduced blood flow of less than 40% of basal levels is termed the penumbra (Back, Zhao et al. 1995). The events in the penumbra are less drastic as compared to the core but without intervention still result in cell death. The penumbra becomes electrically inactive similar to the core but differs ionically and metabolically. The ATP in this region drops to 50-70% of basal levels, which prevents the anoxic depolarization (Nedergaard, Gjedde et al. 1986; Back, Zhao et al. 1995). The neurons in this region largely undergo delayed cell death showing characteristics of both necrotic and apoptotic cell death. The apoptotic and necrotic pathways can function in parallel with one another in neurons exposed to ischemia as shown in Figure 1.

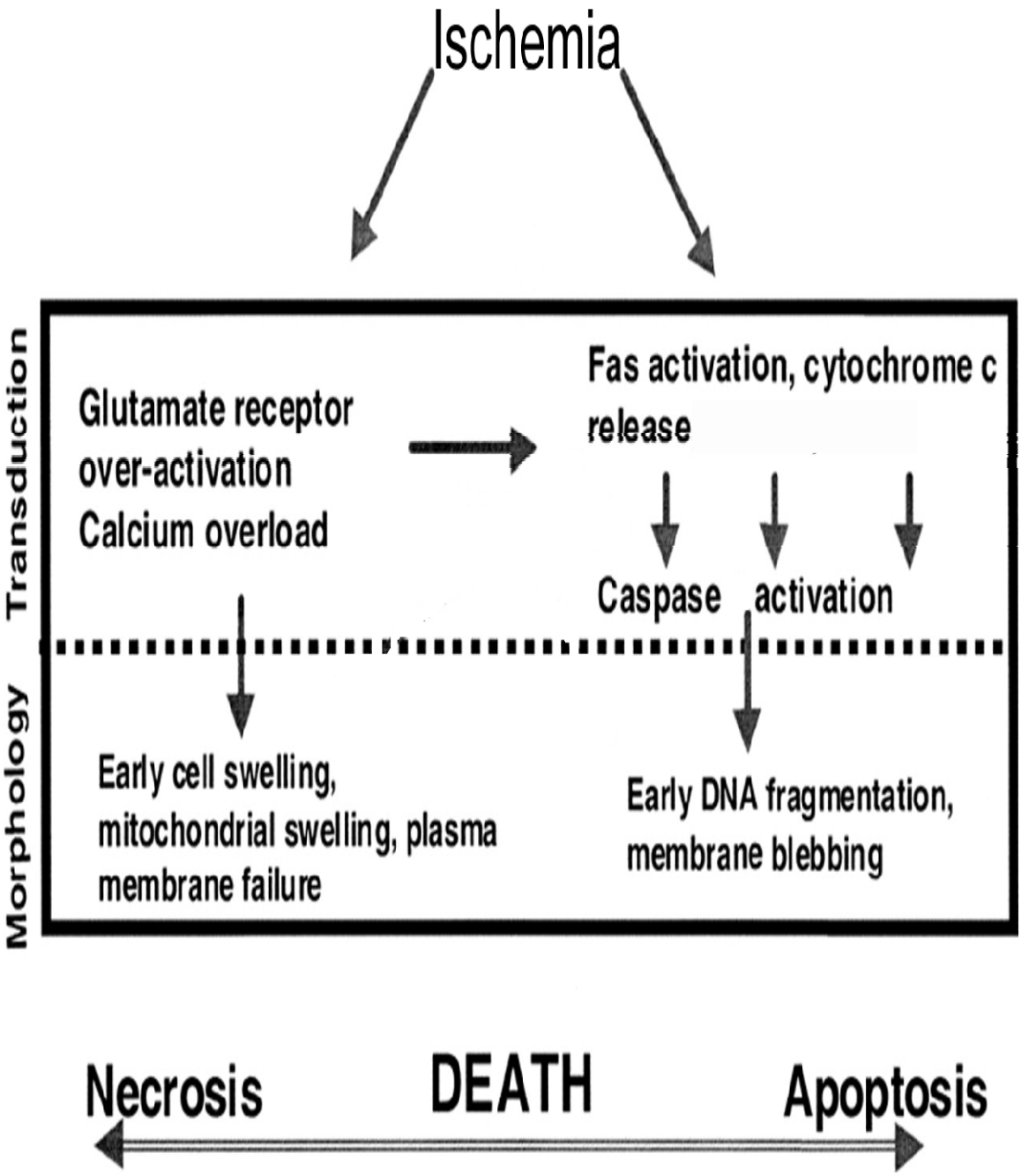

Figure 1. Schematic of cell death pathways that may function in parallel in ischemic neurons. Adapted from (Snider, Gottron et al. 1999).

Anoxic/Ischemic Depolarization

In particular, the hippocampus has been investigated in many stroke models to further understand the mechanisms of cell death and survival during ischemia due to its selective vulnerability to neuronal degeneration. Anoxic depolarization (AD) or ischemic depolarization (ID) is a complex phenomenon that results from a severe prolonged anoxic/ischemic exposure. The resultant depolarization is characterized by a severe acute negative shift in extracellular potential and a drastic redistribution of ions between the intracellular and extracellular compartments. This redistribution is caused by a radical increase in ion permeability which allows a large influx of Na^+, Ca^{2+}, and Cl^- ions and a large efflux of K^+ ions, hence causing the net negative shift in extracellular potential (Somjen 2001). This depolarization has been shown to also cause neuronal cell swelling and shrinkage of the extracellular space (Hansen and Olsen 1980). Anoxic/ischemic depolarization has been proposed as a factor causing neuronal cell death due to the massive influx of Ca^{2+} at the time of depolarization (Balestrino and Somjen 1986). Brain slices experiencing short durations of AD still recover neuronal function whereas prolonged depolarizations cause an irreversible loss of neuronal cell function (Balestrino and Somjen 1986; Kawasaki, Czeh et al. 1988; Balestrino, Aitken et al. 1989; Somjen, Aitken et al. 1990). The time to depolarization has been prolonged by using drugs to reduce the influx of Na^+ during hypoxia/ischemia. Tetrodotoxin (TTX), a voltage-gated sodium channel blocker, caused a postponement in the time to depolarization (Somjen 2001). There is also speculation that the rapid depolarization is correlated with the depletion of ATP and hence the function of the Na^+/K^+ - ATPase. Conditions that have

shown to slow the rate of ATP depletion such as hyperglycemia, creatine preincubation, and hypothermia all increase the time to depolarization. Ouabain, a specific inhibitor of the Na^+/K^+ pump, leads to a depolarization that resembles an anoxic depolarization (reviewed in (Lipton 1999).

The Role of Adenosine in Neuroprotection

When the brain is unable to synthesize enough ATP to meet the energy demands of the cell, there results an accumulation of adenosine (Dunwiddie and Masino 2001). Adenosine has been shown to increase significantly in the extracellular space of the rat hippocampal slice within 8 minutes of an ischemic insult and up to 4-5 fold following an anoxic depolarization (Fowler 1993). Adenosine is a purine nucleoside that acts as a powerful modulator of neuronal activity. This nucleoside has been shown to provide protection from an ischemic insult (Sweeney 1997).

One of the early responses to hypoxic/ischemic conditions is the reversible depression of synaptic transmission. This has been shown to be mediated by the action of adenosine on central A_1 receptors (Dunwiddie 1985; Fowler 1989; Fowler 1990; Gervitz, Lutherer et al. 2001). The activation of A_1 receptors reduces calcium influx by inhibiting voltage-gated Ca^{2+} channels on the presynaptic terminal (Wu and Saggau 1994). This inhibition of the Ca^{2+} influx into the presynaptic terminal is thought to attenuate the release of glutamate from the presynaptic terminal, thus inhibiting synaptic transmission. This action of adenosine is thought to play a protective role by decreasing the energy demand of the neuron during compromised energy production (Fowler 1989).

The activation of A_1 receptors contributes to the inhibition of synaptic transmission by also stimulating the opening of sulfonylurea-sensitive ATP-sensitive K^+ (K_{ATP}) channels. The opening of these K_{ATP} channels causes a hyperpolarization of the neurons. This hyperpolarization is thought to inhibit glutamate release from the presynaptic neuron and attenuate the net depolarization of the postsynaptic neuron, thus decreasing glutamate excitotoxicity (Fredholm and Dunwiddie 1988).

Recent work has also shown another possible mechanism of neuroprotection mediated by the activation of A_1 receptors. The activated form of protein kinase B (PKB), also known as Akt, has been shown to be elevated through an A_1 receptor mediated pathway both *in vivo* and *in vitro* (Gervitz, Nalbant et al. 2002). This finding illustrates another possible protective role of adenosine because Akt is part of an endogenous pathway that exerts neuroprotection from both necrotic and apoptotic cell death (Crowder and Freeman 1998; Datta, Brunet et al. 1999; Gervitz, Nalbant et al. 2002; Taylor, Ali et al. 2005).

Upstream Regulation of Akt/PKB

There are three known isoforms of Akt/PKB in mammalian cells: Akt1(PKBα), Akt2(PKBβ), and Akt3(PKBγ) (Datta, Brunet et al. 1999). These three isoforms have a greater than 85% sequence identity and are ubiquitously expressed in mammals with the Akt1/PKBα isoform being predominant in most tissues including the brain. All three contain an amino-terminal pleckstrin homology (PH) domain, a catalytic domain, and a carboxy-terminal regulatory domain. The phosphorylation of the Thr^{308} and Ser^{473}

residues are critical for the full activation of Akt. Akt has been shown to be activated through phosphatidylinositol 3-OH (PI3K)-dependent and PI3K-independent mechanisms (Datta, Brunet et al. 1999).

The PI3K-dependent pathway is triggered upstream by growth and survival factors. PI3K is a heterodimeric enzyme activated through the binding of its regulatory subunit to phosphotyrosine residues typically located on tyrosine kinase membrane receptors. Once activated, the catalytic subunit of PI3K phosphorylates phosphoinositides at the 3-position of the inositol ring resulting in the formation of phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3). PI(3,4)P2 and PI(3,4,5)P3 bind with high affinity to the PH domain of Akt and induce a conformational change of Akt which recruits it to the plasma membrane and exposes it to phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) at the Thr^{308} residue and subsequent phosphorylation by 3-phosphoinositide-dependent kinase 2 (PDK2) at the Ser^{473} residue. After phosphorylation of both residues, Akt becomes fully activated, detaches from the plasma membrane, and proceeds to the cytosol and nucleus where its function takes place (reviewed in (Coffer, Jin et al. 1998; Datta, Brunet et al. 1999; Kandel and Hay 1999).

Two of the known PI3K-independent pathways of activation of Akt are mediated by cAMP and intracellular Ca^{2+}. Elevated intracellular cAMP can activate Akt via protein kinase A (PKA) in the presence of PI3K inhibitors (Filippa, Sable et al. 1999). Moderate increases in intracellular Ca^{2+} stimulate the activation of Akt through a

calmodulin-dependent pathway that is independent of PI3K. Ca^{2+}/calmodulin-dependent kinase kinase (CaM-KK) directly phosphorylates Akt causing activation (Yano, Tokumitsu et al. 1998).

Downstream Akt/PKB Effectors Involved in Neuroprotection

Serine/threonine kinase Akt/PKB mediates the activity of many proteins associated with cell survival and growth as illustrated in Figure 2. Akt has been shown to be both necessary and sufficient to promote cell survival by growth factors *in vitro* (Crowder and Freeman 1998; Hayashi, Abe et al. 1998). The promotion of cell survival occurs by phosphorylation of components of the apoptotic cascade. Akt directly phosphorylates Bad, caspase 9, and the Forkhead family of transcription factors, all of which promote cell death.

Bad is a pro-apoptotic protein which forms heterodimers with bcl-2 and bcl-x_L, thus causing the inactivation of these prosurvival proteins. Akt directly phosphorylates the Ser^{136} residue of Bad. Phosphorylation of the Ser^{136} residue causes Bad to dissociate from bcl-2 and bcl-x_L and instead bind to 14-3-3 proteins. This sequestration of Bad enables bcl-2 and bcl-x_L to perform their prosurvival functions (Datta, Dudek et al. 1997). Bcl-2 has been shown to prevent the translocation of cytochrome c from the mitochondria into the cytosol (Kluck, Bossy-Wetzel et al. 1997). One of the methods utilized by bcl-2 that prevents the mitochondrial release of cytochrome c is preventing the formation of the PTPC. The formation of the PTPC is thought to be prevented by the maintenance of the mitochondrial membrane potential by enhancing H^+ efflux in the

presence of Δψ-loss-inducing stimuli such as mitochondrial Ca^{2+} influx (Shimizu, Eguchi et al. 1998). Regarding Ca^{2+} influx into the mitochondria, bcl-2 has also been shown to potentiate the maximal calcium uptake capacity of neural cell mitochondria. This potentiation may help protect against glutamate excitotoxicity (Murphy, Bredesen et al. 1996). Bcl-xL enhances cell survival by maintaining ATP/ADP exchange at the mitochondrial membrane during a low rate of cellular metabolism as illustrated in Figure 3. Inaccessibility of ADP to the mitochondrial matrix prevents the utilization of the H+ gradient created by the electron transport chain. The resulting hyperpolarization of the inner mitochondrial membrane and matrix swelling leads to loss of outer membrane integrity and release of cytochrome c into the cytosol (Vander Heiden, Chandel et al. 1999).

Akt can also block cell death even after mitochondrial cytochrome c release by phosphorylating caspase 9 at the Ser^{196} residue. This inhibition of caspase 9 may involve the phosphorylation at an allosteric site which prevents subunit dimerization or alters the catalytic domain through conformational change (Cardone, Roy et al. 1998).

The phosphorylation of Forkhead-like protein 1 (FKHRL1) by Akt leads to FKHRL1 assocation with 14-3-3 protein. The binding of 14-3-3 protein to FKHRL1 sequesters FKHRL1 in the cytoplasm so that it is unable to mediate transcription in the nucleus of apoptotic factors such as Fas ligand (Brunet, Bonni et al. 1999).

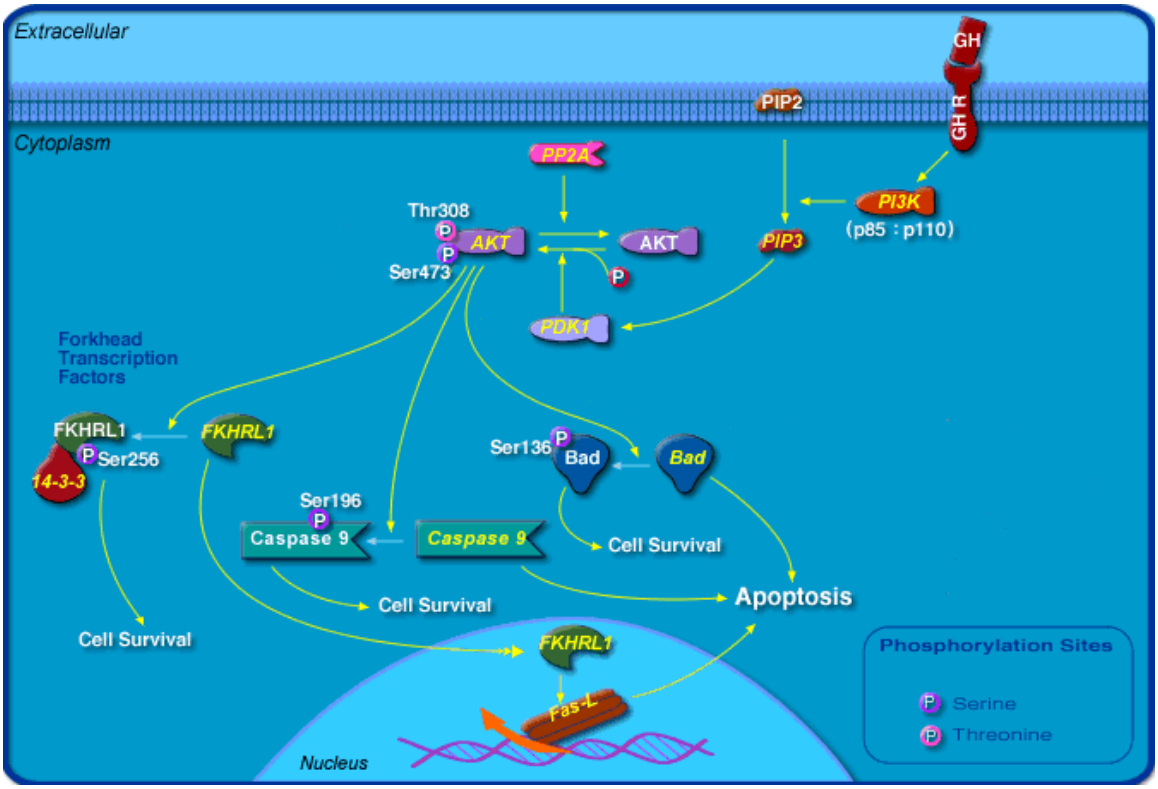

Figure 2. Schematic representation of Akt signaling pathway. Adapted from http://www.biocarta.com/pathfiles/h_aktPathway.gif.

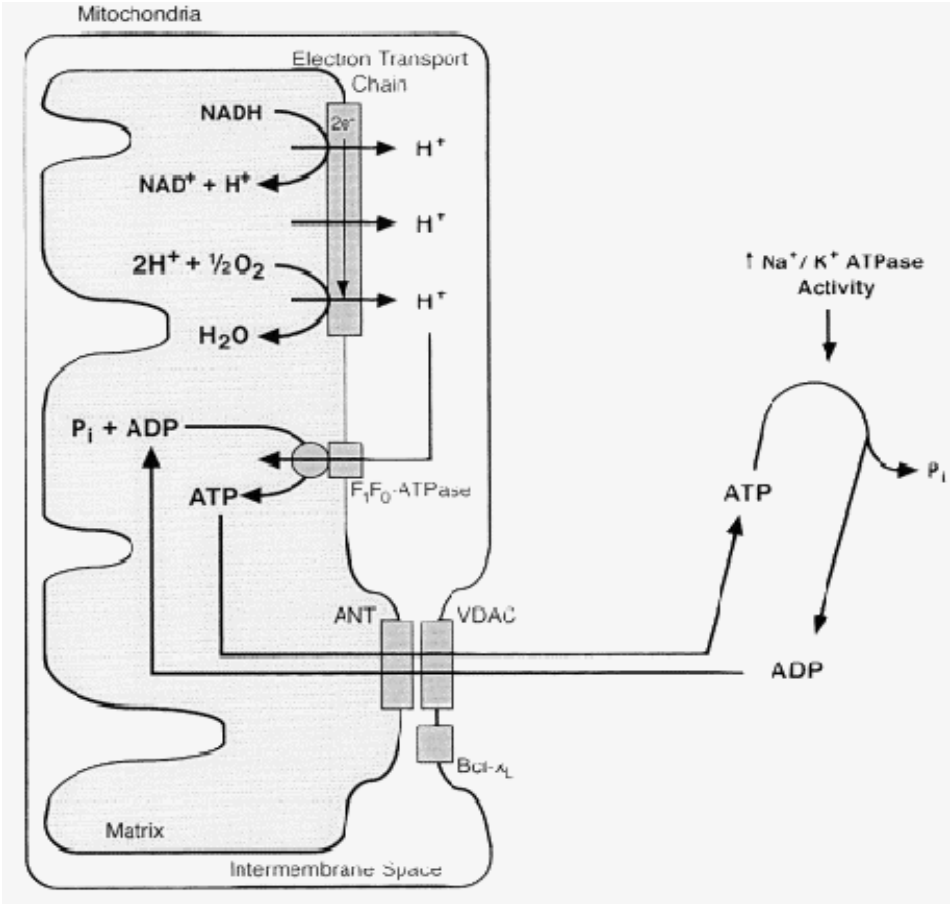

Figure 3. Schematic representation of the mitochondrial pathway for ADP utilization to generate ATP. Adapted from (Vander Heiden, Chandel et al. 1999).

CHAPTER III

MATERIALS AND METHODS

Hippocampal Slice Preparation

Transverse hippocampal slices were prepared from male Fisher 344 rats aged 6-10 weeks. Following decapitation, the dorsal hippocampi were immediately isolated, rinsed in ice-cold artificial cerebrospinal fluid (aCSF) and placed on a mechanical tissue slicer (Mickle Laboratory Engineering Co., London). Slices, 400 μm in thickness, were cut and then incubated for at least 1 hour in a static chamber containing heated (33 ± 0.5°C) aCSF composed of (mM): NaCl 124, KCl 5.9, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 25.6, glucose 10; equilibrated with 95% O_2 / 5% CO_2.

Drug Incubation

Wortmannin, a specific phosphatidylinositol 3-kinase (PI_3-K) inhibitor, was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 200 μM. Hippocampal slices, being exposed to wortmannin, were incubated immediately following isolation from the whole rat brain for at least one hour in a static chamber containing heated (33 ± 0.5°C) aCSF with wortmannin (200 nM or 2 μM). Final DMSO concentrations have previously been shown to have no significant effect when compared to control slices (Fowler 1989). Wortmannin and DMSO were both purchased from Sigma-Aldrich (St. Louis, MO).

Protein Extraction

Hippocampal tissue slice samples for analysis of Akt activation were immediately frozen on dry ice to prevent protein dephosphorylation from occurring after tissue isolation (Ouyang, Tan et al. 1999). Tissue homogenization was performed according to the method described by Ouyang (Ouyang, Tan et al. 1999). Hippocampal slice preparations were homogenized using a Dounce homogenizer (12-15 strokes) in 10 volumes of homogenization buffer (15 mmol/L Tris base/HCL, pH 7.6, 1 mmol/L DTT, 0.25 mol/L sucrose, 1 mmol/L $MgCl_2$, 1.25 μg/ml pepstatin A, 10μg/ml leupetin, 2.5 μg/ml aproptonin, 0.5 mmol/L PMSF, 2 mmol/L ethylenediamine tetra-acetic acid (EDTA), 1 mmol/L ethyleneglycol tetra-acetic acid (EGTA), 0.1 mol/L Na_3VO_4, 50 mmol/L NaF, and 2 mmol/L sodium pyrophosphate). The homogenates were then centrifuged at 1000 x g for 10 minutes at 4°C. The pellets were discarded and the protein concentration of the supernatant was determined by the Bradford method using optical density.

Western Blot Analysis

Western blot analysis was carried out on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method. Equal amounts of protein were loaded into each lane. After electrophoresis, proteins were transferred to an immobilon-P membrane (Millipore). Following the transfer, membranes were blocked with bovine serum albumin in non-fat dry milk for at least one hour. After blocking, membranes were incubated overnight at 4°C with primary antibodies against phospho-

Akt at a dilution of 1:1000 (Cell Signaling Inc, Beverly, MA). Membranes were then washed four times, 15 minutes each in duration, using phosphate buffered saline with tween 20 (PBS-T) and then incubated for one hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. The membranes were then washed another four times as before with PBS-T. Protein blots were developed on X-ray film using Super Signal chemilluminescent substrate marker (Pierce, Rockford, IL). After capturing the bands on X-ray film, membranes were stripped using 0.1M glycine (pH 2.6) at room temperature for 30 minutes, so they could be re-probed with primary antibodies against total Akt. After stripping, the membranes were again washed three times, 15 minutes each, using PBS-T and then incubated overnight at 4°C with primary antibodies against Akt. The antibody against Akt does not distinguish between the phosphorylated and unphosphorylated forms, whereas the phospho-specific antibody detects phosphorylation of Akt at Ser-473. The density of each protein band was evaluated using the Visage 2000 optical densitometer camera and Visage 2000 analysis software (Genomic Solution, Inc. Ann Arbor, MI).

Immunohistochemistry

The 400 μm slices were individually submerged in a 1.5 ml microfuge tube containing ice-cold fixative solution composed of 4% paraformaldehyde in phosphate buffered saline (PBS) with picric acid and maintained at 4°C for 48 hours. The fixative solution was then replaced with 30% sucrose PBS and kept at 4°C for at least 24 hours. The 400 μm slices were then sectioned with a vibratome (UltraPro 5000 Cryostat, St.

Louis, MO) at a thickness of 40 μm and placed in PBS. Tissue sections were then washed 3 times, for 5 min each in duration, with tris buffered saline (TBS) containing 0.1% Triton X-100 (TBS/Triton). Sections were then treated with 1% H_2O_2 TBS for 30 minutes at room temperature. After incubation, the sections were then washed 3 times, 30 min each, with TBS/Triton at room temperature. The nonspecific binding sites were then blocked by incubating the sections with 3% Normal Goat Serum (NGS; purchased from Vector), in TBS/Triton for 1 hour. After blocking, the sections were incubated with the primary antibody against phospho-Akt at a dilution of (1:200) in 3% NGS in TBS/Triton overnight at 4°C. Sections were washed 3 times, 10 min each, with TBS/Triton and then incubated in biotinylated anti-rabbit secondary antibody at a dilution of (1:200) in 1% NGS in TBS/Triton for 1 hour at room temperature. After the one hour incubation, sections were then washed 3 times, 10 min each, in TBS/Triton. The sections were then incubated in avidin-biotin-peroxidase complex (ABC) reagent (using the ABC Elite Kit purchased from Vector) for 1 hour. Sections were then washed 3 more times, 10 min each, in TBS/Triton. Following these 3 washes, the sections were incubated in the 3,3'- diaminobenzidine (DAB) reagent (purchased from Vector) until the desired staining was achieved. To prevent further staining, the sections were washed 3 times, for 5 min each, in TBS. For viewing and storage purposes, the sections were mounted on gelatin coated glass microscope slides. The intensity of staining was determined by the optical density in three random areas of the pyramidal cell layer of CA1 using ImageTool 3.0 software (acquired as freeware from UTHSCSA, San Antonio, TX).

Electrophysiology

For recording, the slices were transferred to a smaller chamber (0.5 ml) and superfused with oxygenated (95% O_2 / 5% CO_2) aCSF (33°C) at a constant flow rate of 2 ml/min. The stimulating electrode, made from a pair of twisted, insulated nichrome wires, was placed in the Schaeffer collateral fibers of the slice (Figure 4). Stimulation was applied at an intensity between 1 – 10 V, with a 140 μsec pulse at 10 second intervals. Capillary glass microelectrodes were fashioned from heat-pulled (Sutter Instrument Corp., Novato, CA) 1.5 mm borosilicate glass micropipettes (WPI) and filled with 2 M solution of sodium chloride. A silver chloride wire was inserted into the recording electrode and led to a WPI DAM 50 differential DC amplifier. Evoked field excitatory postsynaptic potentials (fEPSPs) and presynaptic fiber volleys were recorded in stratum radiatum of the CA1 region (Figure 4). Population spikes and presynaptic fiber volleys were recorded in the pyramidal cell layer of the CA1 region (Figures 4 and 5). Stimulation, recording, and measurement of the amplitude of the population spike were controlled by A/Dvance software (McKellar Designs, U. British Columbia, Vancouver, BC) through an ITC computer interface (Instrutech Corp., Long Island, NY) and recorded by a Macintosh 7100 Power PC computer (Apple Computer Inc., Cupertino, CA). The anoxic/ischemic depolarization was characterized as a large voltage transient recorded by a chart recorder (Linseis Inc., Princeton, NJ).

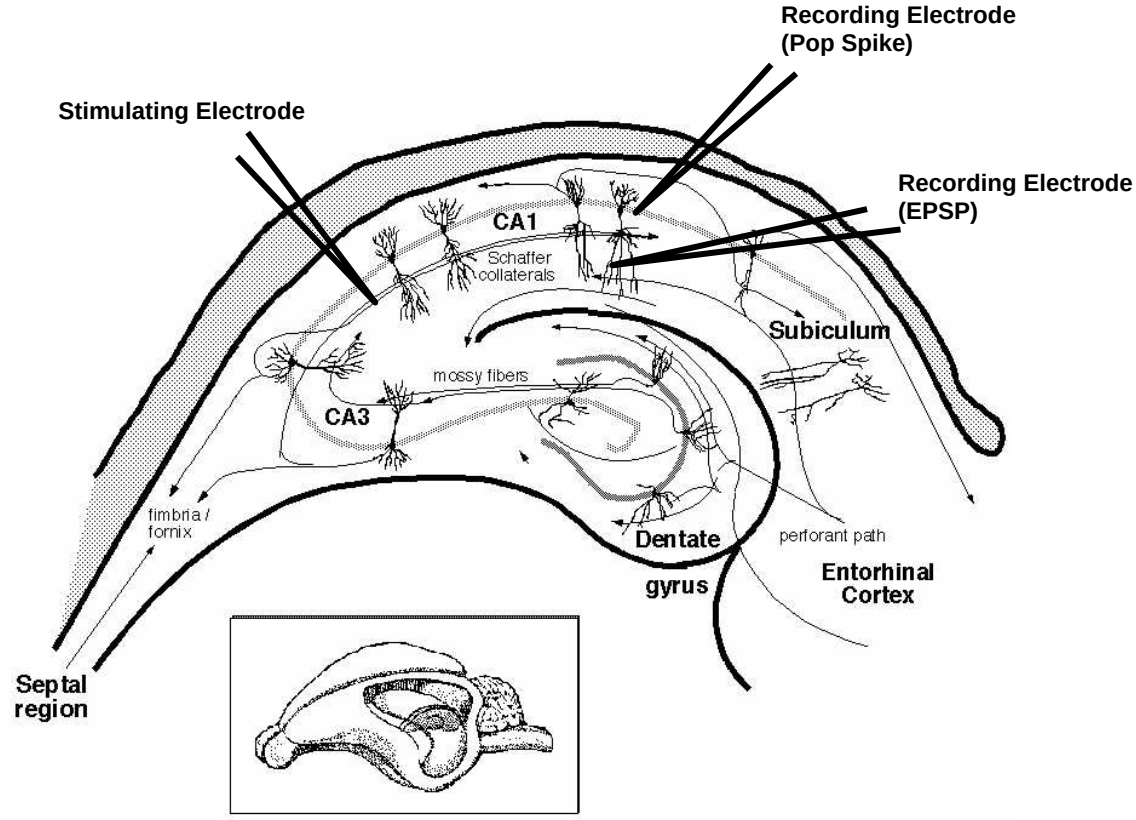

Figure 4. Rat hippocampal slice circuitry and placements of stimulus and recording electrodes for electrophysiological recordings. Adapted from Aging & Plasticity (**http://www.utdallas.edu/~tres/integ**/pla2/16_01.jpg).

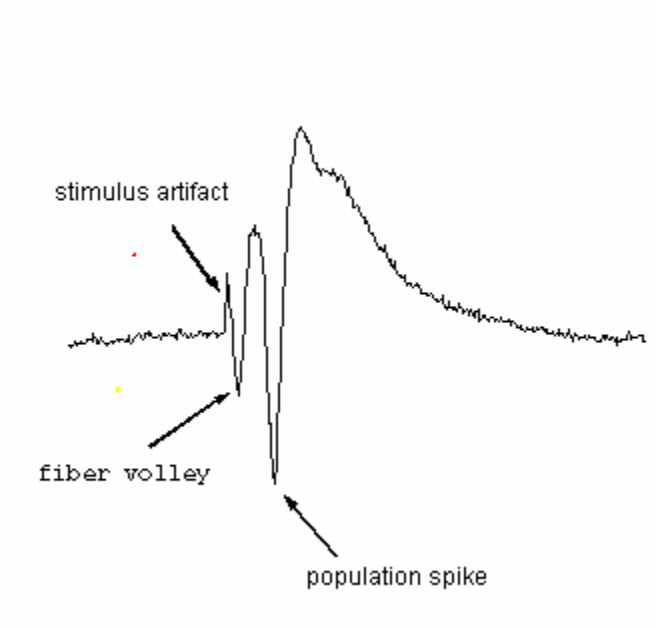

Figure 5. Representative trace of a population spike recording from the pyramidal cell layer. Adapted from Molecular Neurophysiology. (http://www.biocenter.helsinki.fi/bi/physiol/GABA/Experiments.htm).

Hypoxia/Ishemia Protocol

Multiple reservoirs were used to hold differing aCSF solutions. One reservoir contained control aCSF composed of (mM): NaCl 124, KCl 5.9, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 25.6, glucose 10; continuously equilibrated with 95% O_2 / 5% CO_2 gas. Another reservoir contained oxygen-glucose deprived (OGD) aCSF composed of (mM): NaCl 124, KCl 5.9, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 25.6, glucose 0; continuously equilibrated with 95% N_2 / 5% CO_2 gas. Slices were made ischemic by switching the solutions from the control aCSF to the oxygen-glucose deprived aCSF. In the experiments exposing the slices to wortmannin, the appropriate amount of stock wortmannin solution was mixed in with the perfusate. The slices were exposed to the various perfusates for different time durations depending on the hypothesis being tested. The rate of perfusion of the solutions was set at 2 ml/min and took about 90 seconds to reach the slice bath.

Statistical Analysis

Data were analyzed using a Student t-test for comparison between two groups (significance= $p<0.05$). In addition data were analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey post hoc test for comparisons among means of more than two groups (significance= $p<0.05$). All statistical analyses were conducted using Sigma-Stat analysis software. Fitted curves and linear regressions were calculated using Microsoft Excel software.

CHAPTER IV

RESULTS

Hypothesis Overview

The following experiments were performed to determine the magnitude of constitutive phosphorylation of Akt and the role of this activated enzyme in neuronal viability under normal physiological conditions. Also, early protection by Akt from environmental stressors such as ischemia was investigated in the adult Fischer-344 rat hippocampus *in vitro*. In particular, this chapter includes experimental data testing the following three hypotheses: (1) There exists a constitutive activation of Akt *in vitro* mediated by PI3 kinase, (2) Inhibition of the constitutive activation of Akt impairs neuronal cell viability, (3) Inhibition of the phosphorylation of Akt lessens the effectiveness of neuronal survival pathways during ischemia. Each hypothesis with its results will be presented in detail as a subsection with corresponding figures of experimental data concluding each section.

Hypothesis I: There exists a constitutive activation of Akt in vitro mediated by PI_3 kinase

Rationale

We investigated the presence of a constitutively active process for the activation/phosphorylation of Akt in the adult rat hippocampal slice mediated in part by PI3K. We predicted that a PI3K inhibitor such as wortmannin would lessen the amount of activated Akt in rat hippocampal slices as compared to control.

Methods

The phosphorylation of Akt at the serine^{473} site was assessed in Fischer-344 adult rat hippocampal slices incubated for 2 hours in two separate experimental conditions: control aCSF and 2 μM wortmannin aCSF. Western blot and immunohistochemical analyses were conducted to determine the relative amounts of Akt phosphorylated at the serine^{473} site. In order to extract sufficient protein for Western analysis, five slices were treated for each condition from an individual rat. Western blot analyses were performed using equal amounts of protein extract in each well and antibodies against serine^{473} phosphorylated Akt and total Akt. The immunohistochemistry was performed using the aforementioned antibodies and quantification by optical density was conducted on the CA1 region of the pyramidal cell layer.

Results

Constitutive levels of phosphorylated Akt in the whole hippocampal slice are reduced by wortmannin

The effects of incubating hippocampal slices in control aCSF and 2 μM wortmannin aCSF determined by Western analysis as mentioned above are illustrated in Figure 6. The slices incubated in wortmannin showed a significant decrease in phosphorylated Akt levels to 21.2% ± 3.8 of control slices. This decrease in phosphorylated Akt was due to inhibition of phosphorylation of Akt and not a decrease in total Akt protein as seen by no significant differences of total Akt in both control and wortmannin treated slices (Figure 7).

Constitutive levels of phosphorylated Akt are reduced in the pyramidal cell layer of CA1 when treated with wortmannin

The effects of incubating hippocampal slices in control aCSF and 2 μM wortmannin aCSF as determined by immunohistochemistry are illustrated in Figure 8. The slices incubated in wortmannin showed a significant decrease in phosphorylated Akt in the pyramidal cell layer of CA1. The optical density of slices treated with 2 μM wortmannin were 25.1% ± 4.9 of control slices.

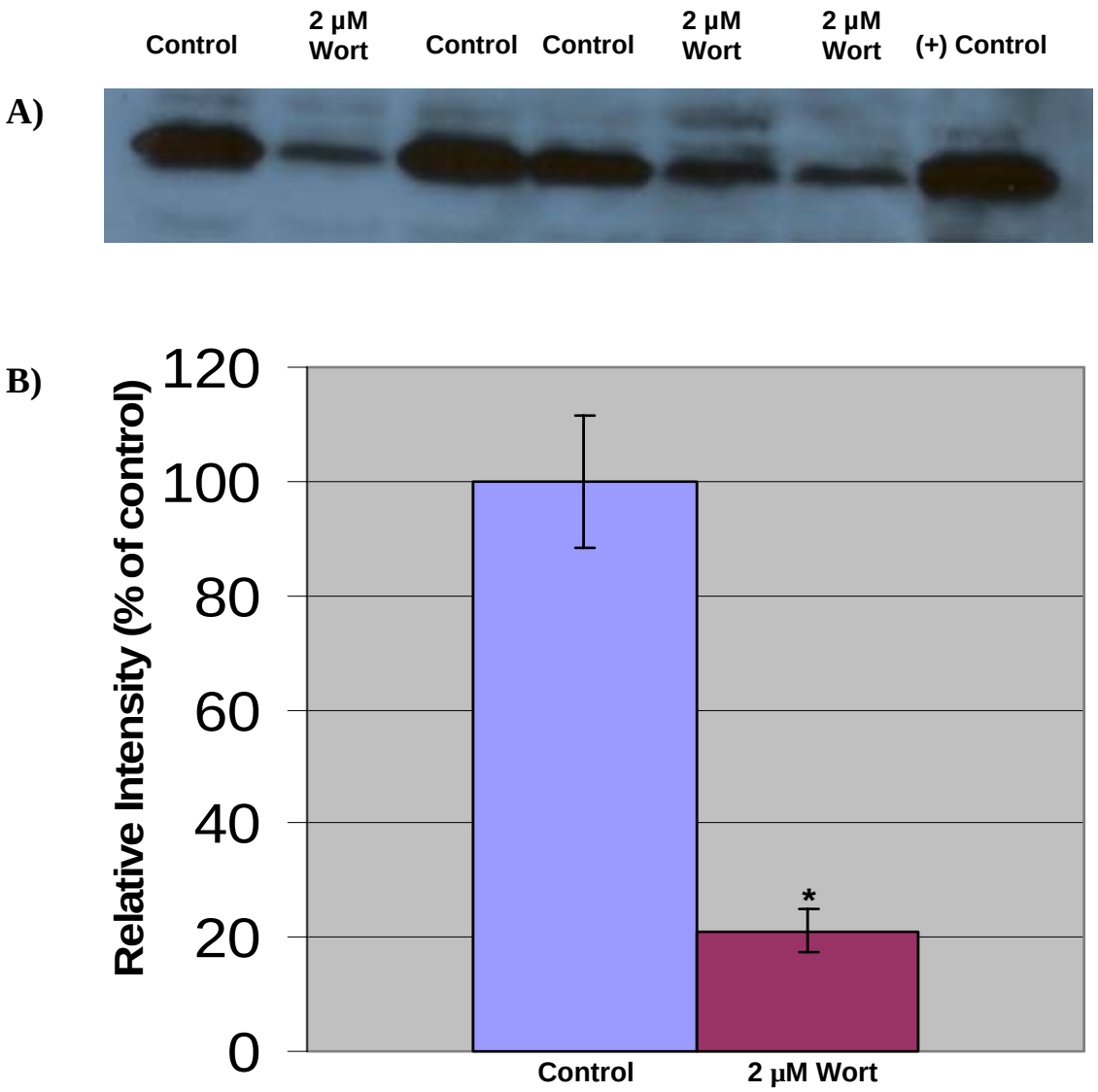

Figure 6. Incubation of slices with wortmannin (2 µM) for 2 hours shows a significant decrease in phosphorylated Akt. (A) Representative Western analysis of phospho-Akt for control and wortmannin exposures in Fischer 344 rat hippocampal slices. (B) Western analysis group data (n=3 for each category) illustrating a significant difference in relative mean intensity of phospho-Akt; control: 100%, 2 µM wort: 21.2% (* significant from control, p=0.003, Student's t-test).

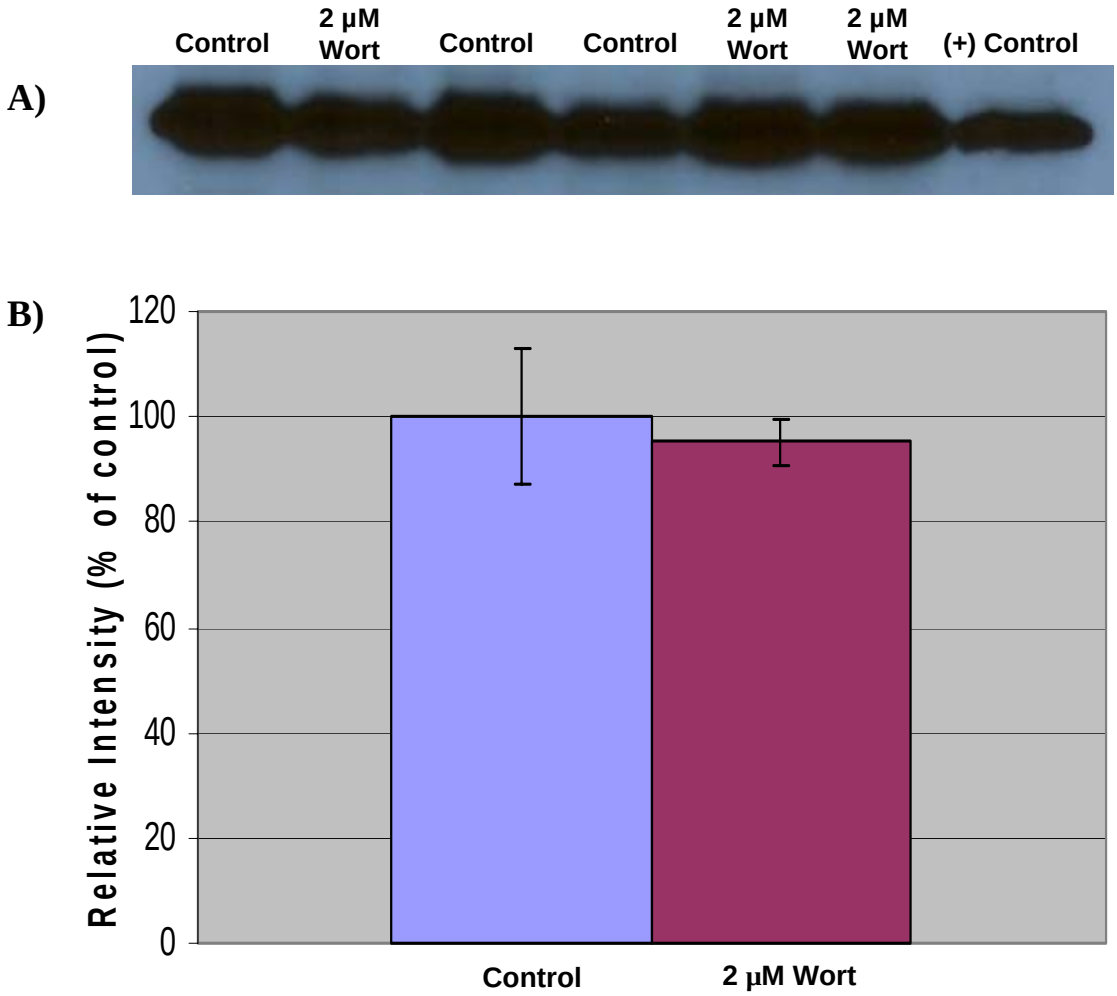

Figure 7. Incubation of slices with wortmannin (2 µM) for 2 hours does not show a significant difference of total Akt. (A) Representative Western analysis of total Akt for control and wortmannin exposures in Fischer 344 rat hippocampal slices. (B) Western analysis group data (n=3 for each category) illustrating no significant difference in relative mean intensity of total Akt; control: 100%, 2 µM wort: 95.2% (p=0.745, Student's t-test).

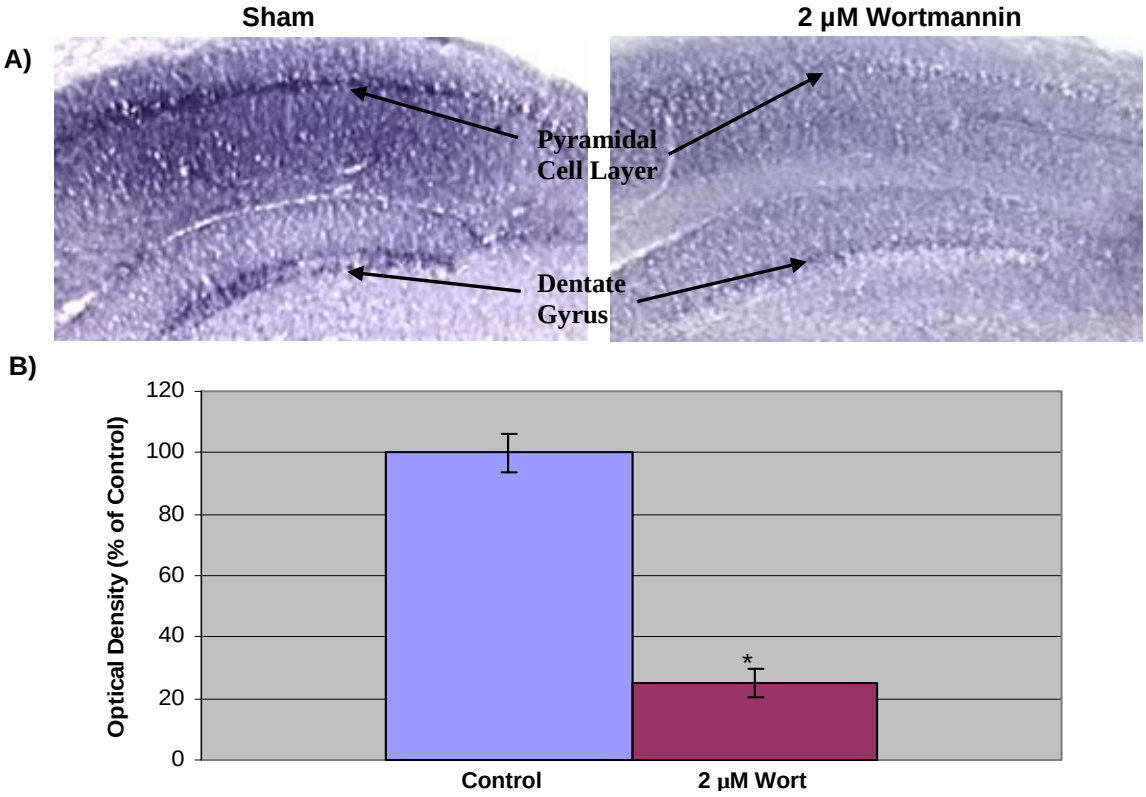

Figure 8. Incubation of slices with wortmannin (2 µM) for 2 hours significantly decreases phosphorylated Akt in the pyramidal cell layer of CA1. (A) Representative rat hippocampal slices treated with or without wortmannin depicting phosphorylated Akt through immunohistochemical staining. (B) Immunohistological group data (n=6 for control category, n=5 for wortmannin category) illustrating mean optical density of slices with phospho-Akt bound DAB stain; control: 100%, 2 µM wort: 25.1%. (* significant from control, $p<0.001$, Student's t-test).

Hypothesis II: Inhibition of the constitutive activation of Akt impairs neuronal cell viability

Rationale

Growth factor-mediated neuronal protection has been shown to be mediated through Akt. In the following experiments, we investigated the role of the constitutive activation of Akt for neuronal cell survival and integrity. We predicted that the inhibition of the constitutive phosphorylation, and hence activation of Akt, would impair the prosurvival mechanisms essential for neuronal cell survival and maintenance of neuronal synaptic activity. Given that, the inhibition of the constitutive activation of Akt would diminish the maximum attainable amplitude of evoked post synaptic potentials and population spikes.

Methods

Three groups of hippocampal slices from the same rat were used to demonstrate the effects of wortmannin on synaptic function. The control group contained slices incubated for 2 hours in aCSF only and the two wortmannin groups contained slices incubated for 2 hours in aCSF containing 200 nM and 2 μM wortmannin, respectively. After incubation, the slices were taken one at a time and placed into the recording chamber for determination of the maximum amplitude of field EPSPs. The optimal depth and location of the recording electrode in stratum radiatum of CA1 was established and the stimulus strength was increased until reaching the maximum amplitude of EPSPs.

Time dependence of the effects of pAkt inhibition was also observed by varying the time of incubation in both groups before attaining the maximum population spike amplitude.

Results

The maximum amplitude of EPSPs recorded in the control, 200 nM wortmannin, and the 2 μM wortmannin groups after two hours of incubation are illustrated in Figure 9. The mean maximum amplitudes of EPSPs for each group were as follows: control group: 2.1 mV ± 0.1, 200 nM wortmannin group: 1.2 mV ± 0.2, 2 μM wortmannin group: 1.4 mV ± 0.2. The mean maximum amplitudes for both the 200 nM wortmannin group and the 2 μM wortmannin group were significantly less than the control group ($p=0.006$ and $p=0.031$ respectively, ANOVA followed by a pairwise comparison Tukey test). There was no significant difference shown in the mean maximum amplitudes between the two wortmannin concentrations ($p=0.747$, ANOVA followed by a pairwise comparison Tukey test). The maximum population spike amplitude for control slices and slices incubated in 2 μM wortmannin for at least 1 hour showed no significant difference (control: 8.9 mV ± 0.7, 2 μM wort: 7.5 mV ± 0.8; $p=0.178$, Student's t-test) as illustrated in Figure 10. However, when comparing the 2 μM wortmannin group with the control group after at least 2 hours of incubation, there was a significant difference in mean maximum population spikes (control: 9.4 mV ± 0.8, 2 μM wort: 7.0 mV ± 0.7; $p=0.029$, Student's t-test) as illustrated in Figure 11.

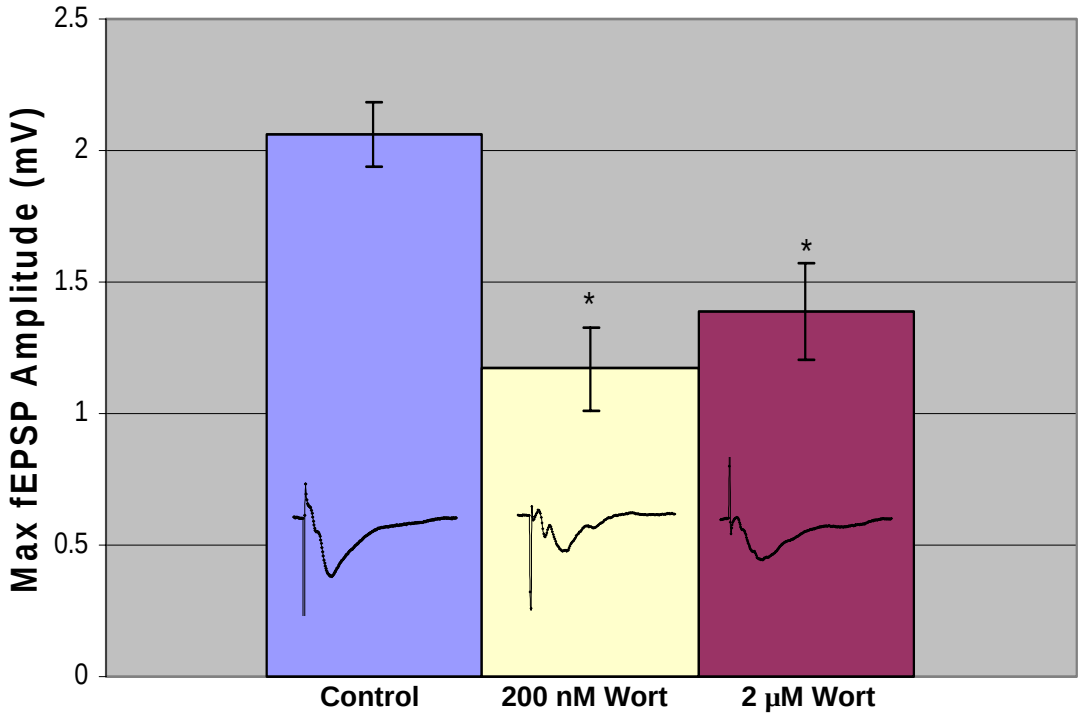

Figure 9. Maximum EPSP amplitude (mV) and representative fEPSP traces attained from slices incubated for 2 hours in control aCSF, 200 nM wortmannin aCSF, or 2 µM wortmannin aCSF. Slices incubated with 200 nM or 2 µM wortmannin aCSF show a significant reduction in maximum attainable EPSP amplitude as compared to slices incubated in control aCSF. Electrophysiological group data (n=10 for control category, n=3 for both 200 nM and 2 µM wort categories) illustrate differences in mean maximum EPSP amplitude; control: 2.1 mV, 200 nM wort: 1.2 mV, 2 µM wort: 1.4 mV (* significant from control, p=0.006 for control vs. 200 nM wort, p=0.031 for control vs. 2 µM wort, and p=0.213 for 200 nM wort vs. 2 µM wort, ANOVA followed by Tukey test for pairwise comparisons).

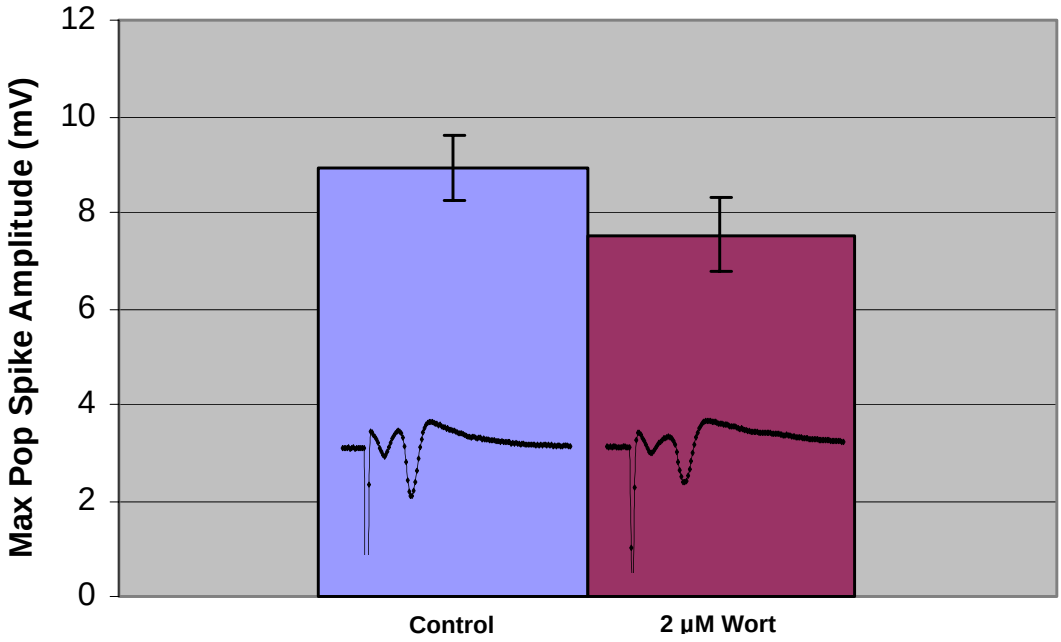

Figure 10. Maximum pop spike amplitude (mV) and representative population spike traces attained from slices incubated for at least 1 hour in control aCSF or 2 µM wortmannin aCSF. Slices incubated for at least 1 hour with 2 µM wortmannin aCSF do not show a significant difference in maximum attainable pop spike amplitude as compared to slices incubated in control aCSF. Electrophysiological group data (n=26 for control category, n=20 for 2 µM wortmannin category) illustrate no significant differences in mean maximum pop spike amplitude; control: 8.9 mV, 2 µM wort: 7.5 mV (p=0.178, Student's t-test).

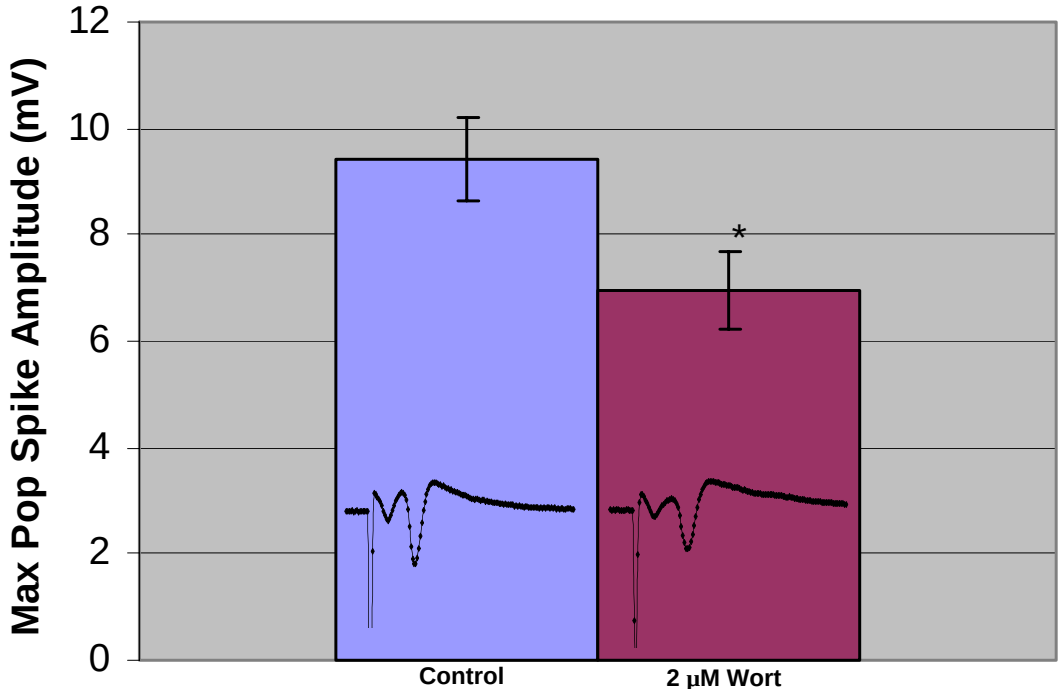

Figure 11. Maximum pop spike amplitude (mV) and representative pop spike traces attained from slices incubated for at least 2 hours in control aCSF or 2 µM wortmannin aCSF. Slices incubated for at least 2 hours with 2 µM wortmannin aCSF show a significant decrease in maximum attainable pop spike amplitude as compared to slices incubated in control aCSF. Electrophysiological group data (n=20 for control category, n=18 for 2 µM wort category) illustrate significant differences in mean maximum pop spike amplitude; control: 9.4 mV, 2 µM wort: 7.0 mV (* significant from control, p=0.029, Student's t-test).

Hypothesis III: Inhibition of the phosphorylation of Akt lessens the effectiveness of neuronal survival pathways during ischemia

Rationale

Since wortmannin did lower the maximum attainable potentials in both dendrites and the cell bodies of the neurons in the CA1 region of the hippocampus as shown in the results of hypthesis II, we wanted to further study its effects on the ability to withstand a survival stressor such as ischemia. We predicted that the slices treated with wortmannin would be more susceptible to an ischemic insult due to inhibiting the prosurvival mechanisms mediated by Akt. To determine if the wortmannin treated slices were more susceptible to ischemia, the time to depolarization in wortmannin treated slices was compared to non-wortmannin treated slices. The onset of ischemic depolarization is a significant event causing irreversible neuronal function and ultimately death without immediate reperfusion (Tanaka, Yamamoto et al. 1997; Kaminogo, Suyama et al. 1998). Therefore, blocking or delaying the depolarization has been shown to be protective (Anderson, Jarvis et al. 2005). The amplitude of depolarization is another significant event of the rapid ischemic depolarization that corresponds to a large ionic flux across the plasma membrane associated with cell swelling and neuronal damage (Tanaka, Yamamoto et al. 1997). Hence, we wanted to also determine if wortmannin would cause an increase in the amplitude of depolarization during a prolonged ischemic insult.

Methods

Hippocampal slices were incubated in control (aCSF only) or 2 μM wortmannin aCSF for 1 to 2 hours before being placed into the perfusion chamber for electrophysiological recordings. The perfusate was switched from aCSF bubbled with 95% O_2/ 5%CO_2 gas to aCSF without glucose bubbled with 95% N_2/ 5% CO_2 gas until depolarization was observed as a large negative DC shift as shown in the representative chart recording in Figure 12 (A). The duration of OGD to cause ischemic depolarization (time to depolarization) was recorded as the time from the start of OGD until the onset of rapid depolarization as shown in Figure 12 (B). The change in DC potential during the depolarization was also determined from the chart recording. The total depolarization amplitude, slow phase depolarization amplitude, fast phase depolarization amplitude, and the rate of depolarization of the fast phase was also determined from the chart recording of the DC potential as shown in Figures 13, 14, 15, and 16.

Results

Slices incubated with wortmannin for at least 2 hours
showed a significant increase in the time to depolarization
during OGD as compared to control slices

There was a significant increase in the mean time to depolarization during the OGD in 2 μM wortmannin slices (8.6 min ± 0.2) as compared to the control slices (7.7 min ± 0.2); p=0.012, Student's t-test as shown in Figure 12. This result contradicted our prediction that the wortmannin would reduce the time to depolarization during OGD.

There was no significant difference in the magnitude and rate of depolarization due to OGD in slices treated with or without wortmannin

There was no significant difference ($p=0.230$, Student's t-test) in the total magnitude of depolarization between control slices (8.4 mV ± 0.8) and 2 μM wortmannin slices (7.2 mV ± 0.6) as shown in Figure 13. To further distinguish between the depolarization of the control slices and wortmannin treated slices, the magnitude of the slow phase of depolarization and the magnitude and rate of the fast phase of depolarization were determined. The magnitude of the slow phase of depolarization for the control slices (1.8 mV ± 0.2) and the 2 μM wortmannin slices (1.9 mV ± 0.2) was not significantly different ($p=0.695$) from one another as shown in Figure 14. The magnitude of the fast phase of depolarization for the control slices (5.4 mV ± 0.6) and 2 μM wortmannin slices (4.8 mV ± 0.4) was also not significantly different ($p=0.389$, Student's t-test) as shown in Figure 15. The rate of DC voltage change during the fast phase of depolarization for the control slices (1.1 mV/sec ± 0.2) and 2 μM wortmannin slices (0.6 mV/sec ± 0.1) were almost significantly different at a p-value of 0.085 as shown in Figure 16. The wortmannin treated slices showed a mean drop of 41.6% in the rate of DC potential change during the fast phase of depolarization as compared to control slices. However, due to the large SEM for both groups, these values were not significantly different at a 95% confidence level.

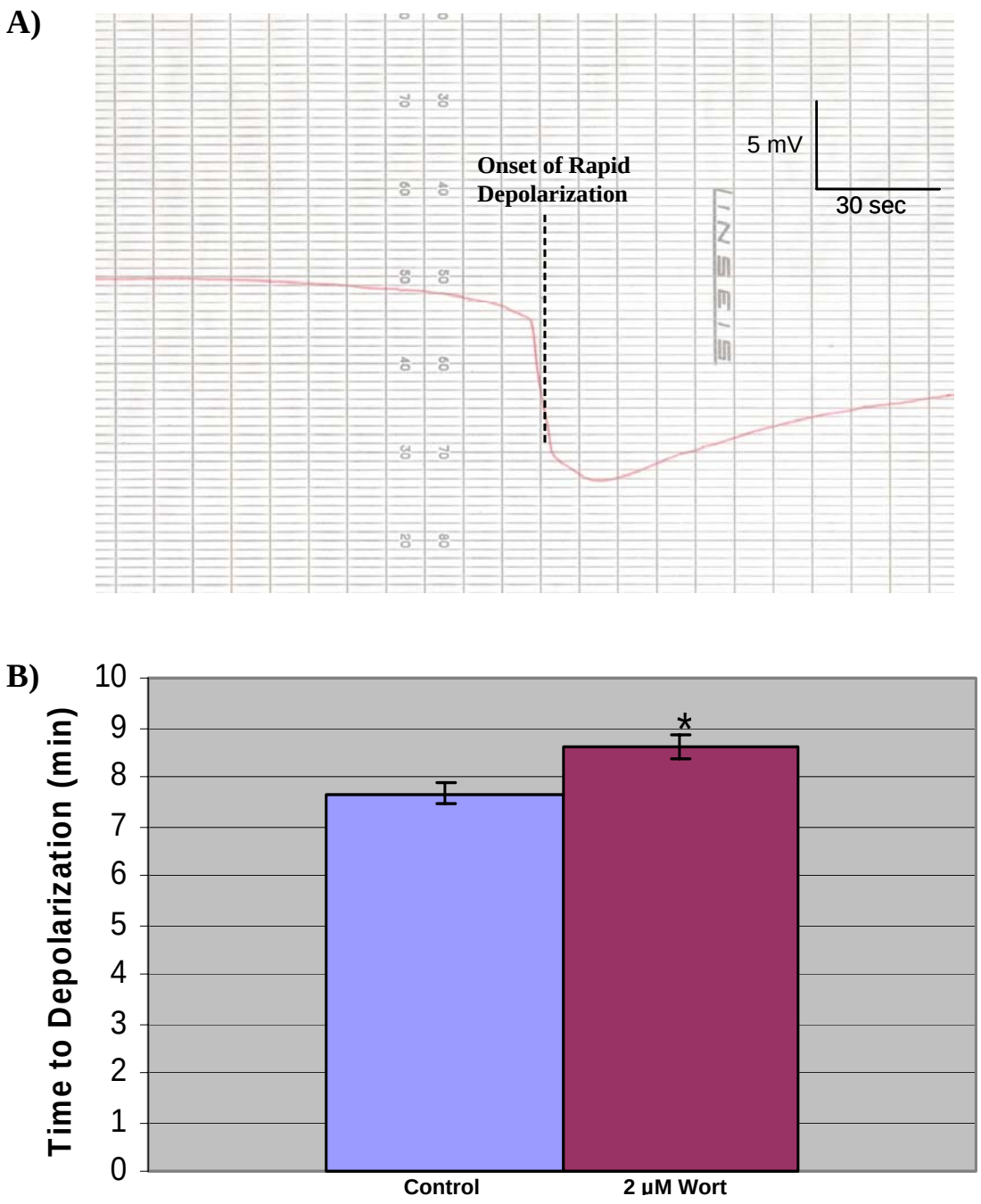

Figure 12. (A) Representative chart recording of a depolarization recorded from the pyramidal cell layer of CA1. (B) Wortmannin treated slices show an increase in the duration of OGD insult to cause ischemic depolarization in hippocampal slices as compared to control. Population spike and fEPSP combined group data (n=15 for control category, n=10 for 2 µM wort category) illustrating differences in mean time to depolarization under OGD; control: 7.7 min, 2 µM wort: 8.6 min (* significant from control, p=0.012, Student's t-test).

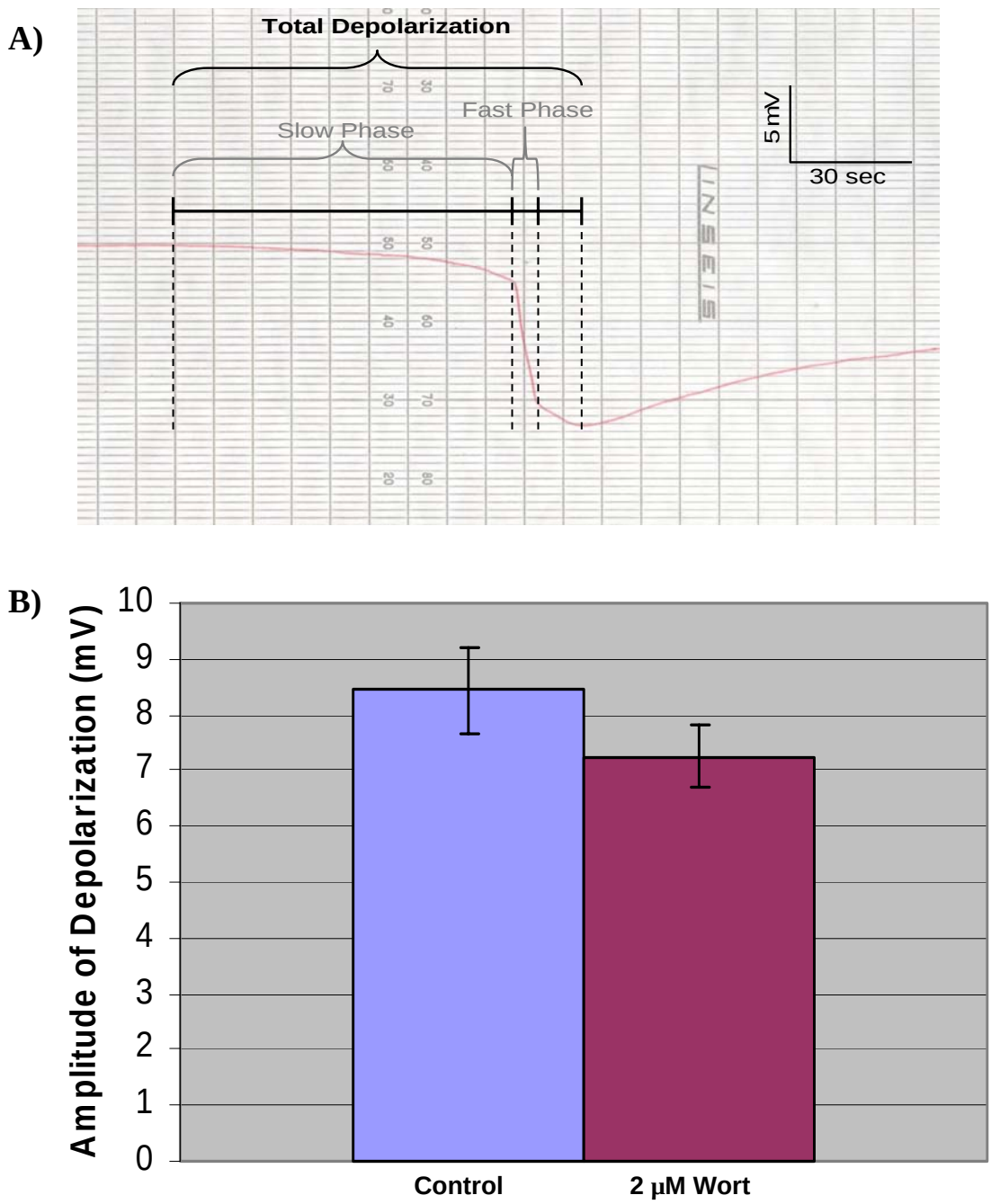

Figure 13. (A) Representative chart recording of a depolarization recorded from the pyramidal cell layer of CA1. (B) No significant difference was shown in the amplitude of depolarization recorded in the pyramidal cell layer of CA1 in slices incubated and perfused in aCSF and 2 µM wormannin aCSF. Population spike group data (n=7 for both categories; control: 8.5 mV, 2 µM wort: 7.2 mV, p=0.230, Student's t-test).

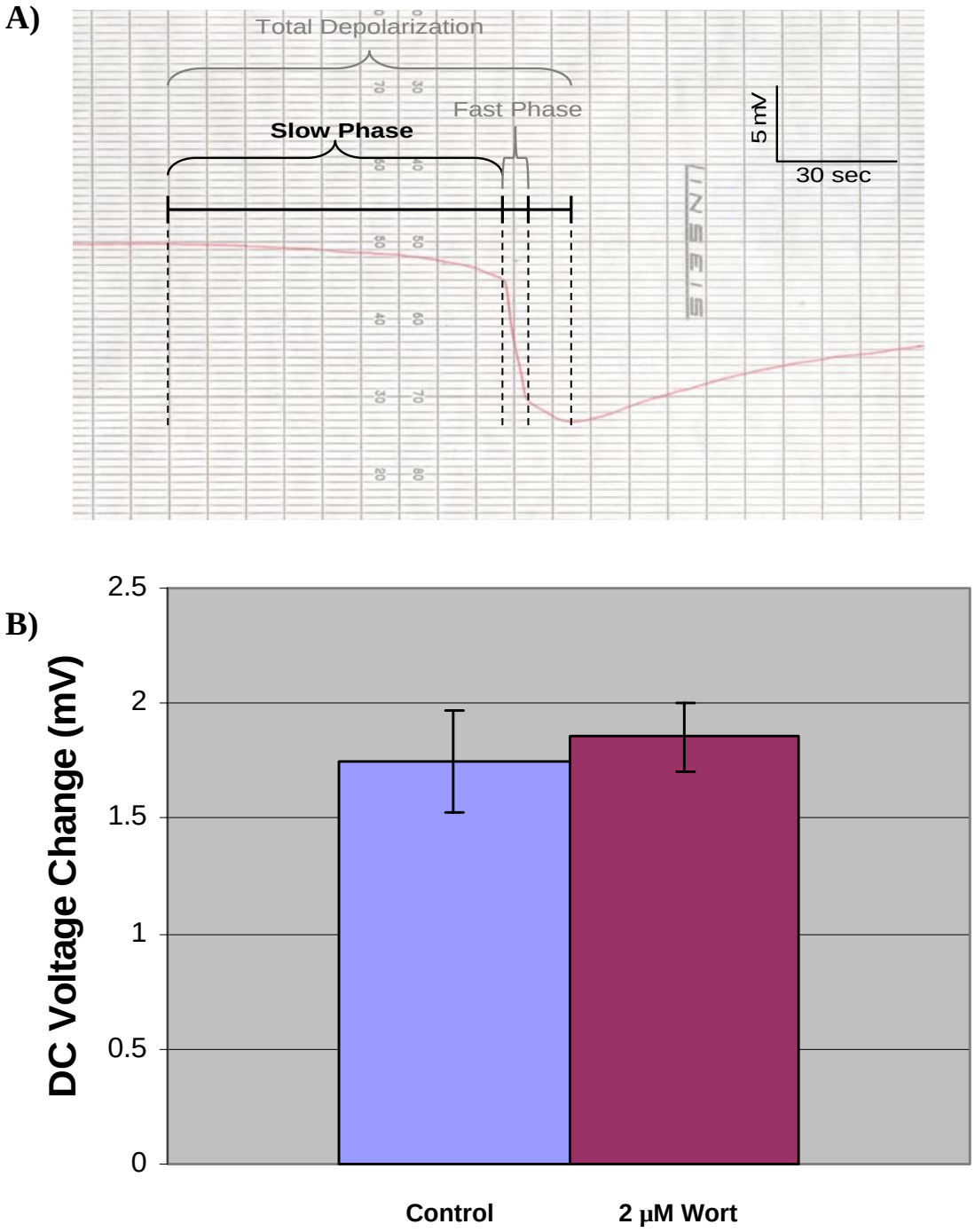

Figure 14. (A) Representative chart recording highlighting the slow phase DC voltage change during depolarization. (B) No significant difference was shown in the DC voltage change during the slow phase of depolarization between control and 2 µM wortmannin aCSF. Depolarization group data (n=7 for both categories; control: 1.8 mV, 2 µM wort: 1.9 mV, p=0.695, Student's t-test).

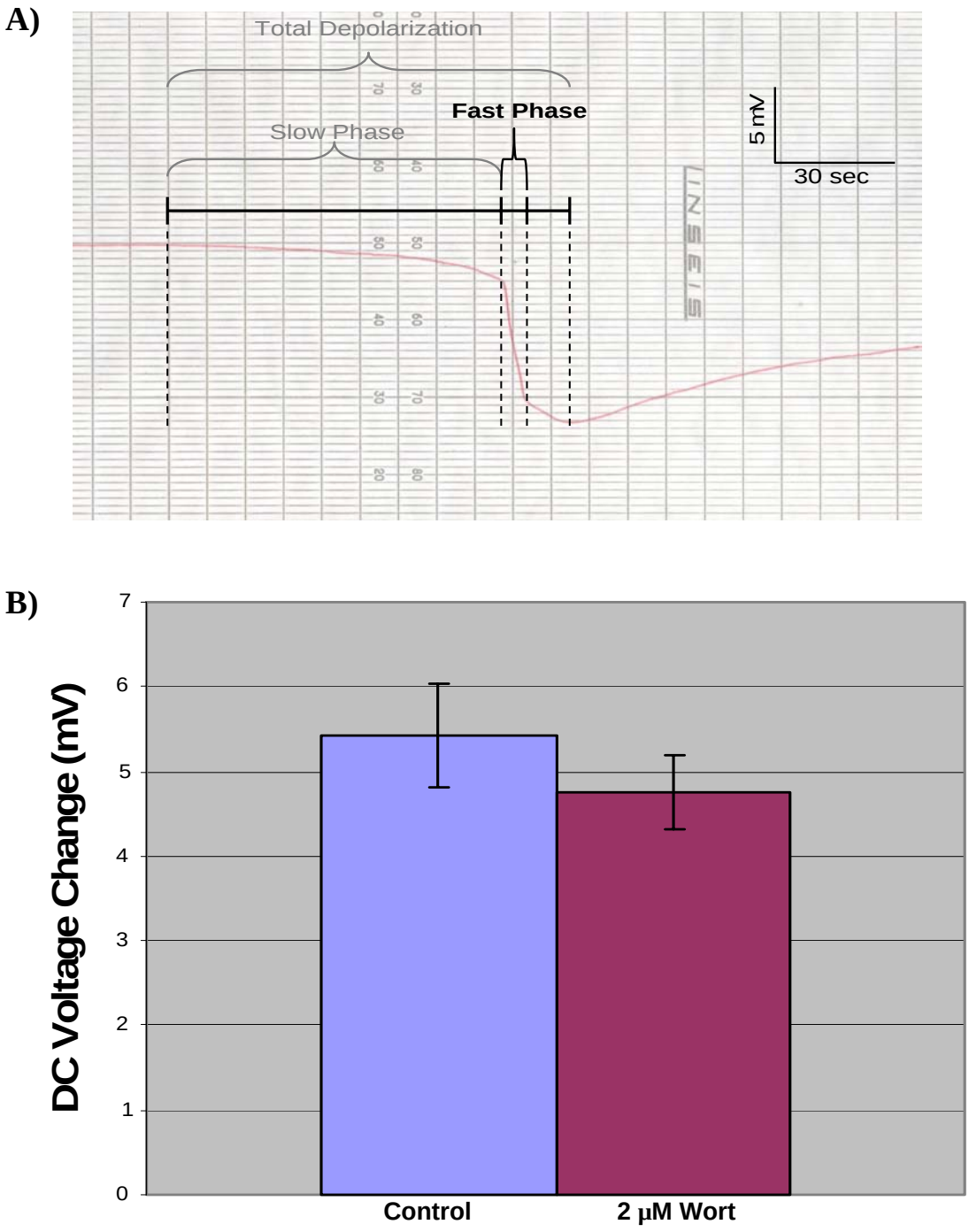

Figure 15. (A) Representative chart recording highlighting the fast phase DC voltage change during depolarization. (B) No significant difference was shown in the DC voltage change during the fast phase of depolarization between control and 2 µM wortmannin aCSF. Depolarization group data (n=7 for both categories; control: 5.4 mV, 2 µM wort: 4.8 mV, p=0.389, Student's t-test).

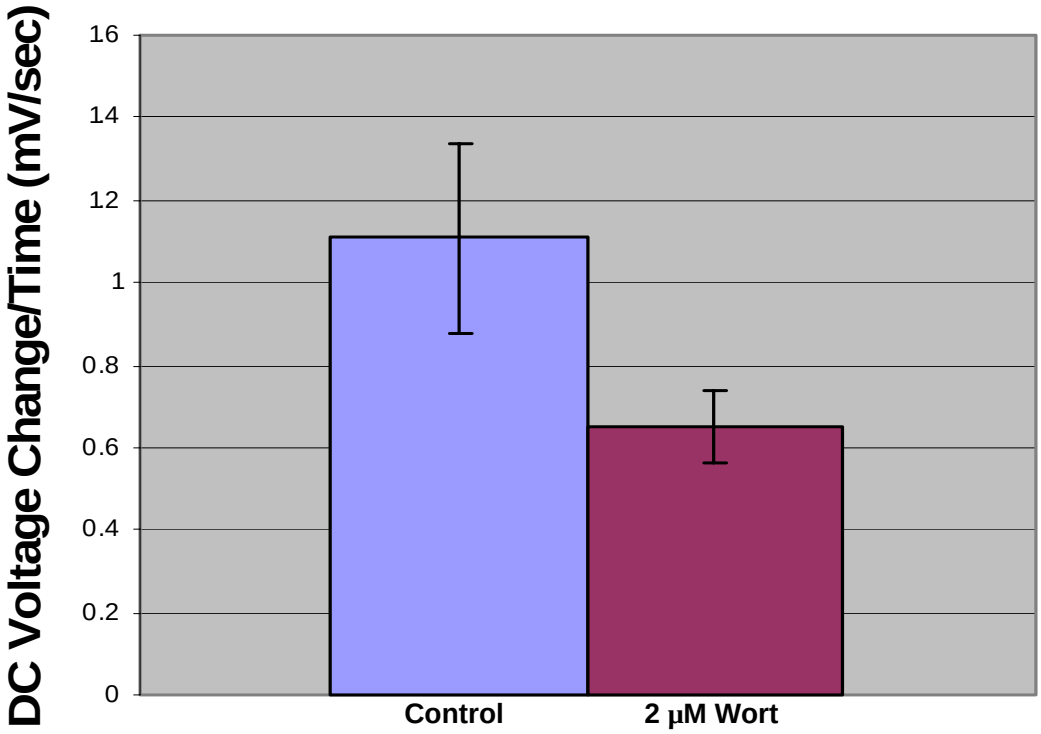

Figure 16. Rate of fast phase DC voltage change (mV/sec) during depolarization recorded in pyramidal cell layer of CA1. No significant difference was shown between control and 2 µM wortmannin aCSF. Depolarization group data (n=7 for both categories; control: 1.1 mV/sec, 2 µM wort: 0.6 mV/sec, p=0.085, Student's t-test).

CHAPTER V

DISCUSSION

In this chapter, each result from every hypothesis will be discussed as separate subsections.

Hypothesis I: There exists a constitutive activation of Akt in vitro mediated by PI_3 kinase

Baseline levels of phosphorylated or activated Akt are reduced in hippocampal slices incubated for 2 hours in aCSF containing 2 μM wortmannin as compared to slices incubated for 2 hours in aCSF only. Wortmannin is a fungal metabolite that specifically inhibits PI3K (Powis, Bonjouklian et al. 1994). The 2 μM concentration of wortmannin used to inhibit PI3K activity was within the working range of PI3K inhibition and well below the high dose of 10 μM used by Okaichi (Okaichi, Suzuki et al. 2002). The reduced levels of phosphorylated Akt due to PI3K inhibition show that the PI3K-dependent pathway is necessary to maintain constitutive levels. Wortmannin inhibition of increased activation of Akt seen in preconditioning studies in the gerbil hippocampus support these results (Yano, Morioka et al. 2001). These results also illustrate that the PI3K-independent pathway does not sufficiently compensate for the activation of Akt to sustain constitutive levels of phosphorylated Akt within a 2 hour time period. Since the levels of phosphorylated Akt in the PI3K-inhibited slices were reduced to 20-25% of

control slices, the PI3K-dependent pathway seems to be the chief mechanism of constitutive activation of Akt.

Hypothesis II: Inhibition of the constitutive activation of Akt impairs neuronal cell viability

The maximum attainable amplitude of evoked population spikes was not significantly different in slices incubated for at least 1 hour in aCSF containing 2 μM wortmannin as compared to slices incubated for at least 1 hour in aCSF only. However, the maximum attainable amplitude of EPSPs and population spikes were significantly reduced in slices incubated for at least 2 hours in aCSF containing 2 μM wortmannin as compared to slices incubated for at least 2 hours in aCSF only. This study shows a time dependence of the duration of incubation in wortmannin that results in a reduction of evoked potentials. The amplitude of evoked potentials seems to be reduced by the inhibition of the constitutive level of activated Akt after a minimum of 2 hours. Since Akt has been shown to mediate the increase in glucose transporters to the plasma membrane of neurons and astrocytes (Bondy and Cheng 2004), the inhibition of Akt through wortmannin decreases glucose utilization (Kandel and Hay 1999). Decrease in glucose utilization could decrease ATP production and inhibit glutamate reuptake and release from the presynaptic membrane. This would cause less glutamate acting on postsynaptic membranes and thus decreased evoked potentials. This reduction could also be caused by a decrease in the number of live neurons that are electrically active. The inhibition of constitutive levels of Akt could cause neurons to undergo necrotic or

apoptotic death due to the removal of prosurvival mechanisms. Further experiments, such as live/dead assays, would need to be conducted to determine if the loss of synaptic transmission is due to cell death.

Hypothesis III: Inhibition of the phosphorylation of Akt lessens the effectiveness of neuronal survival pathways during ischemia

Slices incubated with wortmannin for at least 2 hours
showed a significant increase in the time to depolarization
during OGD as compared to control slices

Neuronal depolarization is characterized by a decrease in membrane resistance and a redistribution of ions across the cell membrane which allows for net movement of Na^+, Ca^{2+}, and Cl^- into the neuronal cells and K^+ out into the interstitial space (Somjen 2001). This redistribution of ions, especially the net accumulation of intracellular Ca^{2+} for a critical length of time, is implicated in causing the cell injury due to hypoxic or ischemic depolarization (Somjen 2001). The result of incubating brain slices in wortmannin prior to OGD contradicted our prediction that the decreased levels of phosphorylated Akt due to wortmannin would impair prosurvival pathways during ischemia leading to a shorter time to depolarization during an ischemic insult. Based on this result, the inhibition of PI3K by wortmannin is beneficial to the slice, tolerating a longer time in OGD before the onset of depolarization occurs. Even though previous studies show that Akt contributes to ischemic tolerance through preconditioning (Yano, Morioka et al. 2001), our results show that Akt does not offer protection by delaying ischemic depolarization. Previous work in the laboratory has shown that transient

hypoxic insults without the onset of anoxic depolarization decrease the level of phosphorylated Akt *in vitro*. Inhibition of PI3K or dephosphorylation of Akt may be a protective mechanism mediating tolerance to depolarization during hypoxia/ischemia.

There was no significant difference in the magnitude and rate of depolarization due to OGD in slices treated with or without wortmannin

The magnitude and rate of depolarization depict the extent and pace at which the ion redistribution is taking place during ischemia (Somjen 2001). Greater magnitudes and rates of depolarization lead to additional ion regulation that must take place within the neuronal cell in order to maintain function and survival before irreversible damage occurs (Somjen 2001). This result contradicted our prediction that there would be a decrease in magnitude and rate of depolarization due to inhibition of phosphorylated Akt by wortmannin. The total magnitude nor the rate of DC potential change during the depolarization in wortmannin and non-wortmannin treated slices were not significantly different. Therefore the activation of bcl-2 by Akt for the enhanced ability of mitochondria to sequester intracellular Ca^{2+} (Murphy, Bredesen et al. 1996; Datta, Dudek et al. 1997; Dudek, Datta et al. 1997) did not attenuate the depolarization due to the ionic flux caused by ischemic depolarization.

CHAPTER VI

CONCLUDING REMARKS

This thesis examined the regulation of Akt by the PI3K-dependent pathway and its importance in neuronal viability and protection from ischemia. Akt is a serine/threonine kinase that has been shown to mediate the actions of many proteins involved with cell maintenance and survival (Crowder and Freeman 1998; Hayashi, Abe et al. 1998). Research on Akt has been done to elucidate its function in metabolism, necrosis, and apoptosis (Datta, Dudek et al. 1997; Crowder and Freeman 1998; Eves, Xiong et al. 1998; Datta, Brunet et al. 1999; Yano, Morioka et al. 2001). The significance of this work contributes toward understanding the various potential roles of mediators in the complex pathways involved in neuronal death following cerebral ischemia.

Following the onset of cerebral ischemia, extracellular adenosine increases significantly in the rat hippocampal slice (Fowler 1993). Adenosine, acting through A_1 receptors, inhibits the increased energy demand to supply ratio during ischemia by attenuating synaptic transmission (Fowler 1989; Dunwiddie and Masino 2001; Gervitz, Lutherer et al. 2001). This neuroprotective mechanism has only transient effects on prolonged ischemic insults. Under ischemic-like conditions, the inhibitory effect of adenosine on glutamate release is lost as seen in the transient increase in population spike amplitude (Fowler 1990). However, adenosine also leads to the activation of Akt mediated by the A_1 receptor (Gervitz, Nalbant et al. 2002). With the elevation of

extracellular adenosine during ischemia (Whittingham, Lust et al. 1984; Fowler 1993; Dale, Pearson et al. 2000; Pearson, Currie et al. 2003), the contribution of the PI3K-dependent activation of Akt was explored in neuronal protection.

It is well documented that wortmannin blocks Akt activation by inhibiting the PI3K-dependent pathway (Powis, Bonjouklian et al. 1994; Alessi and Cohen 1998; Downward 1998; Yano, Morioka et al. 2001; Luo, Hattori et al. 2003). Incubation with wortmannin, an irreversible blocker of PI3K, for 2 hours significantly reduced the baseline levels of phosphorylated Akt in the isolated rat hippocampal slice (Figure 6). Furthermore, phosphorylated Akt is reduced without altering total Akt levels by treatment with PI3K inhibitors such as LY294002, the reversible PI3K blocker, or wortmannin (Luo, Hattori et al. 2003) and as shown in Figures 6 and 7 for wortmannin. Therefore, we conclude that the activation of Akt by the PI3K-dependent pathway is necessary to maintain constitutive levels of phosphorylated Akt.

Studies show that the PI3K-Akt pathway is sufficient and necessary for survival of several neuronal cell types including the hippocampus (Yao and Cooper 1995; Dudek, Datta et al. 1997; Crowder and Freeman 1998; Hetman, Kanning et al. 1999; Luo, Hattori et al. 2003). Our results show that a reduction of maximum attainable evoked population spike amplitudes in slices only occurred when incubated for at least 2 hours with wortmannin as compared to control slices (Figure 11). Inhibition of the PI3K-Akt pathway is associated with hippocampal cell death by both necrosis and apoptosis (Downward 1998; Brunet, Datta et al. 2001; Cantley 2002; Luo, Hattori et al. 2003). Therefore, this reduction in evoked potentials could be caused by a decrease in the

number of live neurons that are electrically active. Live/dead assays should be investigated to determine if the reduction of synaptic transmission in the wortmannin treated slices are due to cell death. On the other hand, wortmannin inhibition of Akt has also been shown to decrease glucose utilization through inhibiting the Akt-mediated increase in glucose transporters to the plasma membrane of neurons and astrocytes (Kandel and Hay 1999; Bondy and Cheng 2004). Decreased glucose utilization could reduce ATP production and thus inhibit glutamate reuptake and release from the presynaptic membrane. This reduction of presynaptic glutamate would in turn cause an attenuation of the evoked potentials due to a smaller pool of glutamate available for synaptic transmission. Further experiments would need to be performed to determine if ATP levels are altered in the hippocampal slice due to wortmannin treatment. Whether the evoked potentials are reduced due to cell death or reduced metabolism, the pro-survival mechanisms of phosphorylated Akt mediated by PI3K are significant in maintaining neuronal cell viability and function.

Several studies have shown that Akt provides neuronal protection from transient ischemic insults (Ouyang, Tan et al. 1999; Yano, Morioka et al. 2001; Hirai, Hayashi et al. 2004). However, in delaying or attenuating the ischemic depolarization, the PI3K-dependent activation of Akt did not show any beneficial effect. In fact, the inhibition of PI3K by wortmannin improved tolerance to ischemia by delaying the depolarization (Figure 12). During ischemia phosphorylated Akt levels are significantly diminished in the *in vitro* hippocampus (Ouyang, Tan et al. 1999; Hirai, Hayashi et al. 2004). Due to this decrease in phosphorylated Akt during ischemia, there may not be a significant

decrease in phosphorylated Akt when treating slices with wortmannin. Therefore, Akt may not have significant effects on prosurvival mechanisms in either of the conditions during prolonged ischemia. The protective effects of Akt in neuronal protection may play more of a role in post-ischemic reperfusion since Akt is rapidly rephosphorylated within 30 minutes of reperfusion and to prehypoxic levels within 24 hours (Ouyang, Tan et al. 1999; Hirai, Hayashi et al. 2004). Western blot analysis of phosphorylated Akt levels should be assessed for control and wortmannin treated slices after the onset of ischemia but prior to the depolarization in order to determine if there is a difference in phosphorylated Akt.

However, this does not explain the delay in depolarization with the inhibition of PI3K by wortmannin. Wortmannin specifically inhibits PI3K by covalently binding to the ATP site of PI3K (Wipf and Halter 2005). This blocking of the ATP site potentially allows ATP to be conserved for use in maintaining Na+/K+ - ATPase activity whose decreased activity during ischemia is implicated in causing the ischemic depolarization (Siesjo 1984; Balestrino 1995; Tanaka, Yamamoto et al. 1997). Furthermore, studies have shown that increased levels of high energy phosphate groups such as ATP and phosphocreatine delay depolarization during ischemic conditions (Balestrino, Rebaudo et al. 1999; Balestrino, Lensman et al. 2002).

To further understand the potential beneficial role of Akt in ischemic conditions, future experiments designed to potentiate activated Akt levels prior to an ischemic insult should be conducted. This can be accomplished by pre-incubating the hippocampal slice in aCSF containing an adenosine agonist such a cyclohexyladenosine (CHA) for 20

minutes, followed by washing out of the agonist for 1 hour to attain a high expression of phosphorylated Akt in the slice (Gervitz, Nalbant et al. 2002). This would be followed by a prolonged ischemic insult to determine if high levels of activated Akt are necessary to promote ischemic tolerance by delaying depolarization. If increased activated Akt levels are shown to protect neurons from the onset of depolarization, manipulation of Akt or downstream mediators of the PI3K-Akt pathway could play a potential role in future therapies.

BIBLIOGRAPHY

Alessi, D. R. and P. Cohen (1998). "Mechanism of activation and function of protein kinase B." Curr Opin Genet Dev **8**(1): 55-62.

American-Heart-Association (2004). Heart Disease and Stroke Statistics - 2005 Update.

Anderson, T. R., C. R. Jarvis, et al. (2005). "Blocking the anoxic depolarization protects without functional compromise following simulated stroke in cortical brain slices." J Neurophysiol **93**(2): 963-79.

Astrup, J., B. K. Siesjo, et al. (1981). "Thresholds in cerebral ischemia - the ischemic penumbra." Stroke **12**(6): 723-5.

Back, T., W. Zhao, et al. (1995). "Three-dimensional image analysis of brain glucose metabolism-blood flow uncoupling and its electrophysiological correlates in the acute ischemic penumbra following middle cerebral artery occlusion." J Cereb Blood Flow Metab **15**(4): 566-77.

Balestrino, M. (1995). "Pathophysiology of anoxic depolarization: new findings and a working hypothesis." J Neurosci Methods **59**(1): 99-103.

Balestrino, M., P. G. Aitken, et al. (1989). "Spreading depression-like hypoxic depolarization in CA1 and fascia dentata of hippocampal slices: relationship to selective vulnerability." Brain Res **497**(1): 102-7.

Balestrino, M., M. Lensman, et al. (2002). "Role of creatine and phosphocreatine in neuronal protection from anoxic and ischemic damage." Amino Acids **23**(1-3): 221-9.

Balestrino, M., R. Rebaudo, et al. (1999). "Exogenous creatine delays anoxic depolarization and protects from hypoxic damage: dose-effect relationship." Brain Res **816**(1): 124-30.

Balestrino, M. and G. G. Somjen (1986). "Chlorpromazine protects brain tissue in hypoxia by delaying spreading depression-mediated calcium influx." Brain Res **385**(2): 219-26.

Barthel, A., S. T. Okino, et al. (1999). "Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1." J Biol Chem **274**(29): 20281-6.

Blumenfeld, H. (2002). Neuroanatomy through clinical cases, Sinauer Associates, Inc.

Bondy, C. A. and C. M. Cheng (2004). "Signaling by insulin-like growth factor 1 in brain." Eur J Pharmacol **490**(1-3): 25-31.

Brunet, A., A. Bonni, et al. (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." Cell **96**(6): 857-68.

Brunet, A., S. R. Datta, et al. (2001). "Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway." Curr Opin Neurobiol **11**(3): 297-305.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-7.

Cardone, M. H., N. Roy, et al. (1998). "Regulation of cell death protease caspase-9 by phosphorylation." Science **282**(5392): 1318-21.

Cheng, C. M., R. R. Reinhardt, et al. (2000). "Insulin-like growth factor 1 regulates developing brain glucose metabolism." Proc Natl Acad Sci U S A **97**(18): 10236-41.

Choi, D. (1998). "Antagonizing excitotoxicity: a therapeutic strategy for stroke?" Mt Sinai J Med **65**(2): 133-8.

Coffer, P. J., J. Jin, et al. (1998). "Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation." Biochem J **335 (Pt 1)**: 1-13.

Cong, L. N., H. Chen, et al. (1997). "Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells." Mol Endocrinol **11**(13): 1881-90.

Crowder, R. J. and R. S. Freeman (1998). "Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons." J Neurosci **18**(8): 2933-43.

Dale, N., T. Pearson, et al. (2000). "Direct measurement of adenosine release during hypoxia in the CA1 region of the rat hippocampal slice." J Physiol **526 Pt 1**: 143-55.

Datta, S. R., A. Brunet, et al. (1999). "Cellular survival: a play in three Akts." Genes Dev **13**(22): 2905-27.

Datta, S. R., H. Dudek, et al. (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." Cell **91**(2): 231-41.

Downward, J. (1998). "Mechanisms and consequences of activation of protein kinase B/Akt." Curr Opin Cell Biol **10**(2): 262-7.

Dudek, H., S. R. Datta, et al. (1997). "Regulation of neuronal survival by the serine-threonine protein kinase Akt." Science **275**(5300): 661-5.

Dunwiddie, T. V. (1985). "The physiological role of adenosine in the central nervous system." Int Rev Neurobiol **27**: 63-139.

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.

Eves, E. M., W. Xiong, et al. (1998). "Akt, a target of phosphatidylinositol 3-kinase, inhibits apoptosis in a differentiating neuronal cell line." Mol Cell Biol **18**(4): 2143-52.

Filippa, N., C. L. Sable, et al. (1999). "Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase." Mol Cell Biol **19**(7): 4989-5000.

Folbergrova, J., H. Memezawa, et al. (1992). "Focal and perifocal changes in tissue energy state during middle cerebral artery occlusion in normo- and hyperglycemic rats." J Cereb Blood Flow Metab **12**(1): 25-33.

Fowler, J. C. (1989). "Adenosine antagonists delay hypoxia-induced depression of neuronal activity in hippocampal brain slice." Brain Res **490**(2): 378-84.

Fowler, J. C. (1990). "Adenosine antagonists alter the synaptic response to in vitro ischemia in the rat hippocampus." Brain Res **509**(2): 331-4.

Fowler, J. C. (1993). "Purine release and inhibition of synaptic transmission during hypoxia and hypoglycemia in rat hippocampal slices." Neurosci Lett **157**(1): 83-6.

Fredholm, B. B. and T. V. Dunwiddie (1988). "How does adenosine inhibit transmitter release?" Trends Pharmacol Sci **9**(4): 130-4.

Gervitz, L. M., L. O. Lutherer, et al. (2001). "Adenosine induces initial hypoxic-ischemic depression of synaptic transmission in the rat hippocampus in vivo." Am J Physiol Regul Integr Comp Physiol **280**(3): R639-45.

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**(2): 175-9.

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

Hajduch, E., D. R. Alessi, et al. (1998). "Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells." Diabetes **47**(7): 1006-13.

Hansen, A. J. and C. E. Olsen (1980). "Brain extracellular space during spreading depression and ischemia." Acta Physiol Scand **108**(4): 355-65.

Hayashi, T., K. Abe, et al. (1998). "Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia." J Cereb Blood Flow Metab **18**(8): 887-95.

Heiss, W. D., A. Thiel, et al. (1999). "Which targets are relevant for therapy of acute ischemic stroke?" Stroke **30**(7): 1486-9.

Hetman, M., K. Kanning, et al. (1999). "Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase." J Biol Chem **274**(32): 22569-80.

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**(1): 51-61.

Kaminogo, M., K. Suyama, et al. (1998). "Anoxic depolarization determines ischemic brain injury." Neurol Res **20**(4): 343-8.

Kandel, E. S. and N. Hay (1999). "The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB." Exp Cell Res **253**(1): 210-29.

Kane, D. J., T. Ord, et al. (1995). "Expression of bcl-2 inhibits necrotic neural cell death." J Neurosci Res **40**(2): 269-75.

Katchman, A. N. and N. Hershkowitz (1993). "Adenosine antagonists prevent hypoxia-induced depression of excitatory but not inhibitory synaptic currents." Neurosci Lett **159**(1-2): 123-6.

Kawasaki, K., G. Czeh, et al. (1988). "Prolonged exposure to high potassium concentration results in irreversible loss of synaptic transmission in hippocampal tissue slices." Brain Res **457**(2): 322-9.

Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.

Kirino, T. and K. Sano (1984). "Selective vulnerability in the gerbil hippocampus following transient ischemia." Acta Neuropathol (Berl) **62**(3): 201-8.

Kluck, R. M., E. Bossy-Wetzel, et al. (1997). "The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis." Science **275**(5303): 1132-6.

Kohn, A. D., S. A. Summers, et al. (1996). "Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation." J Biol Chem **271**(49): 31372-8.

Latini, S., F. Bordoni, et al. (1998). "Temporal correlation between adenosine outflow and synaptic potential inhibition in rat hippocampal slices during ischemia-like conditions." Brain Res **794**(2): 325-8.

Li, P., D. Nijhawan, et al. (1997). "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade." Cell **91**(4): 479-89.

Lipton, P. (1999). "Ischemic cell death in brain neurons." Physiol Rev **79**(4): 1431-568.

Luo, H. R., H. Hattori, et al. (2003). "Akt as a mediator of cell death." Proc Natl Acad Sci U S A **100**(20): 11712-7.

Majno, G. and I. Joris (1995). "Apoptosis, oncosis, and necrosis. An overview of cell death." Am J Pathol **146**(1): 3-15.

Martinou, J. C., M. Dubois-Dauphin, et al. (1994). "Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia." Neuron **13**(4): 1017-30.

McEwen, B. S. and L. P. Reagan (2004). "Glucose transporter expression in the central nervous system: relationship to synaptic function." Eur J Pharmacol **490**(1-3): 13-24.

Murphy, A. N., D. E. Bredesen, et al. (1996). "Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria." Proc Natl Acad Sci U S A **93**(18): 9893-8.

Nedergaard, M., A. Gjedde, et al. (1986). "Focal ischemia of the rat brain: autoradiographic determination of cerebral glucose utilization, glucose content, and blood flow." J Cereb Blood Flow Metab **6**(4): 414-24.

Okaichi, K., K. Suzuki, et al. (2002). "Low dose of wortmannin reduces radiosensitivity of human glioblastoma cells through the p53 pathway." Oncol Rep **9**(4): 859-62.

Ouyang, Y. B., Y. Tan, et al. (1999). "Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases." J Cereb Blood Flow Metab **19**(10): 1126-35.

Pearson, T., A. J. Currie, et al. (2003). "Plasticity of purine release during cerebral ischemia: clinical implications?" J Cell Mol Med **7**(4): 362-75.

Pearson, T. and B. G. Frenguelli (2000). "Volume-regulated anion channels do not contribute extracellular adenosine during the hypoxic depression of excitatory synaptic transmission in area CA1 of rat hippocampus." Eur J Neurosci **12**(8): 3064-6.

Powis, G., R. Bonjouklian, et al. (1994). "Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase." Cancer Res **54**(9): 2419-23.

Schmidt-Kastner, R. and T. F. Freund (1991). "Selective vulnerability of the hippocampus in brain ischemia." Neuroscience **40**(3): 599-636.

Shimizu, S., Y. Eguchi, et al. (1998). "Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux." Proc Natl Acad Sci U S A **95**(4): 1455-9.

Siesjo, B. K. (1984). "Cell damage in the brain: a speculative synthesis." Acta Psychiatr Scand Suppl **313**: 57-91.

Snider, B. J., F. J. Gottron, et al. (1999). "Apoptosis and necrosis in cerebrovascular disease." Ann N Y Acad Sci **893**: 243-53.

Somjen, G. G. (2001). "Mechanisms of spreading depression and hypoxic spreading depression-like depolarization." Physiol Rev **81**(3): 1065-96.

Somjen, G. G., P. G. Aitken, et al. (1990). "Spreading depression-like depolarization and selective vulnerability of neurons. A brief review." Stroke **21**(11 Suppl): III179-83.

Stedman, T. L. (2000). Stedman's Medical Dictionary, Lippincott Williams & Wilkins.

Sugar, O. a. G. (1938). J. Neurochem **1**: 558.

Sun, G. Y., J. P. Zhang, et al. (1995). "Inositol trisphosphate, polyphosphoinositide turnover, and high-energy metabolites in focal cerebral ischemia and reperfusion." Stroke **26**(10): 1893-900.

Sweeney, M. I. (1997). "Neuroprotective effects of adenosine in cerebral ischemia: window of opportunity." Neurosci Biobehav Rev **21**(2): 207-17.

Syntichaki, P. and N. Tavernarakis (2003). "The biochemistry of neuronal necrosis: rogue biology?" Nat Rev Neurosci **4**(8): 672-84.

Tamura, A., D. I. Graham, et al. (1981). "Focal cerebral ischaemia in the rat: 2. Regional cerebral blood flow determined by [14C]iodoantipyrine autoradiography following middle cerebral artery occlusion." J Cereb Blood Flow Metab **1**(1): 61-9.

Tanaka, E., S. Yamamoto, et al. (1997). "Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro." J Neurophysiol **78**(2): 891-902.

Tanti, J. F., S. Grillo, et al. (1997). "Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes." Endocrinology **138**(5): 2005-10.

Taylor, J. M., U. Ali, et al. (2005). "Diminished Akt phosphorylation in neurons lacking glutathione peroxidase-1 (Gpx1) leads to increased susceptibility to oxidative stress-induced cell death." J Neurochem **92**(2): 283-93.

Vander Heiden, M. G., N. S. Chandel, et al. (1999). "Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange." Mol Cell **3**(2): 159-67.

Whittingham, T. S., W. D. Lust, et al. (1984). "An in vitro model of ischemia: metabolic and electrical alterations in the hippocampal slice." J Neurosci **4**(3): 793-802.

Wipf, P. and R. J. Halter (2005). "Chemistry and biology of wortmannin." Org Biomol Chem **3**(11): 2053-61.

Wu, L. G. and P. Saggau (1994). "Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus." Neuron **12**(5): 1139-48.

Yamashima, T. (2000). "Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates." Prog Neurobiol **62**(3): 273-95.

Yano, S., M. Morioka, et al. (2001). "Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus." J Cereb Blood Flow Metab **21**(4): 351-60.

Yano, S., H. Tokumitsu, et al. (1998). "Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway." Nature **396**(6711): 584-7.

Yao, R. and G. M. Cooper (1995). "Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor." Science **267**(5206): 2003-6.

Yuan, J., M. Lipinski, et al. (2003). "Diversity in the mechanisms of neuronal cell death." Neuron **40**(2): 401-13.

Zheng, W. H. and R. Quirion (2004). "Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival." J Neurochem **89**(4): 844-52.

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**(2): 128-31.

Zola-Morgan, S., L. R. Squire, et al. (1992). "Enduring memory impairment in monkeys after ischemic damage to the hippocampus." J Neurosci **12**(7): 2582-96.

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