The roles of Hypoxia Inducible Factor-1, Glutamate and their interaction in Ischemic Tolerance

By

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Abstract

Stroke is a debilitating disorder with no effective treatments. The clinical importance of promoting cerebral ischemic tolerance is apparent; however, our knowledge of the signaling mechanisms involved in the phenomenon of ischemic preconditioning remains insufficient to be able to translate to the clinical environment. This dissertation is aimed at an investigation of endogenous pathways, which are both innate and adaptive (in response to a preconditioning stimulus), and by which the brain might protect itself from ischemia. Defining key proteins and mechanisms involved in those pathways can potentially be used as tolerance-producing drugs. Hypoxia Inducible Factor-1α (HIF-1α) is the major regulator of oxygen homeostasis and functions to induce a wide array of transcripts. HIF-1's downstream targets mediate a number of cellular mechanisms that can facilitate cellular adaptation to hypoxia and promote survival. We have sought to understand the mechanisms of HIF-1 stability and degradation and to determine its use as a potential therapy for the treatment of stroke (Chapter 1). In the brain, HIF-1 is present in neurons as well as astrocytes. Considering their important role in maintaining glutamate homeostasis and providing support necessary for the survival of neurons, we examined the effects of glutamate toxicity on the viability of astrocytes exposed to hypoxia and the role that HIF-1 plays under these conditions (Chapter 2). Moreover, given that glutamate toxicity is responsible for the majority of the damage that occurs after ischemia, we aimed to better understand the mechanisms of tolerance induced by adaptation to chronic levels of glutamate (Chapter3).

Overall, in this thesis we identified novel pathways that promote neuronal and astrocytic survival after ischemia. First, we demonstrated that HIF-1α oxidation by the formation of reactive oxygen species during ischemia increased its susceptibility to degradation by the 20S

proteasome. Proteasome inhibition increased HIF-1 accumulation and reduced ischemia-induced neurotoxicity. Next, we observed that stabilization of HIF-1α under hypoxia has a protective effect on astrocytes in maintaining cell morphology and viability in response to glutamate toxicity. We found that the presence of the antioxidant, glutathione, was correlated with an increased expression of HIF-1α and its inhibition decreased HIF-1α protein levels and cell viability. Finally, for the first time we show that an *in vivo* model of glutamate preconditioning was also able to induce tolerance and decrease brain infarct volume in addition to edema volume in both young adult (9-month) and aged (22-month) mice. Taking advantage of these mechanisms may lead to the development of more feasible and less dangerous therapeutic agents and approaches to treat ischemic strokes.

Dedicated to the memory of my grandmother:

Hanem Ghandar

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List of Abbreviations

·OH	Hydroxyl radical
2Me2	2-methoxyoestradiol
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BSO	l-butathione sulfoximine
CA1	Carbonic anhydrase 1
CA3	Carbonic anhydrase 3
CNS	Central nervous system
CREB	Cyclic AMP responsive element binding protein
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAAT1	Excitatory amino acid transporter-1
ENT-1	Equilibrative nucleoside transporter 1
EPO	Erythropoietin
Epox	Epoxomicin
ETS	Electron transport chain
FBS	Fetal bovine serum
FOXO	Fork-head box protein O4
GABA	Gamma-Aminobutyric acid
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
Glud1	Glutamate dehydrogenase 1
GSH	Reduced glutathione
GSK3β	Glycogen synthase kinase 3 beta
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HIF-1	Hypoxia-inducible factor-1
HIF-OH	Hydroxylated HIF-1α
HO-1	Haem oxygenase 1
HSP	Heat-shock protein
iGluR	Ionotropic glutamate receptors
K^+_{ATP}	ATP-sensitive potassium channel

KO_2	Potassium superoxide
LDH	Lactate dehydrogenase
MAP2	Microtubule-associated proteins-2
MCAO	Middle Cerebral Artery Occlusion
MCB	Monochlorobimane
mGluR	Metabotropic glutamate receptor
mK^+_{ATP}	Mitochondrial potassium ATP channel
MRC	Mitochondrial respiration capacity
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
Nse	Neuron-specific enolase
OGD	Oxygen/glucose deprivation
PBN	N-t-butyl-alpha-phenylnitrone
PHD	Prolyl hydroxylases
PI3	Phosphatidylinositol 3
pVHL	Von Hippel-Lindau
RACK	Receptor of activated protein kinase
ROS	Reactive oxygen species
rt-PA	Recombinant tissue plasminogen activator
siRNA	Small interfering Ribonucleic aci
SO	Stratum oriens
SOD	Superoxide dismutase
SP	Stratum pyramidale
SR	Stratum radiatum
SRC	Spare respiratory capacity
SUMO	Small ubiquitin-like modifier
TBST	Tris-buffered Saline with Tween
Tg	Transgenic
TGFβ	Transforming Growth Factor-β
TIA	Transient ischemic attack
TTC	2,3,5-triphenyltetrazolium chloride monohydrate
UBE	Ubiquitin-Conjugating Enzymes
UPS	Ubiquitin Proteasome System
VEGF	Vascular endothelial growth factor
Wt	Wild type
YC-1	3-(50-hydroxymethyl-20-furyl)-1-benzylindazole
α-KG	α-ketoglutarate

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Chapter 1: Introduction

1.1 Background and Significance

Stroke

Stroke is the third leading cause of death in the United States, resulting in approximately 133,000 deaths every year (Lopez *et al.*, 2006). Worldwide, stroke is responsible for 9% of all deaths (Murray & Lopez, 1997). It claims 6.2 million lives each year and is the leading cause of adult disability. Following a stroke 15-30% of stroke survivors are permanently disabled and many require institutional care because of the extent of the damage (Lakhan *et al.*, 2009). Depending on the brain area that is affected, patients can exhibit a variety of symptoms that include paralysis, loss of vision, deficits in speech and understanding, and difficulties with memory. Stroke is caused by the disruption of blood flow in the brain and can be ischemic or hemorrhagic in nature. Ischemic strokes are the most common type and account for 87% of all strokes (Thrift *et al.*, 2001) and occur as a consequence of prolonged occlusion of a blood vessel by a thrombus or embolism (Frizzell, 2005) .

Ischemic Cascade:

The ischemic cascade is triggered following a stroke and can cause irreversible neuronal injury in the ischemic core within minutes of the onset (Dirnagl *et al.*, 1999). This central core is characterized by severely compromised cerebral blood flow and is surrounded by a much larger volume of brain tissue, known as the penumbra. The penumbra refers to the area of the brain that is damaged, having impaired electrical activity but preserved cellular metabolism and viability (Astrup *et al.*, 1981; Hakim, 1987; Kaufmann *et al.*, 1999). The tissue in the penumbra has a variable outcome and can be salvageable if cerebral blood flow can be restored within 6 to 8

hours (Wise *et al.*, 1983; Baron *et al.*, 1995; Marchal *et al.*, 1996; Kaufmann *et al.*, 1999). The central goal of therapy in acute stroke is to target this area and preserve brain tissue that would reduce post-stroke disabilities.

Glutamate-mediated excitotoxicity represents the major mechanism in the pathophysiology of stroke. Glutamate is the major excitatory neurotransmitter in the central nervous system and plays an important role in mediating developmental plasticity and growth, intracellular communication and learning and memory (Matute *et al.*, 2002). Excitotoxicity is caused by the pathological over-activation of the glutamate receptors (Mattson, 2003). The release of glutamate following ischemia activates metabotropic glutamate (mGluR) and ionotropic glutamate receptors (iGluR).

Metabotropic receptors: mGluRs are G-protein coupled receptors and are divided into three groups. Group I mGluR agonists were shown to downregulate the excitatory amino acid transporter-1 (EAAT1) while Group II mGluR agonists upregulated its expression (Gegelashvili *et al.*, 2000). Group II mGluR are present both pre- and post-synaptically and are also located on glial cells (Petralia *et al.*, 1996). Both Groups II and III mGluRs are negatively coupled to cyclic AMP which is derived from ATP. Therefore, a loss of ATP during cerebral ischemia (Folbergrová *et al.*, 1992) will also lead to changes in mGluR activity and a loss of glutamate regulation (Hazell, 2007).

Ionotropic receptors: There are three known types of iGluRs based on their different agonist specificities; the NMDA (N-methyl-D-aspartate) receptor, AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor and Kainate receptor. The AMPA and Kainate receptors mediate fast synaptic transmission and are permeable to Na^+ and K^+ and low concentrations of Ca^{2+} (Hollmann *et al.*, 1991). NMDA receptors are more permeable to Ca^{2+}

and are voltage dependent as a result of ion channel blockade by extracellular Mg^{2+} (MacDermott *et al.*, 1986). This makes their activation secondary to the activation of the AMPA and Kainate receptors that depolarize the neuron and the release of Mg^{2+} blockade (Lynch & Guttmann, 2002). The iGluRs are the major mediators of excitotoxicity, more specifically, the NMDA receptors because of their high Ca^{2+} conductance and the voltage-dependent fluxes (Hazell, 2007). Studies have established that Ca^{2+} overload following iGluR overstimulation is key in glutamate-mediated toxicity. The removal of Ca^{2+} but not Na^{+} led to a decrease in neuronal death following glutamate treatment (Choi, 1987). Tymianski et al. (1993) demonstrated that the neurotoxicity of glutamate was primarily triggered by Ca^{2+} influx through NMDA receptor channels rather than non-NMDA receptors and voltage-sensitive Ca^{2+} channels. This was not due to its ability to trigger greater amounts of Ca^{2+}. They hypothesized that it was a result of a physical co-localization of NMDA receptors with Ca^{2+}-dependent secondary processes that cause neuronal degeneration (Tymianski *et al.*, 1993).

Some of these downstream processes that ultimately result in cell death include the initiation of the arachidonic acid cascade (Dumuis *et al.*, 1988; Lazarewicz *et al.*, 1990; Sanfeliu *et al.*, 1990) the activation of catabolic enzymes such as endonucleases, proteases (Siman *et al.*, 1989) and lipases (Farooqui & Horrocks, 1991). Ca^{2+} also increases the activity of nitric oxide synthase (Fleming *et al.*, 1997) and the release of nitric oxide (Beckman, 1991; Dawson *et al.*, 1991) and causes the formation of reactive oxygen species (ROS) (Pellegrini-Giampietro *et al.*, 1990). ROS can cause damage through a direct oxidation of proteins, lipids and nucleic acids leading to the breakdown of the cell membrane, the cytoskeleton and the genomic DNA. The accumulation of intracellular Ca^{2+} can also cause mitochondrial dysfunction and the activation of

cell signaling pathways such as the caspase cascade (Sugawara *et al.*, 2002) and the p53 pathway (Trinei *et al.*, 2002) that lead to apoptosis (Pradeep *et al.*, 2012).

Figure 1.1 summarizes the mentioned biochemical pathways in the ischemic cascade that ultimately result in cell death (Mattson *et al.*, 2000).

Astrocytes and glutamate transporters:

The removal of glutamate from the extracellular space by excitatory amino acid transporters (EAATs) is one of the main functions of astrocytes (Drejer *et al.*, 1983), a process that is essential in maintaining the levels of this neurotransmitter within a normal range (Nicholls & Attwell, 1990; Hazell, 2007). There are five subtypes of Na^+ dependent glutamate transporters in the brain, two of which (EAAT1and EAAT2) are predominantly localized in astrocytes (Danbolt *et al.*, 1992) and provide major buffering of glutamate in brain (Danbolt, 2001).

Glutamate transport in astrocytes is altered in response to a stroke. A number of processes contribute to excitotoxicity under cerebral ischemia. These include (1) a decrease in glutamate uptake as a consequence of EAAT1 and EAAT2 downregulation (Rothstein *et al.*, 1996; Rao *et al.*, 2001; Yeh *et al.*, 2005) (2) glutamate efflux as a result of transporter reversal (Szatkowski *et al.*, 1990; Longuemare & Swanson, 1995) that occurs when ATP decreases and suppresses of the Na^+/K^+ ATPase (Lees, 1991) after prolonged ischemia. This causes a loss of the ionic gradients necessary to drive the transporter function. (3) Enhanced glutamate release caused by the dysregulation of vesicular release (Hazell, 2007).

Considering their important role in maintaining glutamate homeostasis, their involvement in excitotoxicity, and lack of understanding of their involvement in ischemia, more emphasis is now being placed on astrocytes and their roles in cerebral injury and neurodegenerative diseases (Maragakis & Rothstein, 2006).

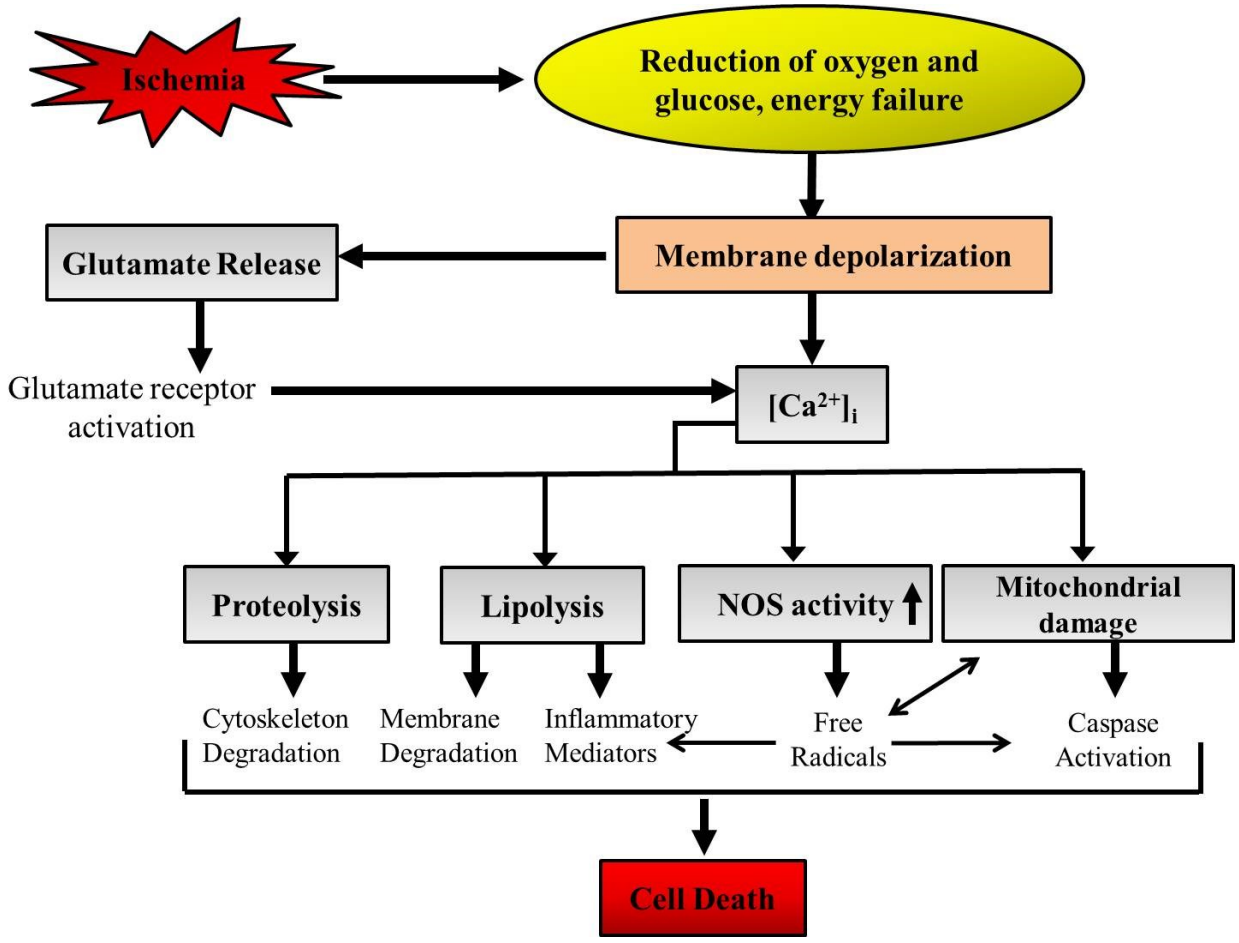


Figure 1.1 Overview of the biochemical cascades that occur in response to ischemia

Adapted from Mattson et al. (2000)

1.2 Treatments for ischemic stroke

More than 1,000 drugs have been tested in various animal models, of these 114 underwent clinical trials (Pérez de la Ossa & Davalos, 2007) that ultimately failed (Lakhan *et al.*, 2009). Consequently, recombinant tissue plasminogen activator (rt-PA) remains the only agent shown to improve stroke outcome. Rt-PA can effectively reduce neurological deficits and improve stoke outcome when administered within three hours after symptom onset (Lakhan *et al.*, 2009). However, a majority of patients do not go to a hospital within these 3 hrs and thus cannot receive this treatment. Rt-PA is also associated with an increased incidence of symptomatic intracranial hemorrhage, which occurs in approximately 6% of patients (Furlan *et al.*, 2003). The optimum treatment of cerebral focal ischemia remains one of the major challenges in clinical medicine. It's been suggested that one of the major reasons for the failure of the clinical trials was an incomplete understanding of the mechanisms responsible for cell death during ischemia (Wang & Shuaib, 2007). Understanding and defining the brains own endogenous neuroprotective mechanisms that occur in ischemic tolerance has been suggested as a good way to unravel the molecular mechanisms and might improve therapeutic strategies for patients with stroke (Dirnagl *et al.*, 2003; Schaller *et al.*, 2003).

Ischemic tolerance and ischemic preconditioning:

Tolerance is a phenomenon in which any stimulus that is applied at a subtoxic level can activate protective mechanisms that can reduce the damage done by a subsequent, more severe exposure. This adaptive capability is fundamental in protecting living cells and allowing them to survive potentially recurrent stressors (Durukan & Tatlisumak, 2010). A solution to reducing ischemic brain damage could be derived from studying the mechanisms by which cells adapt to

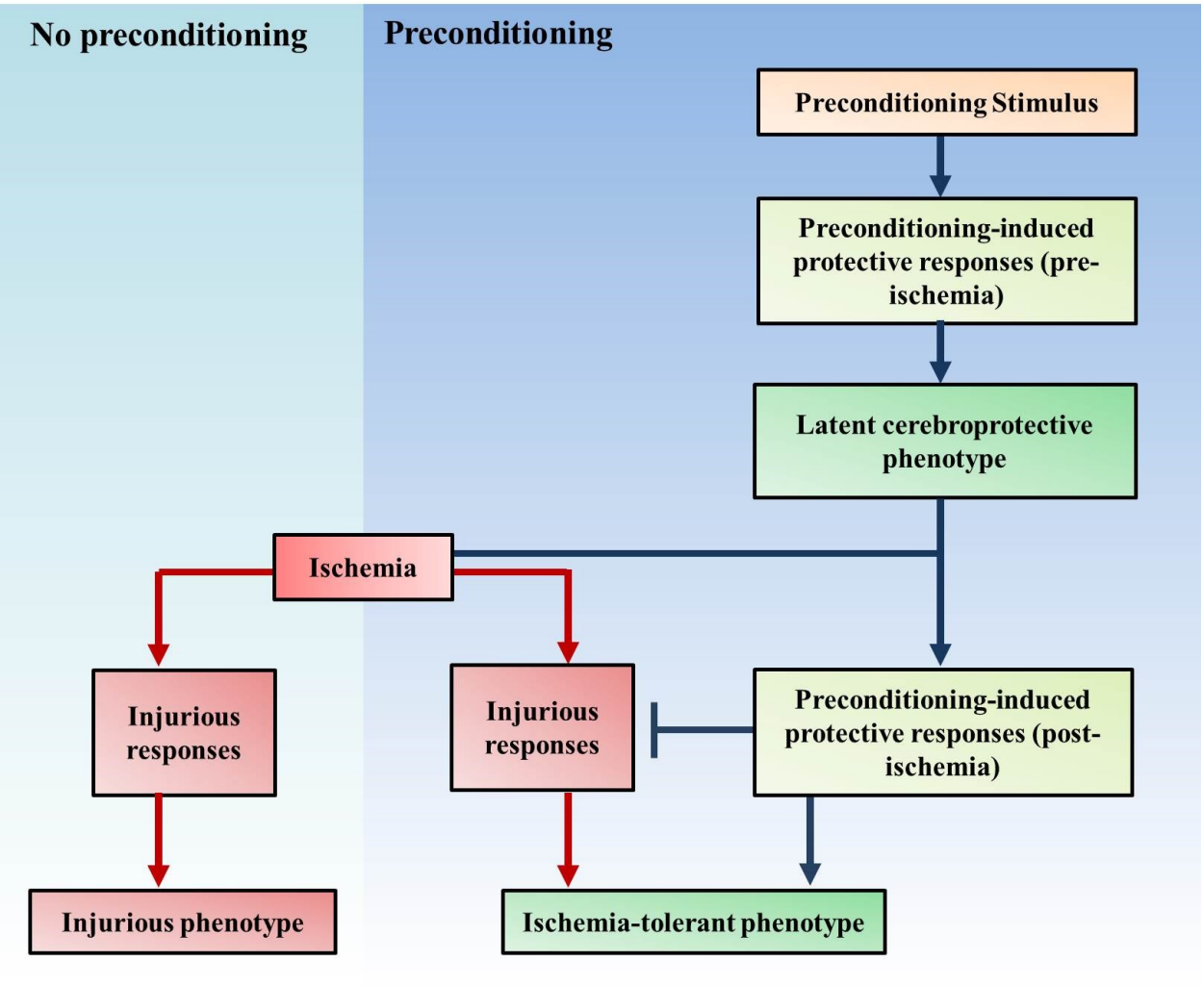
an ischemic stress or ischemic preconditioning. A preconditioning stimulus in the brain will initiate adaptive pathways in cells that will establish a latent cerebroprotective phenotype (Figure.1.2) that prepares the brain for an injurious ischemic event (Gidday, 2006) and promote an ischemic-tolerant phentotype characterized by less tissue damage. This phenomenon has been confirmed in various *in vivo* models of ischemia and in *in vitro* models of primary neurons. Additionally, it has been observed in humans in the form of a transient ischemic attack (TIA) (Wegener *et al.*, 2004) which occurs as a short period of ischemia that does not form an infarct. Studying the molecular and regulatory pathways that participate in preconditioning has provided the opportunity to uncover endogenous neuroprotective mechanisms that can be utilized for the treatment of stroke patients.

1.3 Protective Mechanisms that occur in ischemic tolerance

1.3.1 Excitatory neurotransmitters and receptors:

NMDA receptors:

Preconditioning neurons with exogenous NMDA at subtoxic concentrations was sufficient to induce resistance against subsequent *in vitro* ischemia and glutamate-mediated excitotoxicity (Grabb & Choi, 1999; Tauskela *et al.*, 2001; Jiang *et al.*, 2003). Blocking the NMDA receptor with either MK-801, the non-competitive NMDA receptor antagonist, or LY202157 the competitive NMDA receptor antagonist, during preconditioning eliminated the ischemic tolerance in both *in vivo* and *in vitro* models (Bond *et al.*, 1999). This implies that mild activation of the NMDA receptor plays a role in the protective effect of ischemic preconditioning. One mechanism by which this occurs is that the NMDA receptor mediates rapid



Adapted from Gidday (2006)

Figure 1.1 Cerebroprotection by preconditioning.

Ischemia-induced injurious responses (red arrows) lead to an injurious phenotype in the non-preconditioned brain. However, a preconditioning stimulus promotes the induction of protective responses both pre- and post-ischemia that protect the brain against the injurious responses of ischemia and results in a tolerant phenotype.

adaptation of the voltage-dependent calcium influx. Gerbils subjected to ischemic preconditioning were able to regulate calcium levels and maintain them at concentrations that did not result in neuronal damage whereas non-tolerant gerbils showed an increase in calcium influx (Shimazaki *et al.*, 1998). Another mechanism is through the rapid release of brain-derived neurotrophic factor (BDNF) following the activation of the NMDA receptor (Steiger & Hänggi, 2007). Other key mediators that are involved in NMDA receptor-dependent neuroprotection glycogen synthase kinase 3 beta (GSK3β), phosphatidylinositol 3 (PI3)-kinase and include cyclic AMP responsive element binding protein (CREB) (Soriano *et al.*, 2006; Steiger & Hänggi, 2007). CREB is activated in response to elevated intracellular calcium caused by NMDA receptor activation (Hu *et al.*, 1999). Phosphorylation of CREB is enhanced in the penumbra of preconditioned rats (Nakajima *et al.*, 2002) and is implicated as an important player in NMDA receptor preconditioning (Liu *et al.*, 2009).

AMPA receptors:

The role of the AMPA receptor in ischemic preconditioning is not well established and contradictory results exist (Durukan & Tatlisumak, 2010). Some studies have shown that the AMPA and kainate receptor blocker DNQX only partially attenuates ischemic tolerance (Bond *et al.*, 1999; Gonzalez-Zulueta *et al.*, 2000) in primary neurons while others have shown that the receptor blockade does not eliminate the preconditioning effect (Duszczyk *et al.*, 2005).

Calcium:

The role of Ca^{2+} is not well definied in ischemic tolerance (Tauskela & Morley, 2004), however, the chelation of Ca^{2+} before and during oxygen and glucose deprivation (OGD) prevents ischemic tolerance *in vitro* (Raval *et al.*, 2003; Durukan & Tatlisumak, 2010).

Adenosine and ATP-sensitive K^+ (K^+_{ATP}) channels:

Adenosine is an endogenous neuroprotectant that can inhibit the release of excitatory neurotransmitters. During ischemia, adenosine is formed as a degradation product of ATP. Studies have demonstrated that adenosine A1 receptors are essential in preconditioning-induced protection in the brain (Heurteaux *et al.*, 1995). The activation of A1 receptors leads to an inhibition of synaptic transmission, glutamate release and presynaptic calcium fluxes that would otherwise lead to cell damage and death. Ischemic preconditioning increased the A1 receptor in the rat hippocampus (Zhou *et al.*, 2004). Adenosine A1 receptor antagonists attenuated or abolished the ischemic tolerance phenomenon in rats (Yoshida *et al.*, 2004) and gerbils (Hiraide *et al.*, 2001).

Plasma membrane K_{ATP} channels are found widely throughout the brain (Rodrigo & Standen, 2005) and their activation also plays a role in brain ischemic preconditioning. Treatment with K_{ATP} channel antagonists abolished ischemic tolerance (Reshef *et al.*, 2000) and also canceled the protective effect of adenosine or the use of an A1 receptor agonist (Hiraide *et al.*, 2001). On the other hand, a K_{ATP} channel agonist induced ischemic tolerance (Auchampach *et al.*, 1992; Gross & Auchampach, 1992; Yao & Gross, 1994).

Excitatory amino acid transporters:

Changes in EAATs density can contribute to the adaptive changes that occur during ischemic tolerance. An upregulation of EAATs could be protective by increasing the efficiency of extracellular glutamate removal (Obrenovitch, 2008). However, a downregulation can also be beneficial when ischemia is severe enough to induce anoxic depolarization (Jabaudon *et al.*, 2000) by reducing glutamate efflux caused by reversed uptake (Rossi *et al.*, 2000). Both *in vivo*

and *in vitro* studies have provided contradictory results for the effect of preconditioning on EAATs. Ischemic preconditioning in an *in vivo* stroke model in rats led to an upregulation of EAAT2 and EAAT3 but had no effect on EAAT1 expression (Cimarosti *et al.*, 2005; Pradillo *et al.*, 2006). Another study, also in rats, showed that preconditioning downregulated both EAAT1 and EAAT2 (Douen *et al.*, 2000). EAAT2 and EAAT3 were upregulated in primary cortical neurons that were subjected to sublethal oxygen-glucose deprivation (OGD). The preconditioned neurons also had increased glutamate uptake (Romera *et al.*, 2004). Ischemic preconditioning in a neuron/astrocyte co-culture in different studies showed a downregulation of the EAAT2 (Kosugi *et al.*, 2005; Yamada *et al.*, 2006).

1.3.2 Mitochondria function

The reperfusion period following a stroke in which the blood flow is restored, significantly contributes to injury that occurs. Various studies have shown that mitochondria dysfunction plays a role in this mechanism of injury. Dave *et. al* observed that mitochondrial function after brain ischemia was maintained in the rat hippocampus by ischemic preconditioning. They show that preconditioning restored the deficits in complex I-IV respiration. It also improves respiration (Dave *et al.*, 2008), oxidative phosphorylation (Dave *et al.*, 2001) and allows mitochondria to be more resistant to depolarization (Cho *et al.*, 2005; Zhou *et al.*, 2012). Since mitochondria are the main source of ROS, a preservation of mitochondrial function would reduce ROS generation. An interesting study by Bugger *et. al* showed that mitochondria that were isolated from an atrophied heart was more resistant to ischemia than a normotrophic heart (Bugger *et al.*, 2006). Since atrophied hearts are characterized by an increase in ROS (Muller *et al.*, 2007), that previous exposure to ROS could be the reason for the protection observed after the ischemia.

1.3.3 Heat Shock Response and protein degradation

Heat shock proteins (HSPs)

HSPs are molecular chaperones that assist in the folding of newly synthesized proteins. They are also among the proteins that are synthesized during ischemia when most proteins synthesis is inhibited, suggesting they could have an important role in maintaining cell viability (Liu *et al.*, 2009). HSPs are expressed both constitutively or they can be inducible under conditions of stress. Inducible HSPs contribute to cytoprotection by preventing protein denaturation and improper aggregation (Franklin *et al.*, 2005). In particular, the role of 72-kDa HSPs, or HSP70 has been well characterized and studied extensively with regard to ischemic preconditioning (Obrenovitch, 2008). HSP70 was increased in the hippocampus of gerbils exposed to a brief period of ischemia and was correlated with an increase in neuronal viability. (Kirino *et al.*, 1991). A rat *in vivo* model of ischemic preconditioning also resulted in increased HSP70 protein expression (Liu *et al.*, 1993; Nishi *et al.*, 1993). HSP70 suppression in gerbils 2 hrs before or 3 hrs after the preconditioning stimulus inhibited the induction of ischemic tolerance in the hippocampus (Nakata *et al.*, 1993; Obrenovitch, 2008).

The mechanisms of HSP70-mediated cytoprotection are not only limited to its important role in protein folding and transport. Mutated HSP70s that were able to bind to denatured proteins but not fold them were still protective in both *in vivo* and *in vitro* models of ischemia (Sun *et al.*, 2006). The protective actions were attributed to their role in maintaining mitochondrial function in terms of improving respiration, reduced formation of ROS and inhibiting changes in mitochondrial membrane potential (Ouyang *et al.*, 2006). HSP70 is also capable of binding and sequestering apoptosis protease activating factor-1 (Apaf-1) and

preventing the formation of the apoptosome (Saleh *et al.*, 2000). Deleting the HSP70 gene increased the infarct volume after ischemia through enhanced apoptosis as a result of increased cytochrome *c* release into the cytoplasm and subsequent caspase-3 activation (Lee *et al.*, 2004) (Liu *et al.*, 2009).

Ubiquitin Proteasome System (UPS)

The UPS represents the main pathway responsible for the degradation of abnormal or unwanted proteins. Numerous neuropathological diseases, including stroke, have been implicated with an inability of the proteasome to cope with an overproduction of abnormal proteins. When proteins are damaged or are unfolded they tend to form aggregates that are toxic to cells (Taylor *et al.*, 2002; Dobson, 2003). Neuronal death following brain ischemia does not occur immediately but takes place during reperfusion in which cerebral blood flow gradually recovers (Kirino, 1982; Kirino *et al.*, 1984; Nitatori *et al.*, 1995). Previous studies have indicated that during this period there is an accumulation of unfolded proteins and ubi-protein aggregates in the vulnerable ischemic neurons (Hu *et al.*, 2001; Liu *et al.*, 2005a; Liu *et al.*, 2005b; Ge *et al.*, 2007) that lead to their death. However, preconditioning neurons with a short period of sublethal ischemia resulted in decreased protein aggregation after ischemia (Liu *et al.*, 2005a).

1.3.4 Hypoxia-inducible factor (HIF-1α)

Among the transcription factors that are regulated by hypoxia, HIF isoforms, more specifically HIF-1, have gained the most experimental support, (Durukan & Tatlisumak, 2010). HIF-1 proteins are the primary mechanism by which cells respond to hypoxia (Wang & Semenza, 1995) and have been shown to increase during conditions of ischemic tolerance and preconditioning (Bergeron et al., 2000; Bernaudin et al., 2002). Hypoxic preconditioning can be

mimicked using pharmacological activators of HIF such as deferoxamine or cobalt chloride (Bergeron et al., 2000). The binding of HIF-1α to HIF-1β in the nucleus results in the induction of HIF target genes. Several of these genes contribute to protection from ischemia by improving cellular metabolism, vascularization, proliferation and glucose metabolism (Sharp *et al.*, 2004; Durukan & Tatlisumak, 2010). Studies performed on the heart have shown the protective effect of ischemic preconditioning was lost in mice that are heterozygous for the HIF-1α knockout allele (Cai *et al.*, 2003) when HIF-1α was inhibited using siRNA (Eckle *et al.*, 2008).

1.3.5 Reactive oxygen species and defense mechanisms against oxidative stress

ROS are recognized as important mediators of the ischemic/reperfusion injury (Zweier & Talukder, 2006). However, recent studies suggest that oxygen free radicals can trigger signal transduction pathways in ischemic preconditioning process (Wiegand *et al.*, 1999; Wada *et al.*, 2001). The free radical scavenger, N-t-butyl-alpha-phenylnitrone (PBN) abolished the protective action of hypoxia preconditioning and the hydroxyl radical (·OH) was involved in the development of the hypoxic preconditioning phenomenon (Rauca *et al.*, 2000). Murry et al., (1988) showed that administration of oxygen radical scavengers during the first reperfusion period attenuated the beneficial effect of preconditioning on reducing infarct size in the heart. They proposed that a brief ischemic episode that leads to the generation of a low amount of oxygen free radicals which is not sufficient to cause cell death can modify cellular activity and induce preconditioning effects (Murry *et al.*, 1988).

Under normal conditions, the first line of defense triggered in response to an oxidant insult is the production of antioxidants that can prevent damage by scavenging the free radicals. However, during ischemia oxidative stress is not only due to an increased generation of ROS but

also because of the reduction in the levels of antioxidants due to increased consumption or changes in the expression and activities of antioxidant enzymes that include superoxide dismutase (SOD), catalase and glutathione peroxidase (Lyrer *et al.*, 1991; Mahadik *et al.*, 1993; Love, 1999; Lewen *et al.*, 2000). Another neuroprotective mechanism induced by ischemic preconditioning is the upregulation of antioxidants that reduce oxidative stress caused by ischemia and reperfusion. These include antioxidant enzymes such as SOD (Yuan *et al.*, 2010) and low molecular weight antioxidants such as ascorbic acid (Glantz *et al.*, 2005). Transgenic mice that over-express SOD were resistant to injury following cerebral ischemia and reperfusion (Yang *et al.*, 1994).

1.3.6 Glial Ischemic tolerance

Astrocytes extensively communicate with neurons which they ensheath. Changes in the brain environment are effectively sensed by astrocytes to which they are able to respond immediately at a genomic (e.g., trophic factors) and non-genomic level (e.g., uptake of glutamate) (Trendelenburg & Dirnagl, 2005). The response of astrocytes to a preconditioning stimulus is crucial to the foundation of the overall cerebroprotective phenotype (Gidday, 2006). The supportive function of astrocytes is not limited to their own protection but to neurons as well which they are in intimate contact with. Astrocytes promote ischemic resistance of neighboring neurons through various mechanisms which include improved ionic buffering and transferring energy substrates and intermediates for the de novo synthesis of the neurotransmitters glutamate and gamma-Aminobutyric acid (GABA). Astrocytes are the main source of the hormone erythropoietin (EPO) and are major contributors to the antioxidant defense of the brain and play a central role free radical and reactive metal scavenging. The generation of anti-inflammatory cytokines, heat shock proteins and trophic factors such as Transforming Growth Factor-β

(TGFβ), Brain-Derived Neurotrophic Factor (BDNF) and the Glial Cell Line-Derived Neurotrophic Factor (GDNF) by preconditioned astrocytes contribute to ischemic tolerance (Gidday, 2006).

Activation of astrocytes and microglia/macrophages can be triggered in response to ischemia. Morphologically, they adopt a more immature phenotype (Kajihara *et al.*, 2001) and undergo morphological changes where they extend their processes and thus contribute to brain tissue damage by causing regeneration failure. Studies have shown however, that ischemic preconditioning can attenuate the activation of astrocytes and microglia following cerebral ischemia (Kato *et al.*, 1994; Rosenzweig *et al.*, 2004) through the repression of NF-κB mediated transcription of inflammatory mediators (Ginis *et al.*, 2002).

Further research is needed to understand whether and how astrocyte protection can be therapeutically utilized after cerebral ischemia. This could be either done by boosting astrocytic neuroprotection, or by delivering therapeutics that mimic astrocyte derived neuroprotectants.

1.4 Potential clinical applications

1.4.1 Ischemic preconditioning

A study by Chan et al. (2005) evaluated the effects of temporary artery occlusion on brain tissue gases and acidity during a cerebral aneurysm clipping to produce an ischemic preconditioning model. A multiparameter catheter was inserted to determine oxygen and carbon dioxide tension, and the pH levels in tissue that was at risk of ischemic injury during the temporary artery occlusion. Patients with aneurysmal subarachnoid hemorrhage were used for the study. The preconditioning group was subjected to an initial 2 min occlusion of a proximal artery followed by 30 min of reperfusion before undergoing the cerebral artery occlusion for the

clipping of the aneurysm. The results demonstrated that the preconditioned group showed a significant decrease in the decline of oxygen tension and pH compared to the control, untreated group. This attenuation in tissue hypoxia suggests that a brief occlusion of the proximal artery provides brain protection following the subsequent artery occlusion (Chan *et al.*, 2005).

Another study by Wegener et al. (2004) investigated whether transient ischemic attacks (TIAs) can raise the threshold of brain vulnerability and induce tolerance in response to a subsequent stroke. They concluded that the initial lesions tended to be smaller and final infarct volumes were also drastically reduced in patients with a history of TIA. These patients also exhibited milder clinical deficits (Wegener *et al.*, 2004). Other reports show that TIAs appear to be neuroprotective when they were recent, when multiple attacks occurred (not more than three) and when they were of a shorter duration (less than 20 min) (Moncayo *et al.*, 2000). Elderly patients that were over the age of 65 did not benefit from TIAs (Della Morte *et al.*, 2008). This protective effect of TIAs on reducing infarct size and facilitating thrombolysis (Wegener *et al.*, 2004; Schaller, 2005) suggests the existence of endogenous neuroprotection in the human brain (Dezfulian *et al.*, 2013).

1.4.2 Ischemic postconditioning

Ischemic postconditioning refers to ischemic tolerance that occurs as a result of an interruption of blood flow during the reperfusion period. These short bursts of ischemia that were performed at the time of reperfusion lead to a decrease in the volume of the damaged tissue (Lehotský *et al.*, 2009). Similar to ischemic preconditioning, postconditioning was initially demonstrated in the heart in a cat model of regional cardiac ischemia. The results demonstrated that intermittent reperfusion prevented reperfusion-induced ventricular fibrillation and was as

effective as ischemic preconditioning (Na *et al.*, 1996). This was further confirmed as a protective treatment in the heart (Zhao *et al.*, 2003; Galagudza *et al.*, 2004; Kin *et al.*, 2004), and then demonstrated in other organs that include the liver (Sun *et al.*, 2004), kidney (Liu *et al.*, 2007), muscles (McAllister *et al.*, 2008), skin (Moon *et al.*, 2008) and the intestinal mucosa (Santos *et al.*, 2008). Brain Ischemic postconditioning with a series of interruptions of reperfusion also proved to be protective and increased neuronal survival rate in an *in vivo* model of focal ischemia (Zhao *et al.*, 2006; Xing *et al.*, 2008) and global cerebral ischemia (Pateliya *et al.*, 2008; Wang *et al.*, 2008).

Little is known about the mechanisms of ischemic postconditioning as it is still a novel neuroprotective approach. There is evidence that ischemic postconditioning can improve cerebral blood distribution by reducing the effects of hyperemia and the resulting hypoperfusion (Wang *et al.*, 2008). Loukogeorgakis et. al. (2006) observed that postconditioning can reduce tissue injury when applied at the onset of reperfusion (Loukogeorgakis *et al.*, 2006). This provides a narrow therapeutic window of opportunity and would be challenging in a clinical setting where the interruption of reperfusion would not be performed at the end of ischemia (Lehotský *et al.*, 2009). However, other studies using a delayed postconditioning paradigm that occurred 3-6 hours after focal ischemia (Ren *et al.*, 2008) or 48 hrs after ischemia (Burda *et al.*, 2006) reduced infarct size. Thus, targeting neuronal death that occurs in a delayed manner broadens the potential therapeutic window for inhibiting processes that lead to apoptosis.

1.4.3 Future Clinical Considerations

The delivery of brief ischemia in either a preconditioning or postconditioning paradigm can be technically challenging and there is still much controversy over whether it is possible to

be used as a therapeutic measure to promote neuronal survival in the brain. Even a very short period is bound to have a harmful effect and thus ethical and safety concerns still exist. There was some evidence of a therapeutic benefit in clinical trials of ischemic preconditioning in which patients that were on cardio-pulmonary bypass were subjected to aortic clamping before the procedure (Hausenloy & Yellon, 2009). The heart however, is less sensitive than the brain and can better tolerate such an insult without cell death (Dezfulian *et al.*, 2013). A study by Oppenheim *et al.*(2006) determined that a long-tern MRI follow up of TIA patients revealed infarcts and lesions in attacks that were previously thought of as being harmless (Oppenheim *et al.*, 2006). Hallmarks of injury were also observed in experimental models with a longer follow-up duration and when more sensitive detection methods were utilized (Sommer, 2008). In other experimental animal models, different combinations of ischemic preconditioning were not protective and caused more severe injuries following a subsequent ischemic episode (Tomida *et al.*, 1987). Different human populations also exhibit various levels of sensitivities. For instance, geriatrics, which would be the target population, or females tend to be less tolerant to injury and might not benefit from these protective effects. Furthermore, different pre-existing conditions or medications used by patients will also have to be put into consideration (Lehotský *et al.*, 2009). Therefore, identifying a safer stimulus to induce tolerance would be both more practical and effective.

1.4.4 Stimulus Inducing Tolerance

Different activating mechanisms and paradigms have been shown to induce ischemic tolerance (Table 1.1). Brief periods of global ischemia (Kitagawa *et al.*, 1991), focal ischemia (Stagliano *et al.*, 1999) or cerebral hypoxia represent the prototypical preconditioning stimuli (Lehotský *et al.*, 2009). However, preconditioning can be induced through exposure to

Table 1.1 Various inducing stimuli leading to ischemic tolerance in different *in vivo* and *in vitro* models.

Adapted from Lehotsky et al (2012)

Inducing Stimuli	Experimental models
Sublethal preischemia	G, R, M, C
Sublethal postischemia	G, R, M
Spreading depression/excitoxicity	R, M
Remote ischemia	R, M
pH/ion imbalance	R, M, C
Oxidative/nitrosative stress	G, R, M, C
Other factors (hormones, toxins)	G, R, M, C
Lipopolysaccharides	R
Immunization/inflammatory cytokines	G, R, M, C
Hypoxia or HIF modifiers	G, R, M, C
Hypoperfusion	G, R, M
Hypoglycemia/metabolic inhibitors	G, R, M, C
Hyper/Hypothermia	G, R, C
Exercise	G, R, M
Erythropoietin	G, R, M
Epilepsy	M
Anesthetics	R, M

G, gerbil; R, rat; M, mouse; C, cell culture model; HIF, hypoxia inducible factor

endogenous or exogenous stimuli such as hypoperfusion (Sharp & Bernaudin, 2004), hyperoxia or oxidative/nitrosative stress, cortical spreading depression (Kobayashi *et al.*, 1995), and hyper or hypothermia and heat shock (Nishio *et al.*, 2000). Pharmacological agents such as metabolic inhibitors, lipopolysaccharides and inflammatory cytokines or natural regulators such as EPO or other hormones can also induce ischemic tolerance (Lehotský *et al.*, 2009). The phenomenon in which a noxious stimulus can induce cellular tolerance to a subsequent stimulus that is different in nature from the first is referred to as cross-tolerance.

1.5 In Summary

Our knowledge of the complex pathophysiology and the mechanisms of ischemic preconditioning remain insufficient to be able to translate to a clinical setting. Defining key proteins that proved to be protective can potentially be used as tolerance producing drugs. HIF-1α is the major regulator of oxygen homeostasis and functions to induce a wide array of transcripts. HIF-1's downstream targets mediate a number of cellular mechanisms that can facilitate cellular adaptation to hypoxia and promote survival. Even without preconditioning, brain cells naturally respond to hypoxia by triggering a host of defenses to counter cell injury and death (Sapolsky, 2001); the most critical of these responses being the stabilization of HIF-1α. HIF-1 is part of the fundamental endogenous protective responses that occur after stroke that can be innate but is also adaptive following a preconditioning stimulus. We have sought to understand the mechanisms of HIF-1 stability and degradation and to determine its use as a potential therapy for the treatment of stroke.

Ischemia initiates a complicated biochemical cascade of events that triggers cell death. These events include the release of glutamate, excitotoxicity induced by over-activation of glutamate receptors, Ca^{2+} influx, mitochondrial dysfunction, ROS production, activation of enzymes, inflammation and apoptosis (Figure 1.1). The modulation of any of these molecular targets within the ischemic cascade can induce protection. In our studies, we chose to purse the effects of glutamate as glutamate-induced toxicity is the major cause for the initiation and maintenance of this cascade. Moreover, excitatory neurotransmitter, receptors and transporters are important contributors to ischemic preconditioning. We examined the effects of glutamate toxicity on the viability of astrocytes exposed to hypoxia and the role that HIF-1 plays under these conditions. We also aimed to better understand the mechanisms of tolerance induced by adaptation to chronic levels of glutamate. Taking advantage of these mechanisms may lead to the development of safer therapeutic agents and more feasible approaches to treat ischemic strokes.

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Chapter 2: A comparative study: effects of proteasome and hydroxylation inhibitions on hypoxia inducible factor 1α degradation in cerebral ischemia

2.1 ABSTRACT

Hypoxia inducible factor-1 (HIF-1) is a key regulator in hypoxia and can determine the fate of brain cells during ischemia. However, the mechanism of HIF-1 regulation is still not fully understood in ischemic brains. We previously demonstrated that the presence of glucose maintained the level of HIF-1α protein, the regulatable subunit of HIF-1, in ischemic neurons. In this study, we tested a hypothesis that both the 26S and the 20S proteasomal pathway were involved in HIF-1α degradation under ischemic conditions due to the action of reactive oxygen species (ROS). We observed that superoxide anion radical increased 20S but decreased 26S proteasomal activity in SH-SY5Y cells. In the concentration range of 0-200 μM, H_2O_2 elevated 20S proteasomal activity while it only increased 26S activity at concentrations less than 100 μM. Pre-treatments of the SOD mimic and catalase decreased both 20S and 26S proteasomal activity in SH-SY5Y cells exposed to oxygen and glucose deprivation (OGD). We also demonstrated that proteasome inhibitors increased HIF-1α stabilization and cell viability and were more effective than prolyl hydroxylase inhibitors, which inhibit HIF-1a hydroxylation and thus suppress HIF-1α degradation through 26S proteasomal pathway, indicating the involvement of 20S in the HIF-1α degradation. Furthermore, the administration of the proteasome inhibitor, epoxomicin, to mice subjected to a mouse model of middle cerebral artery occlusion resulted in a reduction in infarct size compared to the untreated animals. Overall, our results indicate that proteasomal inhibition promotes HIF-1α stabilization during ischemia and has a protective effect on neurons in reducing ischemia-induced cytotoxicity.

2.2 INTRODUCTION

Hypoxia inducible factor 1 (HIF-1), a transcription factor, is considered to be the most critical factor involved in the cellular response to hypoxia. This is mainly due to its regulation of 1-2% of human genes that play important roles in cellular adaptation to low oxygen (Mazure *et al.*, 2004). HIF-1 is a heterodimeric protein formed by a continuously expressed subunit HIF-1β and an oxygen regulated subunit HIF-1α (Wang *et al.*, 1995a). Under normal oxygen levels, HIF-1α is degraded through the ubiquitin-dependent proteasomal (26S) pathway, which requires the oxygen for prolyl hydroxylases (PHD) to hydroxylate the protein that is recognized by Von Hippel-Lindau tumor suppressor (pVHL), an E3 ubiquitin ligase. During hypoxia, HIF-1α is stabilized in cells due to reduced hydroxylation resulting from an inactivation of the hydroxylase activities. We and others have shown that low oxygen does not increase the level of HIF-1a protein in cells exposed to oxidative stress such as in hypoxic neurons in the absence of glucose (Guo *et al.*, 2008) The mechanism of low expression of HIF-1a in these conditions remains unclear.

The proteasomal proteolytic pathways are the main mechanisms responsible for the degradation of abnormal or unwanted proteins, which contain 26S and 20S proteasomal pathways. The 26S proteasome is the most abundant form and is composed of a 20S catalytic core and two regulatory 19S caps (Coux *et al.*, 1996). In order for a protein to be targeted to the 26S proteasome, it requires a polyubiquitin tail which can be detected by the 19S for subsequent processing and unfolding. The 20S proteasome complex can exist on its own and unlike the 26S proteasome it does not target ubiquitinated proteins but may act on oxidized proteins (Coux *et al.*, 1996). Although both the 26S and 20S can degrade oxidized proteins, many studies have determined that the contribution of the 20S is significantly higher (Davies, 2001; Breusing &

Grune, 2008; Jung & Grune, 2008)). Since HIF-1α contains residues with redox properties such as cysteine and methionine, it is an oxidizable protein (LE, 1996; Huang *et al.*, 1998b). This potentially makes HIF-1α a target for degradation by the 20S proteasome in an oxidizing environment. Although they do not increase pVHL binding or ubiquitination activities (Jahngen-Hodge *et al.*, 1997), ROS might activate prolyl hydroxylase under hypoxia and promote 26S proteasomal degradation pathways by restoring the hydroxylation (Haddad, 2002; Callapina *et al.*, 2005).

Ischemia is characterized by an increase in ROS formation. We hypothesized that reactive oxygen species (ROS) play a role in HIF-1α degradation during ischemia through activation of not only the ubiquitin-dependent (26S) but also the ubiquitin-independent (20S) proteasomal pathways. To test the hypothesis, we assessed effects of ROS and ischemia on the 26S and 20S proteasomal activities, evaluated the contribution of the proteasomal pathways to HIF-1α degradation during hypoxia, and determined the effect of HIF stabilization through proteasomal inhibition on neuronal viability and brain damage in an *in vitro* and *in vivo* ischemia model. Our results demonstrated that the 20S proteasomal activity was increased by ROS and following ischemia. Furthermore, we indicate a role of the 20S in HIF-1α degradation and that unlike solely inhibiting PHDs, a combined treatment of PHD and proteasome inhibitors is more effective in stabilizing HIF-1α.

2.3 MATERIALS AND METHODS

2.3.1 Culture of SH-SY5Y cells and primary cortical neurons

SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin 1:100) at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2. Medium was changed to DMEM without FBS and without antibiotics at 80% confluence. Cells were then incubated at 37 °C in a humidified hypoxia chamber (Coy laboratory products) with 1% O_2, 5% CO_2 and balance N_2 for 0, 1, 4, or 12 hours (H).

Primary neurons were prepared from the cortical tissue of Sprague–Dawley rat (Charles River Laboratories) brains at embryonic day 16 [E16] to E18. The tissues were washed with Hanks Balanced Salt Solutions (HBSS) and trypsinized for 25 minutes (min) at 37°C. The tissues were then dissociated using a fire polished glass pipette in Dulbecco's Modified Eagle Medium (DMEM) and 10% Fetal Bovine Serum (FBS) transferred into 25 cm^2 flasks in starter medium (DMEM and 10% FBS). After 24 hrs, the medium was replaced by culture medium consisting of 2 mM glutamine and the B27 supplement in Neurobasal media (Gibco). Experiments were conducted 10-12 days following dissection. The University of Kansas Institutional Animal Care and Use Committee approved all procedures.

2.3.2 Assessment of proteasomal activities

Cells were treated with KO_2 at concentrations of 5, 10, 20 and 50 μM, and H_2O_2 at 10, 50,100 and 200 μM in DMEM for 3 hrs. Proteasomal activity was measured as described previously (Fekete *et al.*, 2005). Cells were washed with phosphate buffered saline (pH 7.4) and then lysed by 2 freeze-thaw cycles in lysis buffer (25 mM HEPES (pH 7.8), 0.25 M sucrose,

10mM $MgCl_2$ 1 mM EDTA and 1 mM dithiothreitol (DTT)). The lysates were centrifuged at 11,000 RPM at 4°C for 30 min. 10 µg of cell lysate proteins was incubated with 100 µL of proteasome activity assay buffer. The first assay buffer for evaluation of the 26S proteasome function consisted of 50 mM Tris (pH 7.4), 5mM $MgCl_2$, 2 mM DTT, 2 mM ATP and the fluorogenic substrate Suc-LLVY-AMC (80 µM in 1% DMSO, Sigma-Aldrich). The second buffer for determining 20S proteasome function contained 20 mM HEPES (pH 7.8), 0.5 mM EDTA, 0.03% SDS and 80 µM Suc-LLVY-AMC. The assay monitors the hydrolysis of Suc-LLVY-AMC into AMC (7-amino-4-methly-coumarin) which is then detected with a fluorescence plate reader at ex 380 nm and em 440 nm (24).

2.3.3 *In vitro* protein degradation assays

SH-SY5Y cells were exposed to 1% oxygen for 6 hours. Precipitated HIF-1α protein with or without oxidation by H_2O_2 were incubated with 20S proteasome (Boston Biochem) to determine the proteasome's ability to degrade the HIF-1α. To be able to determine the 26S proteasome's activity, the precipitated HIF-1α was incubated with cytosol fraction from SH-SY5Y cells and the 26S proteasome (Boston Biochem). After HIF-1α was incubated with the proteasome for 1 and 3 hrs, western blotting and ELISA assay were carried out to determine the level of HIF-1α. MG-132 was used to confirm that the degradation was in fact the result of proteasome activity.

2.3.4 Effect of 20S on HIF-1α under hypoxic conditions

To detect the effect of 20S on HIF-1α, proteasome inhibitors MG132 (10 µM, 25 µM, 50 µM) or epoxomicin (2 µM, 4 µM, 8 µM)(Drexler, 1997; Salceda & Caro, 1997; An *et al.*, 1998; Lee & Goldberg, 1998; Adams *et al.*, 1999; Meng *et al.*, 1999b; Zhang *et al.*, 1999; Grune *et al.*, 2002; Zhou *et al.*, 2006) were added in DMEM for 1 hr, and the then cells were treated under

hypoxic conditions for 3 hrs. After treatments, the cells were collected for HIF-1α protein expression by western blotting and ELISA.

2.3.5 Ischemia model

In vitro: Neurons were incubated in 1% O_2/ 5%CO_2 (balanced with N_2) at 37°C for 1 hr in glucose-free medium. Control experiments were conducted at 21% O_2/ 5%CO_2 with medium containing glucose (Goldberg & Choi, 1993). Neuronal viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay kit (Invitrogen) as described previously (Mosmann, 1983).

In vivo: Brain ischemia was induced using the well-established Middle Cerebral Artery Occlusion (MCAO) model in mice (Clark *et al.*, 1997).). Anesthesia for the mice was induced with 3% isoflurane and oxygen and maintained with 1.5% isoflurane, or to desired anesthetic effect, throughout the procedure. Buprenorphine was used as the analgesic and was injected pre-operatively at 0.05 mg/kg. 20-25 g C57/Bl/6 male mice were subjected to MCAO followed by a 24 hr period of reperfusion. TTC (2,3,5-triphenyltetrazolium chloride monohydrate) staining was used to assess brain damage (Ito *et al.*, 1997). Brain edema volume (V_{edema}) was measured from the coronal sections that were stained by TTC by determining the volumes of both the ipsilateral (affected) hemisphere (V_{Ipsi}) and the contralateral hemisphere (V_{contra}) and using the equation: $V_{edema} = V_{contra} - V_{Ipsi}$ (Yan *et al.*, 2011).

2.3.6 Proteasome inhibition and PHD inhibition

Primary cortical neurons were pre-treated for 60 min with the proteasome inhibitors MG-132 (Boston Biochem) (10, 40 and 80 μM) and Epoxomicin (Boston Biochem) (8 μM). Prolyl hydroxylases were inhibited with 2 mM dimethyloxalylglycine (DMOG). For *in vivo* studies

DMOG (50 mg/kg/0.1cc, i.p.) and/or Epox (1.1 mg/kg/0.1cc), i.p.) in DMSO were administered to mice 24 hrs before MCAO.

2.3.7 Immunoblot analysis

Neurons were lysed in 200 μL RIPA buffer (ThermoScientific) containing the protease inhibitor cocktail (Thermo Scientific) and scraped using a cell lifter (Biologix Research Company). The lysates were centrifuged at 12,000 RPM for 10 min at 4°C and the protein concentration of the supernatants was determined using a protein assay kit (Bio-Rad). Proteins were separated by SDS–PAGE and the separated proteins were transferred to a nitrocellulose membrane (BIO-RAD). After being blocked with 5% nonfat milk in Tris-buffered Saline with Tween (TBST), the membrane was incubated with the HIF-1α (1-1000; BD transduction laboratories) or the hydroxyl-HIF (1-1000; Novus) primary antibody overnight at 4°C and the secondary antibody (1-3000 ; goat anti mouse or goat anti rabbit; Santa Cruz) for 1 hr at RT. Western blots were quantified using ImageJ software and protein levels were normalized to β-actin.

2.3.8 Statistical analysis

Data are presented as means ± SD from a minimum of three independent experiments. One-way ANOVA and the Student's *t*-test were used for overall significance. Differences of $p<0.05$ were considered statistically significant. Image-Pro Plus 5.1 (Media Cybernetics), ImageJ, and Excel were used for data analyses.

2.4 RESULTS

2.4.1 ROS alter proteasomal activity in hypoxic SH-SY5Y cells

We first evaluated the effect of ROS on proteasomal activity in SH-SY5Y cells. As shown in Fig. 2.1, ROS had a differential effect on 20S and 26S proteasomal activities. KO_2 generating superoxide anion radical dramatically increased 20S activity at 5, 10, and 20 μM whereas it decreased 26S activity. H_2O_2 in the concentration range of 0-200 μM significantly elevated 20S activity. In contrast, H_2O_2 increased 26S activity only at the lower concentrations of 10 and 50 μM. The results indicate that both 20S and 26S activities can be inhibited or activated by ROS depending on the concentration of ROS and that there is a wider concentration range of $O_2^{\bullet-}$ or H_2O_2 that can increase 20S activity versus the 26S activity.

2.4.2 20S proteasome can degrade HIF-1α under hypoxia

We investigated the effect of 20S on HIF-1α at elevated ROS conditions in a biochemical system. HIF-1α protein was prepared by incubating the cell extracts with anti-HIF-1α antibody and protein A-sepharose beads. Precipitated HIF-1α protein with or without oxidation was incubated with either the 26S or 20S proteasome for 1 or 3 hrs, followed by immunoblotting for HIF-1α. As shown in Fig. 2.2, H_2O_2 treatment alone or in the presence of 26S proteasome had no effect on the level of HIF-1α protein. The level of HIF-1α protein was decreased in the presence of 20S proteasome for 1 hr and there was a further decrease after 3 hrs. In the presence of ubiquitin, 26S proteasome caused HIF-1α degradation whereas 20S proteasome had no effect on the level of HIF-1α.

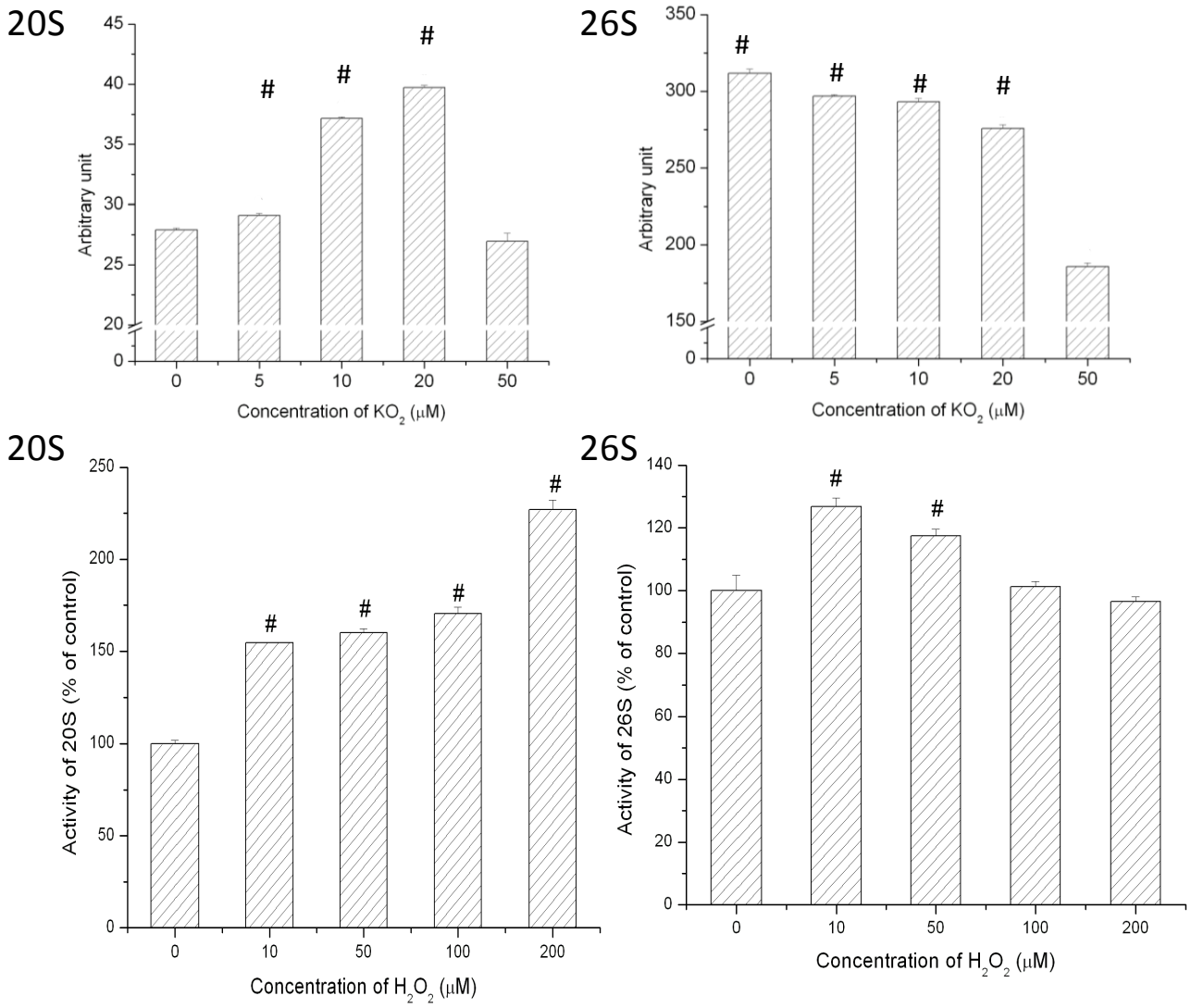


Figure 2.1. Effect of oxidative stress on proteasomal activity. Effect of potassium superoxide (KO_2) and hydrogen peroxide (H_20_2) on the activities of the 26S and 20S proteasome in SY5Y cells exposed to hypoxia for 3 hrs.

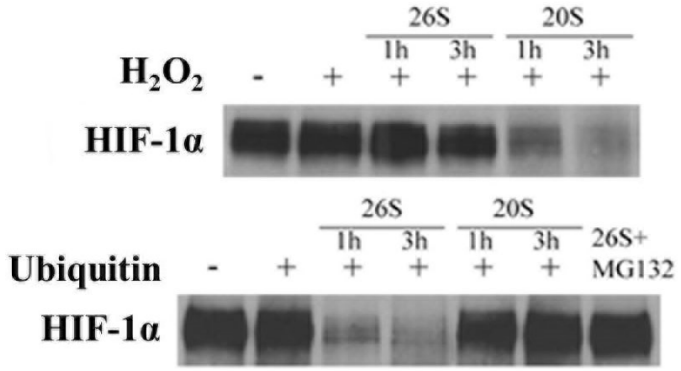


Figure. 2.2. HIF-1α degradation by the 20S proteasome under hypoxia. Immunoblotting for HIF-1α after the HIF-1α protein-beads were incubated with either the 26S or 20S proteasome for 1 and 3 hrs with the addition of H_2O_2 or ubiquitin.

2.4.3 *In vitro* ischemia upregulates proteasomal activity in neurons

Next, we determined whether ischemia could have the same effect on proteasomal activity in neurons. Oxygen-glucose deprivation (OGD) was used as an *in vitro* ischemia model which mimics the loss of oxygen and glucose that occur in a stroke when blow flow is blocked. Primary cortical neurons were exposed to OGD for 30, 60 and 90 min and then the activities of the 26S and 20S proteasome were assessed and compared to neurons at control conditions. Following OGD treatment, there was a mild increase in 26S activity; however, it was not statistically significant (Table 2.1). On the other hand, 20S activity was significantly increased after 30 or 60 min exposure to OGD. The activity began to decrease after 60 min. Since the most significant increase was observed at 60 min, this was the time point at which the rest of the experiments were conducted.

2.4.4 Proteasome inhibition increases HIF-1α stabilization more than hydroxylation inhibition

To determine the contributions of both 20S and 26S proteasomal pathways to the degradation of HIF-1α in ischemic neurons, we carried out the experiments in the presence of proteasome inhibitors and prolyl hydroxylase domain enzyme (PHD) inhibitors. As hydroxylation is a required step for HIF-1α to be degraded through the 26S degradation pathway but not the 20S pathway, PHD and proteasomal inhibitions would differentiate the individual contribution of the two pathways to HIF-1α degradation. MG-132 (10, 40 and 80 μM) was used to inhibit proteasome activities. DMOG at 0.5, 1 and 2 mM was used to inhibit prolyl hydroxylase. These concentrations were selected based on previous reports. As Fig. 2.3 shows, OGD for 60 min increased HIF-1α level in primary cultured cortical neurons. Pretreatment with MG-132 at 10 μM further elevated HIF-1α level (149% increase, compared to OGD only).

Table 2.1. Effect of oxygen-glucose deprivation (OGD) on 26S and 20S proteasomal activity in primary cortical neurons.

OGD duration	Proteasomal Activity (% of control)	
	26S	20S
0 min	100	100
30 min	106.9 ± 5.51	104.8 ± 2.47*
60 min	107.1 ± 3.86	114.4 ± 4.10*
90 min	105.2 ± 2.75	100.5 ± 5.52

Primary cortical neurons were exposed to OGD for 30, 60 or 90 min and then the 26S and 20S proteasomal activity was determined from the lysates. The increase in the 20S proteasome activity following both 30 and 60 min OGD exposure was found to be significant. *$p < 0.05$ vs. 0 min (n=3).

MG-132 at 40 and 80 μM induced a 172% and 174% increase of HIF-1α over OGD only, respectively. A smaller increase in HIF-1α level in the presence of higher levels of MG-132 indicated that at 10 μM, MG -132 was able to effectively inhibit proteasomal activity in primary cultured neurons under OGD condition. Proteasomal inhibition has been linked to cell death, apoptosis and neurotoxicity with prolonged exposure (Williams *et al.*, 2003; Williams *et al.*, 2004; Williams *et al.*, 2005), therefore we chose to use the inhibitor at the lowest effective dosage and shortest incubation time in these experiments. Since there was no significant difference between MG-132 pre-treatment at 40 and 80 μM, the lower dose was used for evaluating the HIF-1α protein levels. DMOG was also able to further increase HIF-1α level, indicating that even under low oxygen conditions the 26S proteasomal pathway was involved in degrading HIF-1α protein. We observed a 57% increase in HIF-1α protein levels in cells pretreated with 0.5 mM DMOG. At 1 and 2 mM, DMOG caused an 83% and 93% increase, respectively, in the protein level. A higher concentration of DMOG did not cause a further increase in HIF-1α stabilization (Supplementary Fig.2.1C), indicating that inhibition of hydroxylation by DMOG at 2 mM is close to its maximal effect. Taken together, these data suggested that inhibiting the proteasome with MG-132 stabilized HIF-1α levels more than DMOG did under an OGD condition.

Next we treated the neurons with a combination of both a proteasome inhibitor (40 μM MG-132 or 8 μM epoxomicin) and a prolyl hydroxylase inhibitor (DMOG 2 mM). Neuronal viability was significantly increased with the combination treatment compared to the control normoxia conditions, OGD, and the individual drug treatments (Fig. 2.3B). Western blot analysis also showed that the combined drug treatments lead to a greater stabilization of HIF-1α (Fig. 2.3C). Immunoblotting for hydroxylated HIF-1α (HIF-OH) confirmed that DMOG was reducing

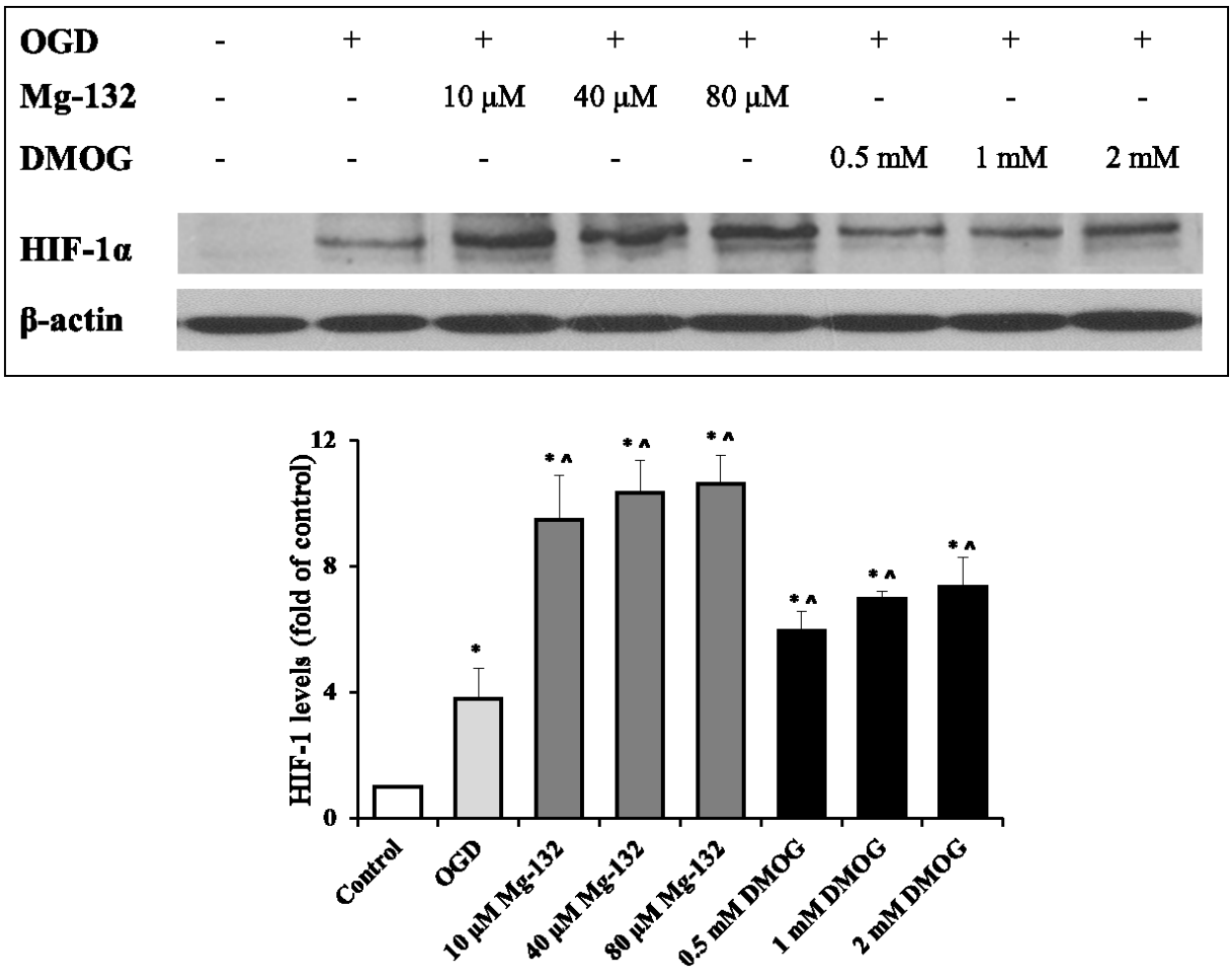


Figure 2.3A. HIF-1α protein stabilization with the treatment of proteasome and prolyl hydroxylase inhibitors. (A) Immunoblotting showing HIF-1α protein levels in neurons and the quantitative results for Western blot data. Equalization of protein loading was determined using β-actin as the housekeeping protein. *p<0.05 versus normoxia (N), $^{\#}p$<0.05 versus oxygen/glucose deprivation (OGD) (n= 3).

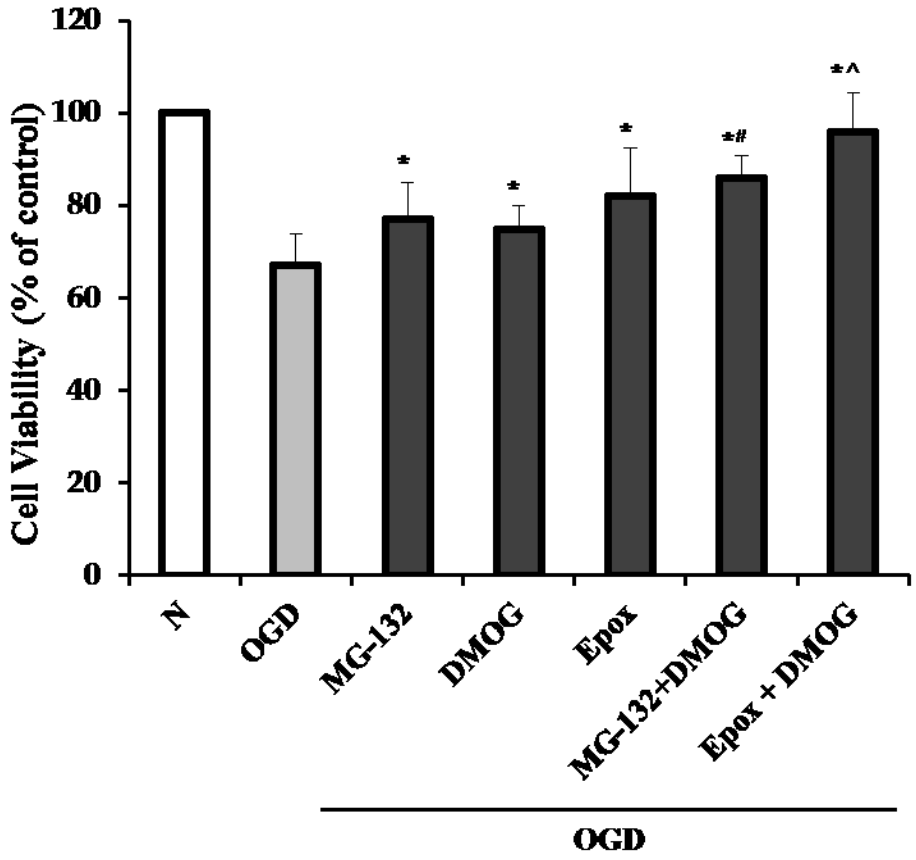


Figure 2.3B. Neuronal viability with the treatment of proteasome and prolyl hydroxylase inhibitors. Neurons were pre-treated with 40 μM MG-132, 2 mM Dimethyglycine (DMOG) or 8 μM of Epoxomicin (Epox) and then exposed to oxygen and glucose deprivation (OGD). Cell viability was assessed using the MTT assay. *p <0.05 OGD, ^p <0.05 versus DMOG. (n = 3).

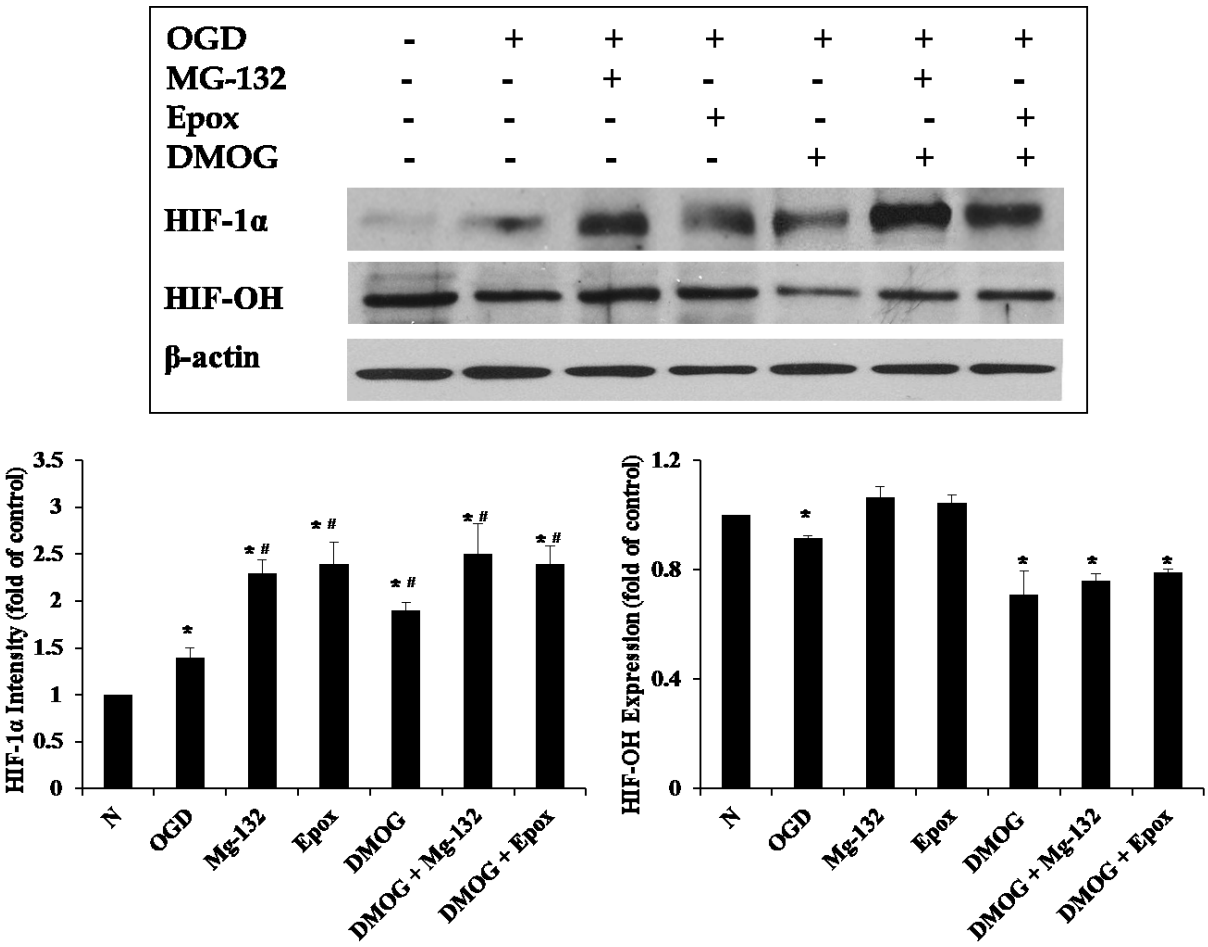
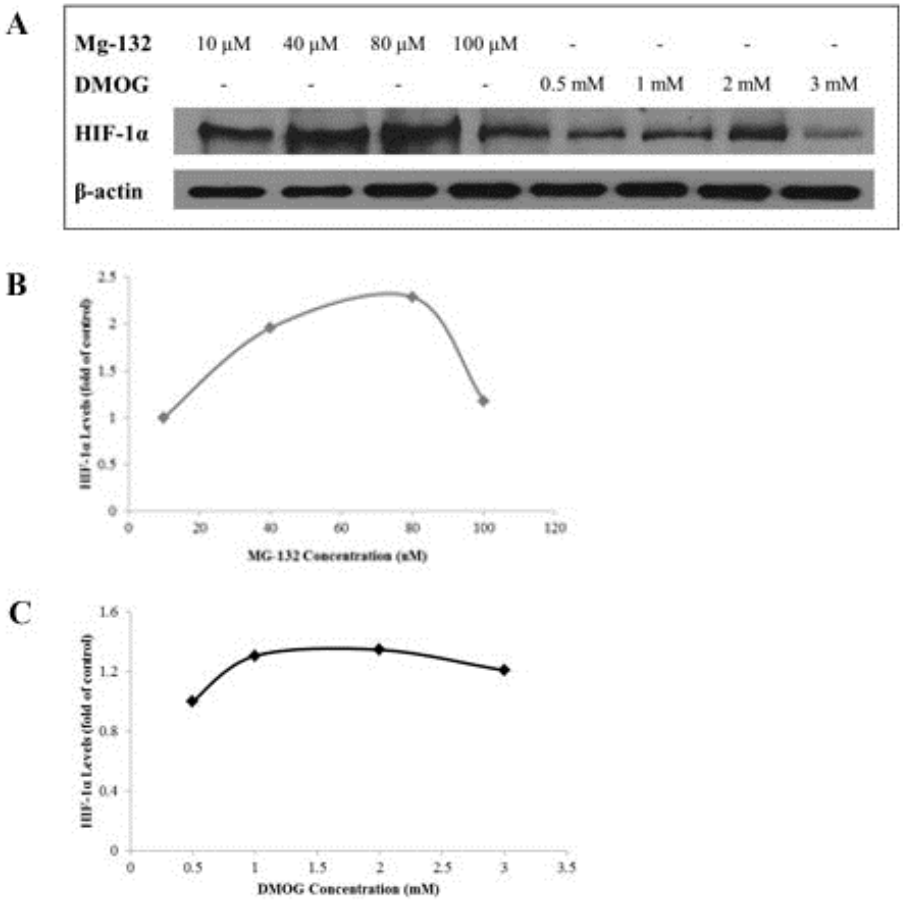


Figure 2.3C Immunoblotting showing HIF-1α and hydroxyl-HIF-1α (HIF-OH) protein levels in neurons with the treatment of proteasome and prolyl hydroxylase inhibitors. Equalization of protein loading was determined using β-actin as the housekeeping protein. Quantitative results for Western blot data. *p<0.05 versus N, $^{\#}p$<0.05 versus OGD (n= 3)



Supplementary Figure 2.1. HIF-1α protein stabilization following OGD with the treatment of MG-132 and DMOG. (A) Immunoblotting showing HIF-1α protein levels in neurons treated with either MG-132 (10, 40, 80, 100 μM) or DMOG (0.5, 1, 2, 3 mM). Equalization of protein loading was determined using β-actin as the housekeeping protein. (B) Curve demonstrating the HIF-1α protein levels with the pre-treatment of MG-132. (C) Curve demonstrating the HIF-1α protein levels with the pre-treatment of DMOG (n= 3)

hydroxylation of HIF-1α by inhibiting PHDs. The level of HIF-OH was highest under normoxia. It was decreased under OGD exposure and slightly increased with the inhibition of the proteasome. The combined drug treatment resulted in HIF-OH levels that were more than that of the DMOG treatment only but still lower than that with the proteasome inhibitors alone.

2.4.5 Proteasomal inhibition is more effective than hydroxylase inhibition in reducing brain infarct size in an *in vivo* stroke model

We then evaluated effects of proteasomal inhibition and hydroxylase inhibition on brain damage in an *in vivo* mouse stroke model. Mice were subjected to MCAO followed by a 24 hr period of reperfusion. Four animal groups were examined: (1) Control, (2) Epoxomicin, (3) DMOG, and (4) Epoxomicin + DMOG. Epoxomicin was chosen as the proteasome inhibitor for the *in vivo* studies as it is able to cross the blood-brain barrier unlike MG-132 (Stefanis & Keller, 2007) It is also more selective and potent than MG-132. Epoxomicin was administered at 1.1 mg/kg/0.1cc as shown by Meng et al. (Meng *et al.*, 1999a) to inhibit the proteasome. DMOG was administered at 50 mg/kg/0.1cc as shown by Ogle et al. (Ogle *et al.*, 2012) to inhibit the PHD. Brain damage was assessed by calculating infarct and edema volume using TTC staining. The results revealed that epoxomicin was more effective at reducing infarct size compared to DMOG (Fig. 2.4B). We also assessed the infarct size following MCAO with the pretreatment with higher concentrations of DMOG to insure that a sufficient amount of DMOG was given to the mice to inhibit the PHD (Supplementary Fig. S2.2). Epoxomicin was more effective than the increased concentrations of DMOG. Brain edema volume was also measured from the coronal sections. As shown in Fig. 2.4C, all 3 drug-treated animal groups had a significant decrease in edema volume following MCAO compared to the control mice. However, there was no significant difference

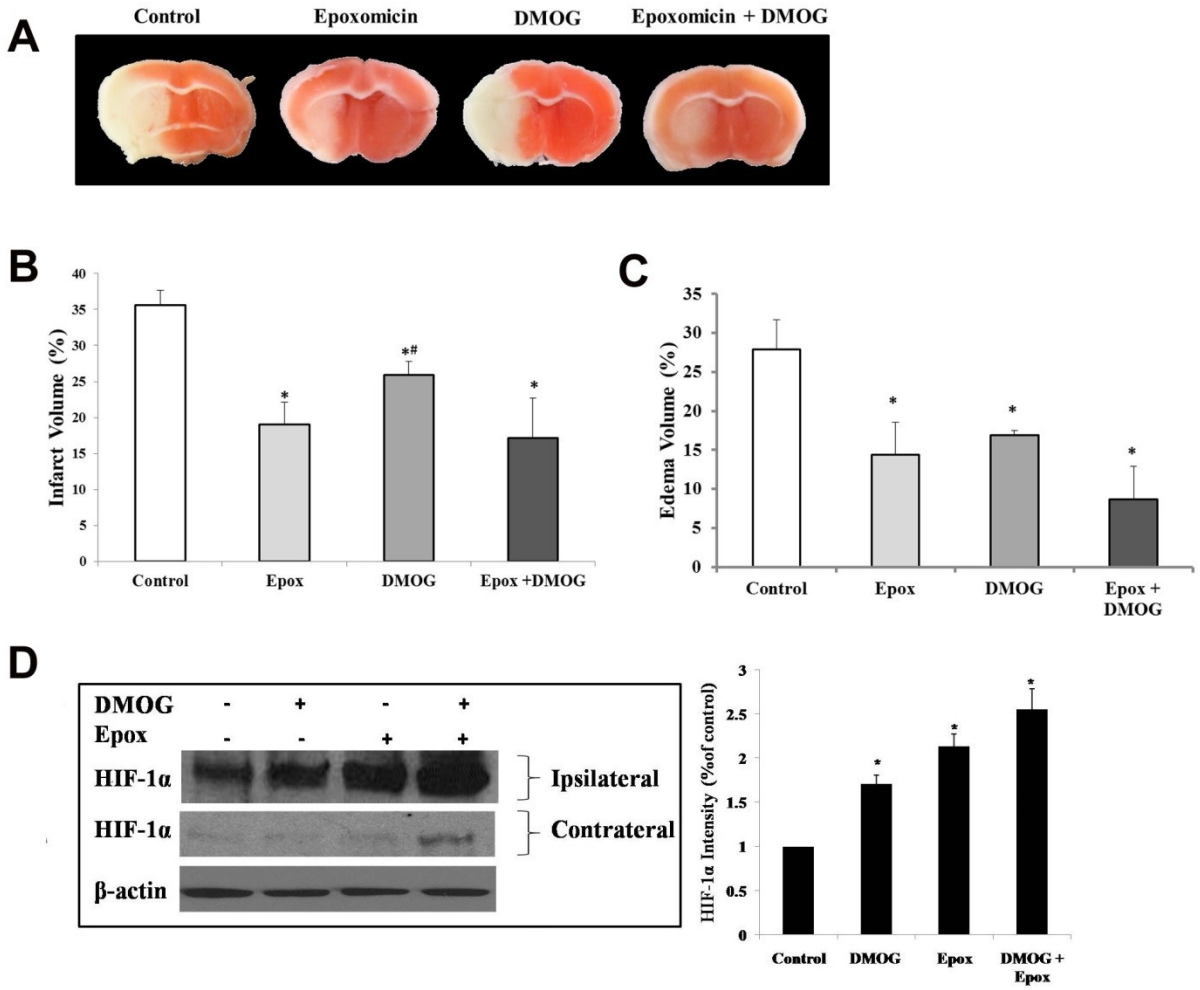
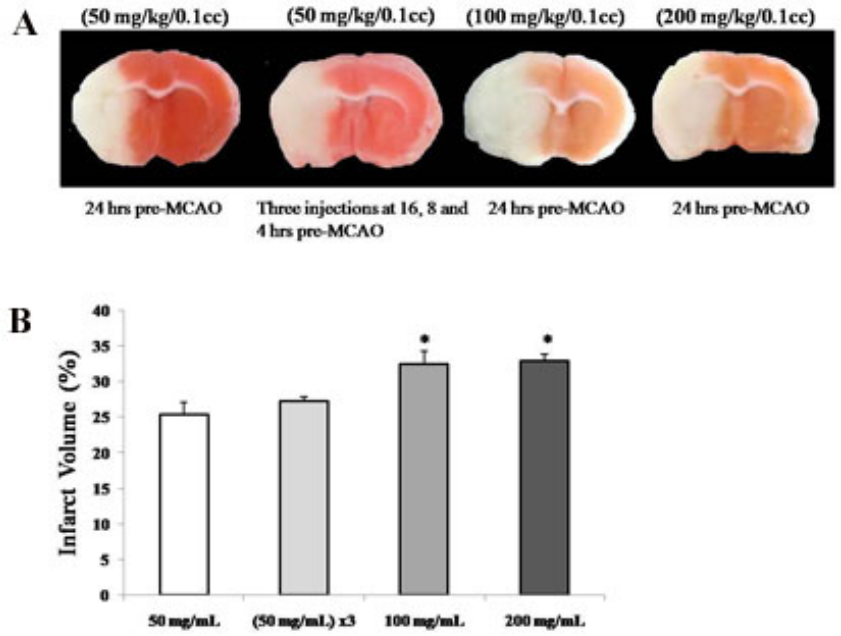


Figure 2.4. Effect of DMOG and epoxomicin on HIF-1a expression and brain damage induced by cerebral ischemia. Brain damage as determined by TCC staining after mice were subjected to 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections taken from the 3 mm position of the frontal pole. (B) Quantification of infarct volume determined by TTC stained sections (n=5). Data presented as means ± SD $*p < 0.05$, vs. control untreated mice. $^{\#}p < 0.05$, vs. mice treated with Epoxomicin. (C) Quantification of brain edema volume estimated from TTC stained sections (n=5). Data presented as means ± SD $*p < 0.05$, vs. control untreated mice. (D) Immunoblotting showing the levels of HIF-1α from the ipsilateral and contralateral brain hemispheres of untreated mice and mice treated with dimethyloxalylglycine (DMOG), Epoxomicin (Epox) or a combination of both (n=3).



Supplementary Figure 2.2. Brain damage as determined by TCC staining after mice were pretreated with DMOG and then subjected to 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections taken from the 3 mm position of the frontal pole. Mice were either pre-treated with a single dose of 50 mg/mL (24 hrs pre-MCAO), a triple dose of 50 mg/mL (16, 8 and 4 hrs pre-MCAO), a single dose of 100 mg/mL (24 hrs pre-MCAO) or a single dose of 200 mg/mL (24 hrs pre-MCAO) (B) Quantification of infarct volume determined by TTC stained sections (n=5). Data presented as means ± SD *$p < 0.05$, vs. mice treated with a single dose of 50 mg/mL

between the groups. Furthermore, immunoblotting showed that HIF-1α protein levels were increased in the ipsilateral hemisphere and that it is significantly stabilized with the combined administration of Epoxomicin and DMOG.

2.5 DISCUSSION

The induction of HIF-1 is very important following cerebral ischemia. Dimerization of HIF-1α to HIF-1β leads to the expression of various genes that can promote cellular adaptation to conditions of low oxygen. Its targets include genes that code for molecules that participate in vasomotor control, angiogenesis, erythropoiesis, cell proliferation, and energy metabolism (Semenza, 2003a; b; Sharp & Bernaudin, 2004). Each of these functions potentially contributes to the survival of neuronal cells. Neuron-specific HIF-1 deficient mice showed increased brain damage following MCAO (Baranova *et al.*, 2007). The neuroprotective effects of iron chelators were also attributed to the activation of HIF-1 *in vivo* (Prass *et al.*, 2002; Hamrick *et al.*, 2005; Freret *et al.*, 2006). HIF-1 can also promote adenosine production which has been shown to be neuroprotective (Heurteaux *et al.*, 1995; Wardas, 2002; Lin *et al.*, 2008). We investigated the mechanisms of HIF-1α degradation in neurons exposed to low oxygen conditions. The results for the first time demonstrated that both 20S and 26S proteasomal pathways were involved in HIF-1α degradation in ischemic neurons. The results provide important information for not only understanding the pathophysiology of cerebral ischemia but also designing potential strategies for stroke treatment.

Under normoxic conditions, HIF-1α is rapidly degraded via the ubiquitin-dependent proteasomal (26S) degradation pathway after hydroxylation and ubiquitination. Under hypoxic conditions, it is generally regarded that HIF-1α is accumulated due to hydroxylation inhibition

(Wang *et al.*, 1995b; Jiang *et al.*, 1996; Wood *et al.*, 1996; Salceda & Caro, 1997; Huang *et al.*, 1998a; Kallio *et al.*, 1999; Huang & Bunn, 2003). However, recent research suggests that HIF-1α can also be degraded under hypoxia. Apart from the PHD hydroxylation pathway, there are other pVHL-dependent pathways that can degrade HIF-1α (Koh *et al.*, 2008). During hypoxia HIF-1α levels can be regulated by the small ubiquitin-like modifier (SUMO)-1. SUMOylation can induce pVHL binding to HIF-1α thus promoting its ubiquitination and degradation by the 26S proteasome (Cheng *et al.*, 2007). There are also pVHL-independent pathways that lead to HIF-1 degradation (Koh *et al.*, 2008). These signaling pathways involve the 90 kDa heat shock protein (HSP90) and the receptor of activated protein kinase (RACK1) (Liu *et al.*, 2007) that lead to HIF-1α ubiquitination and degradation. Glycogen synthase kinase 3 (GSK3) (Flügel *et al.*, 2007) as well as fork-head box (FOXO4) (Tang & Lasky, 2003) overexpression can also lead to pVHL-independent ubiquitination and HIF-1α degradation. All these mechanisms regardless of whether they are pVHL- dependent or – independent result in ubiquitination and therefore a likely degradation by the 26S proteasome. Demidenko *et al.* suggested that under hypoxia HIF-1α may transcriptionally activate its own degradation, possibly mediated by acetylation (Demidenko *et al.*, 2005).

Recently, Kong *et al.* suggested that HIF-1α's turnover under hypoxia was possibly regulated by the ubiquitin-independent proteasomal (20S) degradation pathway (Kong *et al.*, 2007). Consistent with their observation, our current data clearly show that 20S proteasome can degrade HIF-1α in ischemic neurons. For HIF-1α to be a target of the 20S proteasome it would have to be oxidized as the 20S preferably degrade oxidized protein. HIF-1α was found to be an oxidizable protein soon after it was discovered (Wang *et al.*, 1995c; Huang *et al.*, 1996) due to its residues with redox properties such as cysteine and methionine. Oxidative modification of cellular

proteins was reported to occur within 10 min and peak at 1 or 2 hrs after the ischemic insults (Oliver *et al.*, 1990; Hall *et al.*, 1995). Yet, it is not known which residue of HIF-1α is oxidized by ischemia. Moreover, our data suggest that the 26S proteasomal pathway also contribute to the degradation of HIF-1α in ischemia, at least in our experimental setting. As shown in Figure 2.3, PHD inhibition did prevent HIF-1α degradation. Our observation that proteasomal inhibition increased HIF-1α levels more than a PHD inhibitor under a low O_2 condition is in accordance with a previous report (Demidenko *et al.*, 2005). These results indicate that HIF-1α is, at least partly, degraded via a hydroxylation-independent proteasome pathway, and suggest that besides 26S, the 20S proteasome is involved in the degradation of HIF-1α under hypoxia

Currently PHD enzymes are being targeted for drug discovery in the treatment of stroke. PHD enzymes regulate the HIF-1α degradation pathway by acting as oxygen sensors (Epstein *et al.*, 2001). Therefore, the inhibition of PHD enzymes leads to the activation of HIF and its downstream genes. PHD inhibitors have been shown to be protective when administered prior to or upon reperfusion in brain ischemia models (Gu *et al.*; Gidday *et al.*, 1994; Prass *et al.*, 2002; Siddiq *et al.*, 2005; Liu *et al.*, 2009). However, inhibiting PHD may only provide a partial effect because HIF-1α can be degraded by other pathways such as the 20S proteasomes as shown here (Figures 2.3A). Our results reveal a novel mechanism of HIF-1α degradation mediated by ROS in the ischemic brain. We demonstrate that both the 26S and 20S proteasomal pathways are involved in HIF-1α degradation. Thus, PHD inhibitors are not as effective as the proteasome inhibitors because the PHD inhibitors will block hydroxylation of HIF-1α and the 26S pathway activity, but not the 20S pathway. Several studies have shown that the proteasome inhibitor MLN519 is neuroprotective in stroke injuries (Berti *et al.*, 2003; Williams *et al.*, 2003; Williams *et al.*, 2004; Williams *et al.*, 2005; Williams *et al.*, 2006). The application of proteasome

inhibitors in this study was mainly to investigate the mechanism of ROS mediated HIF-1α degradation in neurons during ischemia. An additional increase in HIF-1α levels when both the proteasome and prolyl hydroxylase are inhibited indicates a role of the 20S in HIF-1α degradation (Figure 2.3C). We also found that the combined treatment and the stabilization of HIF-1α was protective in both the *in vitro* primary neuron ischemia model and in the *in vivo* mouse stroke model in terms of maintaining cell viability (Figure 2.3B) and in the reduction of infarct size (Figure 2.4).

The proteasome is a protein by itself, so it can be damaged by oxidative stress. The effect of oxidants on the function of the proteasome is still not very well understood. Its seems as though proteasome regulation is very complex and cells often give contradicting responses depending on the type and severity of the insult. Overall, studies report that the reactive oxygen species can alter the activities of the 26S and 20S proteasome. Although both the 26S and 20S can degrade oxidized proteins, many studies have determined that the contribution of the 20S is significantly higher (Davies, 2001; Breusing & Grune, 2008; Jung & Grune, 2008). This has been attributed to the fact that the 20S is more resistant to oxidative insults The 26S proteasome was in general more sensitive than the 20S proteasome to oxidants such as H_2O_2, hypochlorite, and peroxynitrite (Reinheckel *et al.*, 1998a). The activity of the 20S proteasome after moderate oxidative stress did not change significantly (Grune *et al.*, 2004). The 20S proteasome activity remained unchanged after H_2O_2 exposure of up to 2 mM, while the 26S proteasome was completely inhibited under these conditions (Reinheckel *et al.*, 1998b). There is evidence that suggests that the chymotrypsin-like activity of the 20S proteasome is increased in response to ROS (Ullrich *et al.*, 1999). Other studies have shown that 26S activity was also initially stimulated following oxidative stress and lead to cell protection. However, the mechanism of

how that occurred was unknown (Ding *et al.*, 2003; Grune *et al.*, 2004). Furthermore, the inhibition of the deubiquitinating enzyme, Usp14 (Lee *et al.*, 2010) and the overexpression of the proteasome assembly protein, UMP1(Chen *et al.*, 2006) both resulted in increased proteasomal function and increased cell viability in response to oxidative stress.

In summary, HIF-1 plays an important role in the fate of ischemic neurons. Understanding the mechanism of HIF-1 induction is very important in determining its role in cerebral ischemia and providing potential approaches to regulate its expression. Our study reveals a novel mechanism of redox regulated HIF-1α stabilization during cerebral ischemia (Figure 2.5). We show that ROS can alter proteasomal activities in SH-SY5Y cells and that HIF-1α can be oxidized which results in a significant increase in its degradation by the 20S proteasome. ROS consist of several unique species, which have dramatically different reactivities and half-lives. Actions of ROS on HIF-1α degradation may be through specific reactive oxygen species, rather than ROS in general. Understanding the different ROS species involved will provide us with a better understanding of the mechanisms. It will also help us design more effective intervention strategies, such as efficient antioxidants, to inhibit or remove the specific species. We also provide evidence that the inhibition of proteasomal activity with MG-132 and Epoxomicin restored the attenuated accumulation of HIF-1α that resulted from increased ROS and provided neuroprotection (Figure 2.6). Regulating HIF-1α induction and the genes induced by HIF-1 under ischemia are highly promising therapeutic targets for cerebral ischemia (Giaccia *et al.*, 2003; Williams *et al.*, 2004; Shi, 2009). Defining this mechanism for HIF-1α degradation will make it possible to design more efficient agents for inhibiting HIF-1α degradation and promoting its neuroprotective properties.

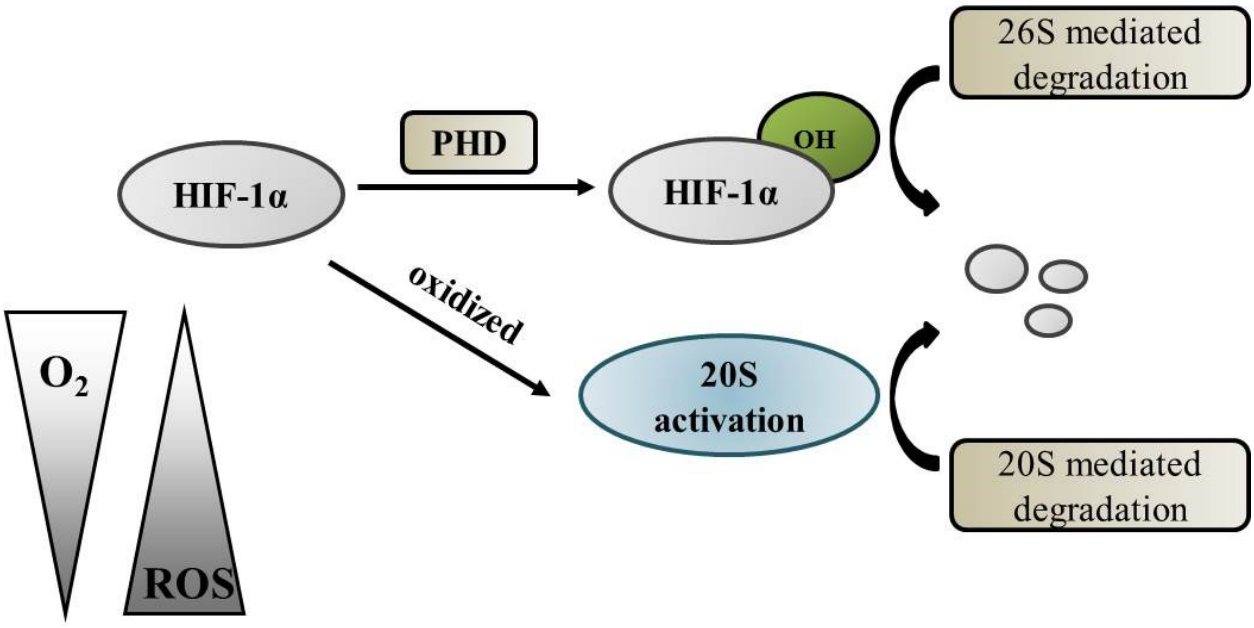


Figure 2.5 Schematic diagram of HIF-1α degradation mechanisms under conditions of low oxygen and high oxidative stress.

HIF-1α, hypoxia inducible factor-1α; ROS, reactive oxygen species; PHD, prolyl hydroxylase

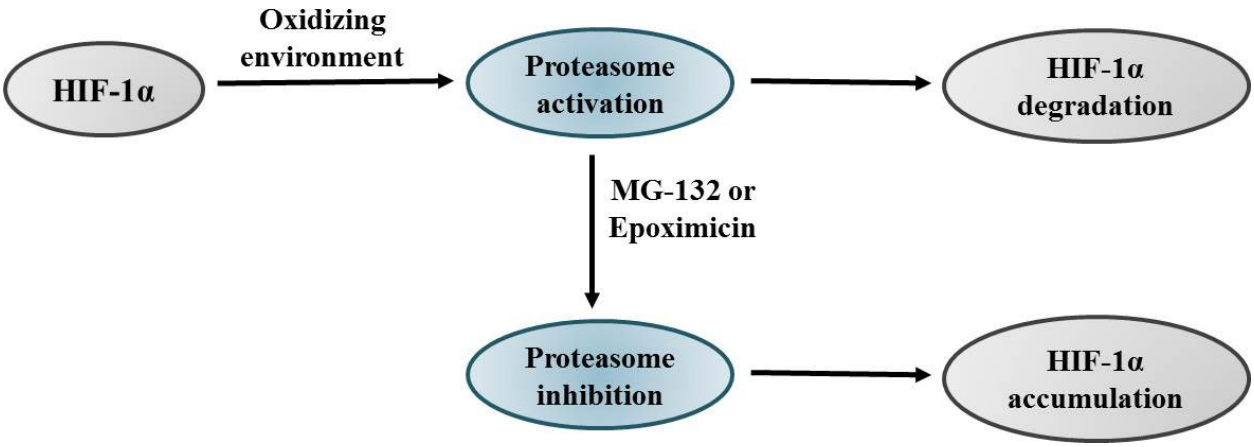


Figure 2.6 Schematic diagram of proposed mechanisms of HIF-1α stailization. An oxidizing environment promotes HIF-1α degrdation through the activation of proteasomal pathways. Inhibiting the proteasome with MG-132 or Epoxomicin will lead to the accumulation of HIF-1α protein accumulation.

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Chapter 3: Hypoxia-inducible factor 1 protects hypoxic astrocytes against glutamate toxicity

3.1 ABSTRACT

Stroke is a major neurological disorder characterized by an increase in the Glu (glutamate) concentration resulting in excitotoxicity and eventually cellular damage and death in the brain. HIF-1 (hypoxia-inducible factor-1), a transcription factor, plays an important protective role in promoting cellular adaptation to hypoxic conditions. It is known that HIF-1α, the regulatable subunit of HIF-1, is expressed by astrocytes under severe ischemia. However, the effect of HIF-1 on astrocytes following Glu toxicity during ischemia has not been well studied. We investigated the role of HIF-1 in protecting ischaemic astrocytes against Glu toxicity. Immunostaining with GFAP (glial fibrillary acidic protein) confirmed the morphological modification of astrocytes in the presence of 1 mM Glu under normoxia. Interestingly, when the astrocytes were exposed to severe hypoxia (0.1% O_2), the altered cell morphology was ameliorated with up-regulation of HIF-1α. To ascertain HIF-1's protective role, effects of two HIF-1α inhibitors, YC-1 [3-(50-hydroxymethyl-20-furyl)-1-benzylindazole] and 2Me2 (2-methoxyoestradiol), were tested. Both the inhibitors decreased the recovery in astrocyte morphology and increased cell death. Given that ischemia increases ROS (reactive oxygen species), we examined the role of GSH (reduced glutathione) in the mechanism for this protection. GSH was increased under hypoxia, and this correlated with an increase in HIF-1α stabilization in the astrocytes. Furthermore, inhibition of GSH with BSO (l-butathione sulfoximine) decreased HIF-1α expression, suggesting its role in the stabilization of HIF-1α. Overall, our results indicate that the expression of HIF-1α under hypoxia has a protective effect on astrocytes in maintaining cell morphology and viability in response to Glu toxicity.

3.2 INTRODUCTION

Brain ischemia induces a cascade of events that involve a loss of glucose and oxygen, membrane depolarization, and Glu (glutamate) release, leading to excitotoxicity. This release of the neurotransmitter Glu and subsequent calcium influx is considered to be the most significant event in the pathogenesis of ischaemic brain damage. Astrocytes play an important role in maintaining extracellular Glu that is released from neurons below toxic levels. They do so by clearing up Glu from the synaptic region through excitatory amino acid transporters and converting the Glu into glutamine by glutamine synthetase. Glutamine is then shuttled back to neurons and is re-used for Glu synthesis (Lehmann et al., 2009). Astrocytes are also involved in the metabolic support to neurons and provide them with nutrients such as lactate to supplement energy requirements. In addition, astrocytes appear to be the main source of EPO (erythropoietin) and GSH (reduced glutathione) in the CNS (central nervous system), having a GSH concentration twice as high that in neurons (Bolanos et al., 1995). There is significant evidence of astrocytes providing GSH and EPO to neighbouring neurons against various stresses (Gabryel and Malecki, 2006). Previous studies have shown that, in ischaemic infarcts, neurons do not survive if neighbouring astrocytes are not viable (Takano et al., 2009). Therefore it is important to examine how ischemia affects the function and viability of astrocytes.

Under ischaemic conditions, HIF-1 (hypoxia-inducible factor-1) is expressed to promote cell survival. HIF-1 is a heterodimeric protein formed by a continuously expressed subunit HIF-1β and an oxygen-regulated subunit HIF-1α that is stabilized under low oxygen levels. Activation of HIF-1 leads to the transcription of various genes that contribute to the cellular adaptation to these conditions. Some of the genes that play an important role in the protective effect of HIF-1 are those that are involved in angiogenesis such as VEGF (vascular endothelial

growth factor), erythropoiesis such as EPO and genes involved in glucose metabolism such as glucose transporters (Siddiq et al., 2007). It has been shown that HIF-1 induces high levels of EPO expression in astrocytes (Masuda et al., 1994), making them the main source of EPO in the CNS. This demonstrates that HIF-1 plays an important role not only in neurons but also in astrocytes. In addition, Glu release during cerebral ischemia causes the formation of ROS (reactive oxygen species) by the disruption of the mitochondrial electron transport chain and the activation of NAPDH oxidases (Brennan et al., 2009). Activation of HIF-1 has been shown to protect astrocytes against oxidative damage (Chu et al., 2010).

Since astrocytes play an important role in maintaining brain homoeostasis and providing neuroprotection, their response to Glu toxicity and ischaemic insult requires further understanding. The main objective of this study was to investigate the effects of Glu on the viability and morphology of astrocytes exposed to hypoxia and the role that HIF-1 plays under these conditions. Our results demonstrate that HIF-1 has a protective effect on primary rat cortical astrocytes in terms of increasing cell viability and maintaining cell morphology in response to Glu toxicity and severe oxygen deprivation. Furthermore, we show that GSH may contribute to this protection by providing optimal conditions for HIF-1 stabilization.

3.3 MATERIALS AND METHODS

3.3.1 Primary culture of astrocytes

All experiments were conducted with the approval of the University of Kansas Institutional Animal Care and Use Committee. Cortical tissue were dissected from the Sprague–Dawley rat (Charles River Laboratories) brains at postnatal day 0 (P0) to P4. The tissues were washed with HBSS (Hanks balanced salt solution) and trypsinized for 50 min at 37°C. The

tissues were then dissociated using a fire polished glass pipette in a dissociation medium (HBSS, 0.1% BSA and 8 mM $MgCl_2$), and centrifuged at 4000 ***g*** for 4 min at room temperature (22°C). The cells were transferred into and grown in 25 cm^2 flasks with DMEM (Dulbecco's modified Eagle's medium) and 10% FBS (fetal bovine serum). After 3–4 weeks the flasks were shaken to purify the astrocytes by dislodging other cell layers. Following purification, astrocytes were plated on coverslips with DMEM and 10% FBS and used for experiments after 10–12 days.

3.3.2 *In vitro* hypoxia model

Hypoxia was induced by incubating the astrocytes in 0.1% O_2/5% CO_2 (balanced with N_2) in a hypoxia chamber (COY Laboratories) for 3 h. To mimic the high levels of Glu release during ischemia, astrocytes were treated with 0, 0.001, 0.01, 0.1 and 1 mM of Glu in serum-free medium (DMEM) at 37°C for 3 h. Control experiments were conducted at 21% O_2/5% CO_2.

3.3.3 Drug treatments

YC-1 [3-(50-hydroxymethyl-20-furyl)-1-benzylindazole] and 2Me2 (2-methoxyoestradiol; Cayman Chemical Company) were used for HIF-1α inhibition studies. Prior to hypoxia exposure, the astrocytes were incubated with 0.1 mM of the inhibitors for 1 h. Preliminary experiments showed that these conditions were sufficient for HIF-1α inhibition during severe hypoxia, as shown in Figure 3.3. For GSH depletion, astrocytes were pre-incubated with 5 mM BSO (l-butathione sulfoximine; Sigma–Aldrich) for 12 h as described by Noda et al. (2001). The BSO was present for an additional 3 h during the hypoxia treatment to inhibit the re-synthesis of GSH.

3.3.4 Immunocytochemistry

Following treatments, astrocytes were washed with PBS and fixed with 4% PFA (paraformaldehyde) for 20 min at room temperature. The cells were then permeabilized using 0.3% Triton X-100 for 15 min at room temperature and incubated in a blocking solution (0.05% Triton X-100 and 0.25% BSA dissolved in PBS) for 30 min at room temperature. Astrocytes were incubated with primary antibodies of GFAP (glial fibrillary acidic protein) (1–500, MAB3402, Millipore) and HIF-1α (1–100, sc-8711, Santa Cruz Biotechnology) overnight at 4°C. Cells were washed and incubated with the appropriate secondary antibodies [GFAP: donkey anti-mouse TRITC (tetramethylrhodamine β-isothiocyanate) (1–50; Jackson ImmunoResearch) and HIF-1α: goat anti-rabbit conjugated to Alexa Fluor® 488 (1–100; Molecular Probes)]. Coverslips were washed and mounted by using Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). Images were acquired on a Leica DMI4000 microscope with a ×40 objective and a Leica DFC340 FX digital camera.

3.3.5 GSH measurement

The GSH level was measured by using the MCB (monochlorobimane) method (Chatterjee et al., 1999). Following the treatments, astrocytes were incubated with 0.1 mM of MCB for 30 min at 37°C. Fluorescence images were then taken immediately, directly from the culture dish. For co-localization studies, the astrocytes were fixed after the MCB treatment and double-stained for GFAP and HIF-1α using the immunocytochemistry procedure described above. The intensity of the fluorescent GSH conjugate (GSH–MCB) of single cells was measured from the images using ImageJ software. Readings of whole-cell intensity were taken from 15 cells from three different culture preparations.

3.3.6 Cytotoxicity assessment

Cell death was assessed by measuring the activity of LDH (lactate dehydrogenase) in the culture medium using an LDH cytotoxicity assay kit (Cayman) as described by Bonfoco et al. (1995). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay kit (Invitrogen) was also used to assess cell survival and to quantify the Glu-mediated cytotoxicity as described by Mosmann (1983).

3.3.7 Immunoblot analysis

Astrocytes were lysed in 200 µl of RIPA buffer (Thermo Scientific) and the protease inhibitor cocktail kit (Thermo Scientific) and scraped with the aid of a cell lifter (Biologix Research Company). The lysates were centrifuged at 15300 ***g*** for 10 min at 4°C, and the protein concentration of the supernatants was determined using a protein assay kit (Bio-Rad). Proteins were separated by SDS/PAGE and the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). After being blocked with 5% (w/v) non-fat dried skimmed milk powder in TBST (Tris-buffered saline with Tween), the membrane was incubated with the primary antibody (HIF-1α: 1–1000; BD Transduction Laboratories) overnight at 4°C and the secondary antibody (1–3000; goat anti-mouse; Santa Cruz Biotechnology) for 1 h at room temperature. Immunoblots were quantified using ImageJ software and HIF-1α levels were normalized to β-actin.

3.3.8 Texture analysis

Changes in astrocyte texture were determined using CellProfiler cell image analysis software as described previously by Haralick et al. (1973) and Carpenter et al. (2006). Quantification of texture was done from fluorescence images from three different culture

preparations. Five microscopic fields were obtained from each culture dish and readings from six to eight cells were taken for further analysis.

3.3.9 Statistical analysis

Data are presented as means±S.D. from a minimum of three independent experiments. One-way ANOVA and the Student's *t* test were used for overall significance. Differences of $P<0.05$ were considered statistically significant. Image-Pro Plus 5.1 (Media Cybernetics), ImageJ and Excel were used for data analyses.

3.4 RESULTS

3.4.1 Severe hypoxia-protected astrocytes from Glu toxicity

Excessive Glu accumulation is a major cause of neuronal death in the brain during ischemia. Astrocytes are very important for the clearance of excessive Glu from the extracellular space; however, high concentrations of Glu also affect astrocytes and can lead to their death under normal conditions. Here, we studied the morphological changes in primary rat cortical astrocytes exposed to Glu at various concentrations (0, 0.001, 0.01, 0.1 and 1 mM) for 3 h. The morphology was assessed based on GFAP expression. Lower concentrations (0.001 and 0.01 mM) of Glu had no effect on the morphology. Increased concentrations (0.1 and 1 mM) caused changes in the structure of the astrocytes (Figure 3.1A). Under control conditions, astrocytes appeared fibrous. However, high Glu resulted in a disrupted or diffuse-like structure. To quantify the GFAP-based structural/morphological changes, we conducted texture analysis by using CellProfiler which measures the amount of local variation present (Carpenter et al., 2006). A higher value with more contrast suggests a more complex structure. The results demonstrate that Glu altered the astrocyte morphology under normoxia, compared with the control (no Glu).

Interestingly, when astrocytes were exposed to severe hypoxia (0.1% O_2) in the presence of 1 mM Glu, the astrocyte morphology was improved when compared with 1 mM Glu under normoxia. These data are evidence that hypoxia protects astrocytes against Glu toxicity. As the low concentrations of Glu had no effects on astrocyte morphology, the following studies were carried out with 0.1 and 1 mM Glu.

To further examine the protective effect of hypoxia on astrocyte against Glu, we measured cell death with the LDH assay as shown in Figure 3.1(C). Under normoxia, there was a Glu-concentration-dependent decrease trend in astrocyte viability. Under severe hypoxia there was a sign of recovery. When cell viability was determined with the MTT assay, we observed a significant decrease in cell viability when astrocytes were exposed to 1 mM Glu under normoxia and a significant recovery in astrocyte survival under hypoxia. The difference in the cell viability may be due to the sensitivity of the two cytotoxicity assays. The LDH assay requires a more severe insult that causes damage to the cell membrane. The MTT assay on the other hand works by measuring the metabolic activity of the mitochondria. Nevertheless, results from both assays indicate that hypoxia reduces astrocyte damage caused by Glu.

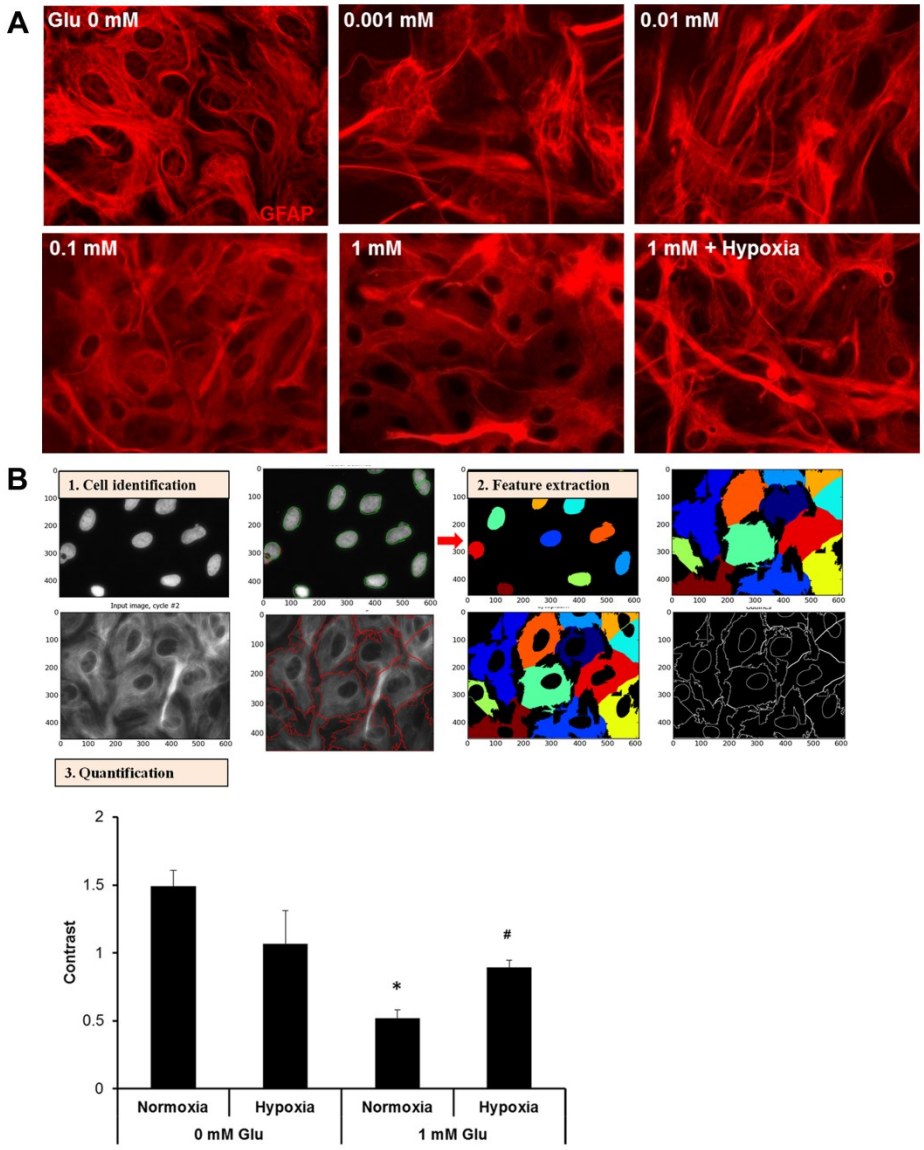

A
Glu 0 mM
0.001 mM
0.01 mM
GFAP
0.1 mM
1 mM
1 mM + Hypoxia
B
1. Cell identification
2. Feature extraction
3. Quantification
Contrast
Normoxia
Hypoxia
Normoxia
Hypoxia
0 mM Glu
1 mM Glu
C
LDH Assay
MTT Assay
Normoxia
Hypoxia
Cell viability (% of control)
0 mM
0.1 mM
1 mM
Glu concentration

Figure 3.1. Hypoxia ameliorated astrocyte damage induced by glutamate (Glu). (A) Immunostaining characterization of cultured rat cortical astrocytes. Representative images depicting GFAP (red) in astrocytes treated with 0, 0.001, 0.1, 0.1 and 1 mM Glu under normoxia (21% O_2) and 1mM exposed to severe hypoxia (0.1% O_2) for 3hrs. (B) Morphologic profiling of astrocytes stained for GFAP and DAPI (for nucleus staining). Individual cells were identified using CellProfiler software and divided with clear boundaries (1). Representation (3) of quantification of morphology using one of the 13 features computed from each cell to measure and compare texture (2). *$p < 0.05$ vs. 0 mM Glu under normoxia. (C) Astrocyte viability assessed using the LDH and MTT assay. *$p < 0.05$ vs. 0 mM Glu under normoxia, $^{\#}p < 0.05$ vs. 1 mM Glu under normoxia. (n=3)

3.4.2 HIF-1α is highly expressed in astrocytes under severe hypoxia

Next, we aimed to address the mechanism by which the astrocytes recovered from the Glu toxicity during severe hypoxia. It is known that HIF-1α is stabilized under low oxygen levels and can contribute to cellular protection under these conditions. HIF-1α expression was first analysed by immunostaining. As expected, there was no HIF-1α expression under normoxia, while treatments with severe hypoxia increased HIF-1 expression in the nuclei of the astrocytes (Figure 3.2A). HIF-1α was also expressed in the nucleus of the astrocytes following severe hypoxia treatment in the presence of Glu. For further confirmation, Western-blot analysis demonstrated a significant increase in HIF-1α protein levels under severe hypoxia, both with and without Glu treatments (Figure 3.2B). However, with 1 mM Glu under severe hypoxia there was a slight decrease in the HIF-1α protein levels. This could be due to increased proteasomal degradation induced by an increase in ROS (see the Discussion for further explanation).

3.4.3 HIF-1α inhibition attenuates the recovery of astrocytes from Glu toxicity under severe hypoxia

To confirm whether the recovery of the cell morphology and cell viability under severe hypoxia was in fact due to the expression of HIF-1α, the effect of two HIF-1α inhibitors, YC-1 and 2Me2, were examined on astrocyte damage. Although the mechanism of HIF-1α inhibition by YC-1 and 2Me2 is not fully understood, it appears as though YC-1 acts at a post-translational level and inhibits HIF-1α activation (Li et al., 2008), while 2Me2 inhibits HIF-1α at the level of translation (Mabjeesh et al., 2003). Astrocytes were pre-treated with a 0.1 mM concentration of the inhibitors for 1 h and then subjected to severe hypoxia with the addition of 1 mM Glu for an additional 3 h. Astrocytes treated with YC-1 or 2Me2 showed much less HIF-1α expression in

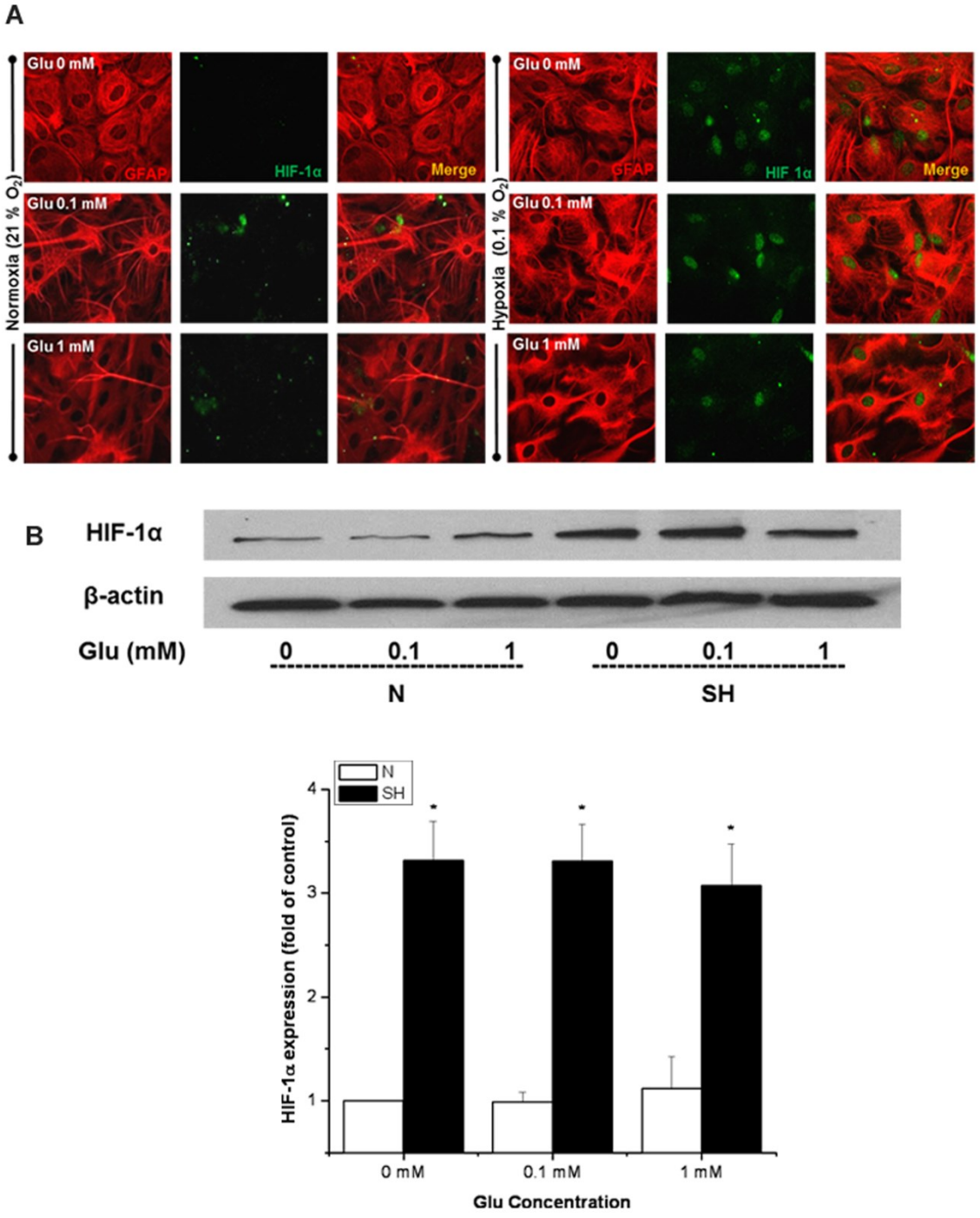

A
Glu 0 mM
GFAP
HIF-1α
Merge
Glu 0.1 mM
Glu 1 mM
Normoxia (21 % O2)
Hypoxia (0.1 % O2)
B
HIF-1α
β-actin
Glu (mM)
0
0.1
1
N
SH
HIF-1α expression (fold of control)
0 mM
0.1 mM
1 mM
Glu Concentration

Figure 3.2. HIF-1α expression was increased in astrocytes exposed to severe hypoxia and glutamate (Glu). (A) Representative immunofluorescent images showing GFAP (red) and HIF-1α (green) labeling in astrocytes treated with 0, 0.1, and 1 mM Glu under normoxia (N) or severe hypoxia (SH) for 3 hrs. (B) Protein stabilization of HIF-1α determined by Western blot analysis. Equalization of protein loading was determined using β-actin as the housekeeping protein. *$p <$ 0.05 vs. 0 mM Glu under N. (n=3)

the nuclei of astrocytes under severe hypoxia in the presence of 0 and 1 mM Glu (Figure 3.3A). The immunostaining also reveals that the addition of either YC-1 or 2Me2 resulted in an attenuation of the recovery of the cell morphology when compared with the controls. Western blotting confirmed that the inhibitors did decrease the HIF-1α protein level (Figure 3.3B).

Table 3.1 shows the effects of the two HIF-1 inhibitors on cell morphology quantified by the texture analysis and cell viability assays. Both YC-1 and 2Me2 treatments resulted in a significant change in cell morphology and significant increase in cell death. Astrocytes treated with the HIF-1α inhibitors under normoxia showed no significant effect (Table 1). The increase in cell death was consistent with no recovery in texture with either YC-1 or 2Me2 treatment under severe hypoxia. This suggests that the recovery in astrocyte morphology is caused by the stabilization of HIF-1α under low oxygen levels.

3.4.4 GSH stabilizes HIF-1α expression

GSH is the most abundant small molecule anti-oxidant that suppresses free radical levels and protects cells against various stress conditions. Previous data from our laboratory has shown that GSH increases HIF-1α expression during oxygen deprivation (Guo et al., 2008). To determine whether GSH plays a role in the HIF-1α stability and protective effect in astrocytes, we compared GSH levels between the different treatments. The cellular GSH level was measured using the MCB method**.** Fluorescence intensities of GSH-MCB were quantified to differentiate between the levels of GSH present in astrocytes treated with 0.1 and 1 mM Glu under normoxia versus severe hypoxia. The results showed no change in GSH levels in the presence of Glu lower than 0.1 mM under normoxia; however, 1 mM Glu caused a significant increase in GSH (Figures

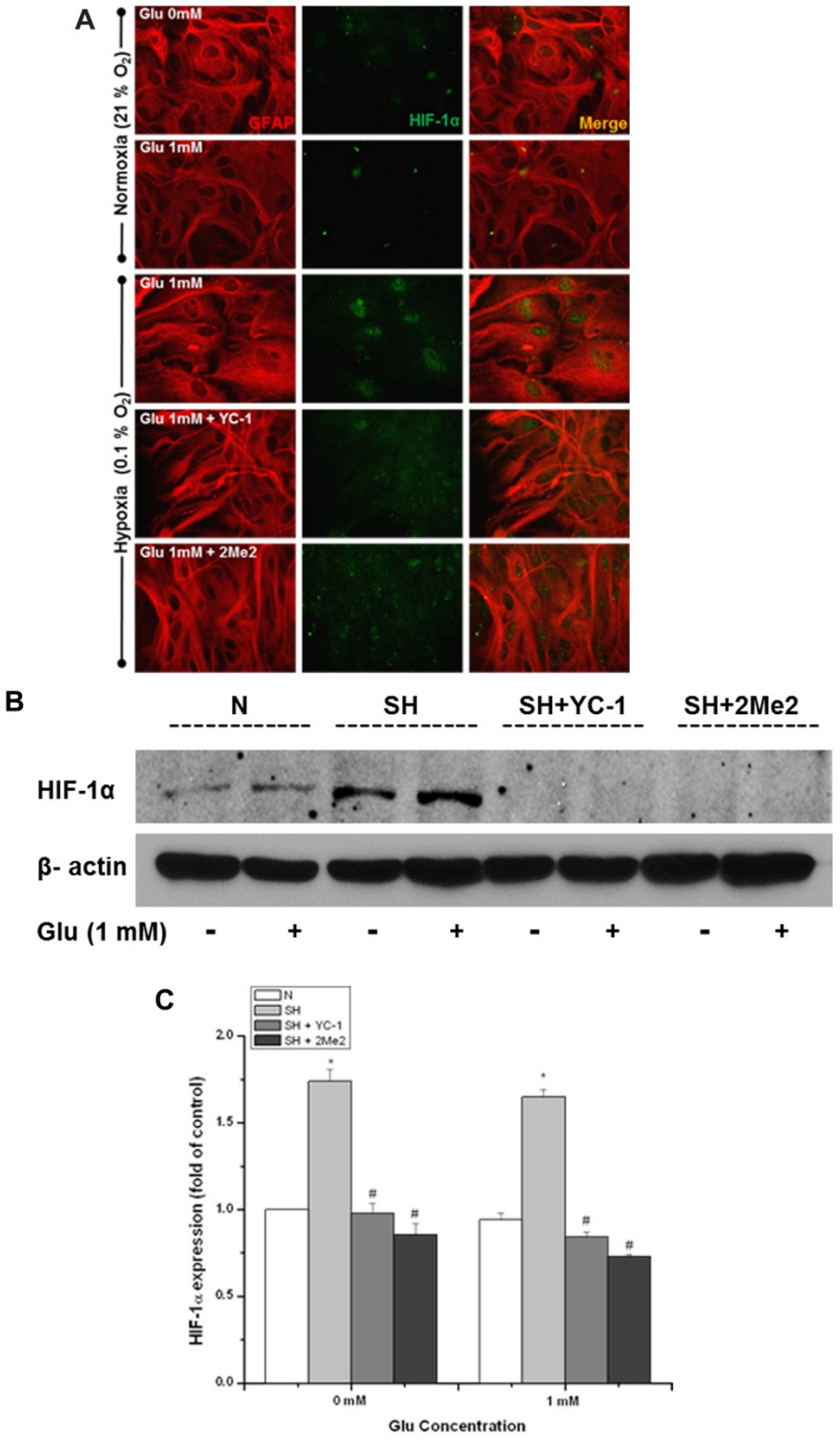

A
Glu 0mM
GFAP
HIF-1α
Merge
Normoxia (21 % O2)
Glu 1mM
Glu 1mM
Glu 1mM + YC-1
Glu 1mM + 2Me2
Hypoxia (0.1 % O2)
B
N
SH
SH+YC-1
SH+2Me2
HIF-1α
β- actin
Glu (1 mM) - + - + - + - +
C
N
SH
SH + YC-1
SH + 2Me2
HIF-1α expression (fold of control)
2.0
1.5
1.0
0.5
0.0
0 mM
1 mM
Glu Concentration

Figure 3.3. YC-1 and 2Me2 attenuated the protection provided by hypoxia in astrocytes. (A) Representative immunofluorescent images demonstrating the effect of YC-1 and 2Me2 on HIF-1α (green) expression and astrocyte morphology (GFAP, red). Astrocytes were pre-treated with 0.1 mM YC-1 and 2Me2 followed by 1 mM Glu with exposure to normoxia (N) or severe hypoxia (SH) 3 hrs. (B) Protein stabilization of HIF-1α determined by Western blot analysis. Equalization of protein loading was determined using β-actin as the housekeeping protein. *$p < 0.05$ vs. 0 mM Glu under N, #$p < 0.05$ vs. 0 mM Glu under SH. (n=3)

Table 3.1. Effect of HIF-1α inhibitors on astrocytes exposed to Glu and hypoxia

Glu concentration	Treatment	Texture Analysis	Cell Viability	
			LDH Assay (%)	MTT Assay (%)
0 mM	N	1.49 ± 0.12	100	100
	N + YC-1	1.19 ± 0.04	99.12 ± 3.09	98.22 ± 1.74
	N + 2Me2	1.13 ± 0.03	99.47 ± 2.52	97.95 ± 2.57
	SH	1.07 ± 0.24	97.35 ± 1.79	90.82 ± 2.93
	SH + YC-1	0.97 ± 0.31	66.15 ± 6.42$^{\#}$	62.74 ± 2.20$^{\#}$
	SH + 2Me2	0.89 ± 0.28	66.34 ± 1.96$^{\#}$	64.41 ± 3.09$^{\#}$
1 mM	N	0.52 ± 0.06*	94.09 ± 1.49	82.84 ± 1.77*
	N + YC-1	0.44 ± 1.08*	98.29 ± 1.51	80.42 ± 5.03*
	N + 2Me2	0.48 ± 1.11*	97.30 ± 3.24	81.03 ± 4.12*
	SH	0.89 ± 0.05**	95.17 ± 3.42	89.86 ± 4.02
	SH + YC-1	0.55 ± 0.11*	59.44 ± 3.59$^{\#}$	55.91 ± 5.24$^{\#}$
	SH + 2Me2	0.51 ± 0.05*	65.56 ± 5.14$^{\#}$	58.62 ± 3.37$^{\#}$

Results were presented as mean ±SD (n=3)

N, normoxia; SH, severe hypoxia
* $p < 0.05$ vs. N
** $p < 0.05$ vs. N 1mM
$^{\#}$ $p < 0.05$ vs. SH 0 mM

3.4A and 3.4B) Hypoxia increased the GSH levels in astrocytes. The presence of Glu further increased the GSH level in astrocytes exposed to hypoxia.

To determine whether an increase in GSH levels contributed to the up-regulated HIF-1α expression, we fixed the cells after MCB treatment with or without severe hypoxia in the presence of Glu and then co-stained for GFAP and HIF-1α. The results showed a co-localization of increased GSH with HIF-1α expression in astrocytes treated with both 0.1 and 1 mM Glu (Figure 3.4C). This suggests that GSH might help stabilize HIF-1α expression in the astrocytes. To confirm the role of GSH in the HIF-1α up-regulation, astrocytes were pre-treated with 5 mM BSO, which decreases cellular GSH levels, for 12 h and then exposed to Glu and hypoxia. This resulted in a significant attenuation of HIF-1α stabilization (Figure 3.5). Furthermore, inhibition of GSH and consequently HIF-1α, decreased astrocyte survival and abolished the morphological recovery under hypoxia (Table 3.2). These results are consistent with the results of HIF-1α inhibition using YC-1 and 2Me2; indicating that HIF-1 provides protection against Glu in hypoxic astrocytes.

3.5 DISCUSSION

To date, there are no effective neuroprotectants for human stroke, and the development of neuroprotective strategies is considered extremely challenging. Although many agents have been tested for the treatment of ischaemic stroke, such as anti-oxidative, anti-apoptotic, anti-excitotoxic and anti-inflammatory drugs, they have all proved unsuccessful. One of the reasons for this may be our incomplete understanding of the mechanisms that are responsible for cellular death. Furthermore, the study of cell death following cerebral ischemia has been primarily focused on neurons. In addition to neurons, ischemia also causes damage to astrocytes (Martin et al., 1997; Yu et al., 2001; Lukaszevicz et al., 2002; Giffard and Swanson, 2005), which are

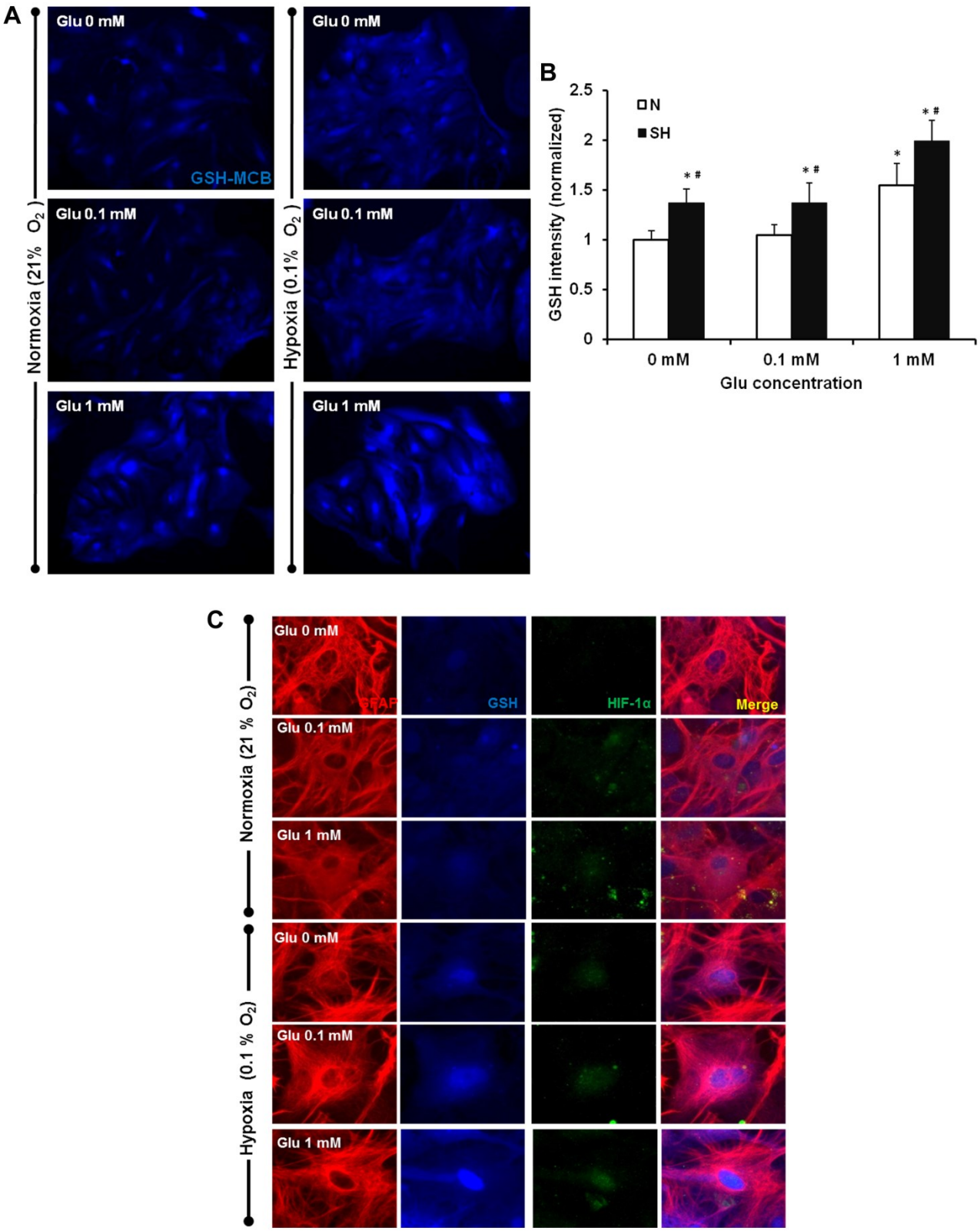

A
Glu 0 mM
GSH-MCB
Glu 0.1 mM
Glu 1 mM
Normoxia (21% O2)
Hypoxia (0.1% O2)
B
N
SH
GSH intensity (normalized)
0 mM
0.1 mM
1 mM
Glu concentration
C
GFAP
GSH
HIF-1α
Merge
Normoxia (21 % O2)
Hypoxia (0.1 % O2)

Figure 3.4. Astrocyte GSH levels were increased with Glu and severe hypoxia treatments. Astrocytes were treated with 0.1 mM or 1 mM Glu with exposure to normoxia (N) or severe hypoxia (SH) for 3 hrs. Astrocytes were loaded with 0.1 mM MCB following treatments. (A) Representatives of GSH-MCB labeling in astrocytes exposed to various conditions. (B) Quantification of the cytosolic GSH intensity in astrocytes exposed to various conditions. $^{*}p < 0.05$ vs. 0 mM Glu under N, $^{\#}p < 0.05$, N vs. SH for each Glu concentration (C) Colocalization of GFAP (red), HIF-1α (green) and GSH (blue) in astrocytes treated with 0, 0.1 and 1 mM Glu under normoxia or severe hypoxia. (n=3)

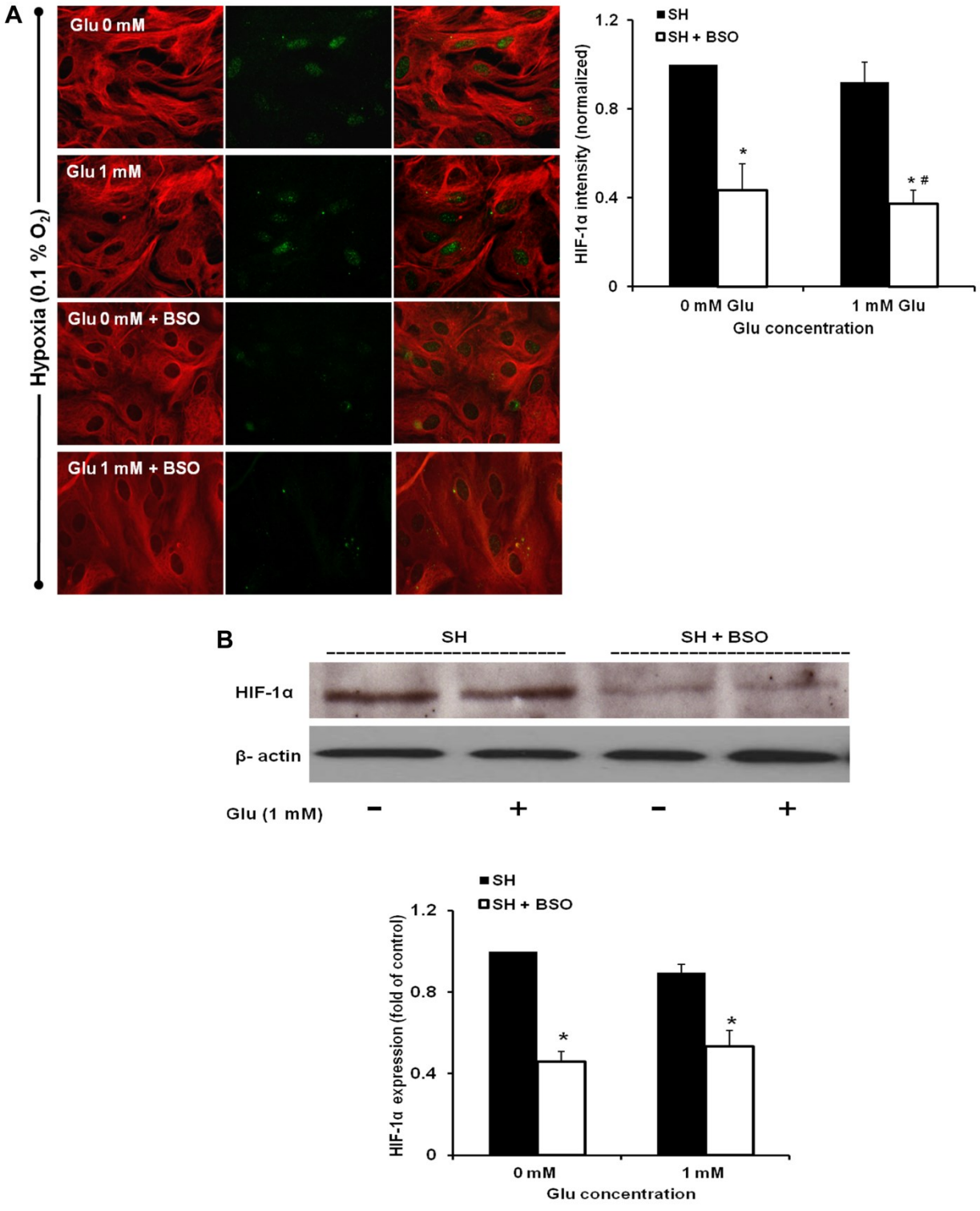

A
Glu 0 mM
Glu 1 mM
Glu 0 mM + BSO
Glu 1 mM + BSO
Hypoxia (0.1 % O2)
SH
SH + BSO
HIF-1α intensity (normalized)
1.2
0.8
0.4
0
*
* #
0 mM Glu
1 mM Glu
Glu concentration
B
SH
SH + BSO
HIF-1α
β- actin
Glu (1 mM)
-
+
-
+
SH
SH + BSO
HIF-1α expression (fold of control)
1.2
0.8
0.4
0
*
*
0 mM
1 mM
Glu concentration

Figure 3.5. GSH inhibition reduces HIF-1α expression. (A) Representative immunofluorescence images demonstrating the effect of buthionine sulfoximine (BS0) on HIF-1α (green) expression and astrocyte morphology. Astrocytes were pre-treated with 5 mM BSO followed by 1 mM Glu with exposure to severe hypoxia (SH) and compared to astrocytes treated with 0 and 1 mM Glu under severe hypoxia only. HIF-1α intensity was measured and normalized to the control. $*p < 0.05$ vs. 0 mM Glu under N, $^{\#}p < 0.05$ vs. 1 mM Glu under SH. (B) Immunoblotting showing HIF-1α protein levels. Equalization of protein loading was determined using β-actin as the housekeeping protein. Quantitative results for Western blot data. $*p < 0.05$ vs. 0 mM Glu under N. (n=3)

Table 3.2. Effect of L-butathione sulfoximine (BSO) on HIF-1α induced protection of astrocytes

Glu concentration	Treatment	Texture analysis	Cell viability	
			LDH assay (%)	MTT assay (%)
0 mM	SH	1.10 ± 0.13	100	100
	SH + BSO	0.59 ± 0.18*	63.70± 3.29*	57.36 ± 4.69*
1 mM	SH	0.96 ± 0.13*	96.14 ± 1.17	97.06 ± 2.37
	SH + BSO	0.54 ± 0.17*#	60.68 ± 2.79*#	54.35 ± 5.26*#

Results were presented as mean ±SD (n=3)

N, normoxia; SH, severe hypoxia

* $p < 0.05$ vs. SH

# $p < 0.05$ vs. SH 1mM

critical in maintaining neuronal viability and functions under ischaemic conditions. Therefore targeting astrocytes can be an important strategy to enhance neuronal survival.

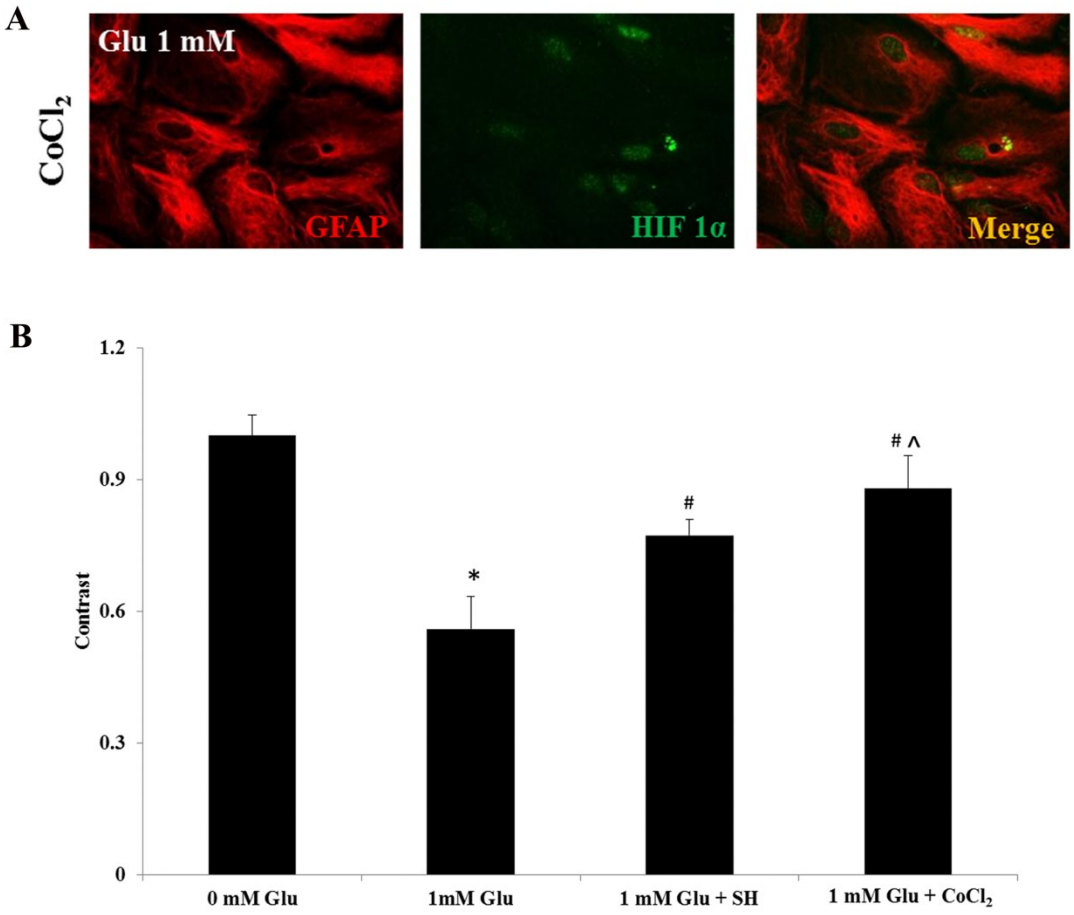
Recently, HIF-1 is being focused on as a potential target for stroke therapy as it appears to have many beneficial roles in the ischaemic brain (Baranova et al., 2007). Although HIF-1 has been shown to have a protective effect specifically in astrocytes in neurological diseases such as Alzheimer's disease (Schubert et al., 2009), few studies have been done to investigate the effect of HIF-1α expression on astrocytes during stroke.

Given the increase in Glu in the ischaemic brain and the scavenging function of astrocytes on Glu, we examined the effects of Glu on the viability and morphology of astrocytes exposed to hypoxia. Compared with neurons, astrocytes are very resistant to hypoxia due to several factors. First, astrocytes have large glycogen stores (Phelps, 1972) that can be metabolized to glucose and lactate and supply energy during ischemia and glucose deprivation (Swanson and Choi, 1993). Secondly, astrocytes have a low energy demand, unlike neurons, which have a high density of ion channels and require more ATP to maintain ionic gradients. Thus ionic deregulation occurs slowly in astrocytes (Silver et al., 1997). Thirdly, astrocytes have a higher level of GSH, an important antioxidant, than neurons and provide GSH or substrates for GSH synthesis to neurons (Makar et al., 1994, Chen and Swanson 2003). In our experiments, astrocytes were exposed to severe hypoxia (0.1% O2) because a milder hypoxic exposure (1% O2) was not sufficient to cause any significant changes in HIF-1α expression (data not shown). As the results demonstrated, Glu caused a concentration-dependent change in astrocyte morphology, which resembled a more diffuse-like structure from a fibrous one, under normoxia. This was correlated with a decrease in cell viability determined by LDH and MTT assays. Interestingly, when the astrocytes were treated with the same Glu concentrations under severe

hypoxia there was a recovery in cell morphology and cell viability. Our observation is consistent with a previous study that demonstrated that pre-treatment with hypoxia reduced astrocyte damage by 45–55% (Chen et al., 2000).

One of the remarkable observations in our study is that increased GSH is not sufficient to protect astrocytes from damage induced by Glu under normoxia. As shown in Figures 3.4(A) and 4(B), Glu at 1 mM significantly elevated the GSH level in astrocyte under normoxia and induced significant cell damage detected by cellular texture and viability (Figure 3.1). In contrast, under hypoxic conditions the increase of GSH was accompanied by improved cellular texture and astrocyte viability (Figures 3.1 and 3.4). This protective effect provided in the hypoxic conditions is largely ascribed to the expression of HIF-1, since inhibiting HIF-1 abolished the protection (Figure 3.3 and Table 3.1). Furthermore, stabilizing HIF-1α expression under normoxia with cobalt chloride appeared to have an even more significant effect in the recovery of cell morphology (Supplementary Figure S3.1B) Taken together, these findings strongly support that the activation of HIF-1 is responsible for promoting the astrocytic survival and protection against Glu toxicity under hypoxia.

Many factors may contribute to HIF-1-mediated protection in hypoxic astrocytes. First, HIF-1 up-regulates EPO (Semenza et al., 1997), which provides cellular protection under different stresses. Many studies have examined the effect of EPO on astrocytes, since they are the main source of EPO in the brain. For example, EPO has been shown to protect astrocytes from damage in response to oxidative stress (Liu et al., 2006) and other agents that induce apoptosis (Diaz et al., 2005). Secondly, HIF-1 leads to the induction of VEGF in astrocytes (Sinor et al., 1998). VEGF has been shown to play an important role in cellular protection in hypoxic preconditioning (Wick et al., 2002) by promoting angiogenesis (Jin et al., 2000). A



Supplementary Figure 3.1. Cobalt chloride ($CoCl_2$) further reduced astrocyte damage induced by glutamate (Glu). (A) Immunostaining characterization of cultured rat cortical astrocytes. Representative images depicting GFAP (red) and HIF-1α (green) in astrocytes treated with 0.3 mM $CoCl_2$ and 1 mM Glu under normoxia (N, 21% O_2) for 3 hrs. (B) Quantification of morphology using one of the 13 features computed from each cell to measure and compare texture. Astrocytes were treated with 0 and 1 mM Glu under normoxia (N, 21% O_2), 1 mM Glu exposed to severe hypoxia (SH, 0.1% O_2) and 0.3 mM CoCl2 and 1 mM Glu under normoxia (N, 21% O_2) for 3 hrs. *$p < 0.05$ vs. 0 mM Glu under N, #$p < 0.05$ vs. 1 mM Glu under N, #$p < 0.005$ vs. 1 mM Glu under N (n=3).

study by Mani et al. (2005) has revealed that exogenous VEGF induces astrocyte proliferation. In addition, HIF-1 may promote the production of adenosine that offers neuroprotective properties (Heurteaux et al., 1995; Wardas 2002; Lin et al., 2008). Adenosine binds to the presynaptic A1 receptor and may lead to a decrease in Ca2+ influx. This further decreases the release of Glu and excitation of the NMDA (N-methyl-D-aspartate) receptors, thus preventing cellular damage caused by the subsequent increases in Ca2+ influx (Monopoli et al., 1998;Wardas 2002). A study by Batti et al. (2010) has shown that the stabilization of HIF-1α through prolyl hydroxylase inhibition protected against Glu-induced damage in the hippocampus of the rat ischaemic brain mainly through adenosine accumulation in response to hypoxia. A review article by Vangeison and Rempe (2009) clearly describes how hypoxia and HIF-1 can regulate various proteins, including connexin 43, CD73 and the ENT-1 (equilibrative nucleoside transporter 1), which ultimately leads to enhanced adenosine levels. Of these, both CD73 and ENT-1 have been shown to be regulated by HIF-1 in intestinal epithelia (Synnestvedt el al. 2002) and endothelial cells (Eltzschig et al., 2005) respectively. CD73 and ENT-1 are expressed in astrocytes (Vangeison and Rempe 2009); therefore it is possible that HIF-1 can regulate their activity and increase adenosine in astrocytes. In fact, it has been shown that adenosine has a direct protective effect on rat primary astrocytes by reducing death induced by glucose deprivation (Shin et al., 2002) and in reducing damage in human astroglioma D384 cells following oxygen deprivation through the preservation of ATP levels (Bjorklund et al., 2008).

The results presented in this paper clearly demonstrate that GSH increases the HIF-1α level in astrocytes exposed to hypoxia. As shown in Figure 3.4, there was an increase in GSH following the 3 h Glu and severe hypoxia treatment. In addition, BSO, which inhibits GSH synthesis, decreased HIF-1α expression. Our results are consistent with previous studies that

have shown that altering redox status can effect HIF-1α expression in other types of cells. Inhibition of GSH synthesis by BSO reduced HIF-1α expression in lung epithelial cells (Haddad and Land, 2000) and in hepatic cells (Jin et al., 2011). Treatment with N-acetylcysteine, the GSH precursor, increased HIF-1α expression in lung epithelial cells (Haddad et al., 2000) and in hepatic cells (Sommani et al 2007; Jin et al., 2011). Our previous results have also demonstrated that HIF-1α stability favours a reducing environment in neurons (Guo et al., 2008). Recently, Tajima et al. (2009) showed that the induction of HIF under hypoxia is regulated by the redox state of GSH in HSC-2 (human oral squamous cells). They suggest that GSH can regulate the activation of HIF by directly binding to the thiol groups of regulatory cellular proteins. Since HIF-1α is sensitive to redox status and can be degraded by increased ROS (Liu et al., 2004; Wellman et al., 2004), it is reasonable to consider that the mechanism by which GSH increases HIF-1α is through the clearance of excessive ROS and by promoting a suitable reducing environment that prolongs its stabilization. Our immunoblot results showed that, under normoxia, there was an increase in HIF-1α levels when astrocytes were treated with 1 mM Glu. Even though this increase was not significant, it did correlate with increased GSH levels. It seems as though GSH is able to stabilize HIF-1α and reduce its degradation when it is normally expressed during hypoxia. However, during normoxia, the oxygen levels are sufficient to maintain prolyl hydroxylase activity and target HIF-1α for ubiquitination.

Meanwhile, HIF-1 may increase the level of GSH in hypoxic astrocytes, as shown in our results. Others have also reported that HIF-1 is able to maintain GSH levels in brains of rats exposed to hypoxia (Shrivastava et al, 2008). In addition, previous studies have shown that HIF-1 can protect astrocytes from ROS-induced injury (Chu et al., 2010) and that GSH depletion induces astrocytic death in response to ROS (Im et al., 2006). All these results indicate that

maintaining GSH levels and reducing ROS toxicity is part of HIF-1-mediated neuroprotection. Based on our results and others, we postulate the GSH–HIF-1 crosstalk in hypoxic astrocytes (Figure 3.6). Hypoxia induces accumulation of HIF-1, which subsequently switches on the expression of genes that promote cell survival such as glucose transporters and glycolytic enzymes, EPO, VEGF, HSPs (heat-shock proteins) (Baird et al., 2006), HO-1 (haem oxygenase 1) (Shrivastava et al., 2008), etc. Some of the genes such as EPO and VEGF provide direct cell protection against hypoxic stress. Others, such as those of glucose metabolism, HSPs and HO-1, may increase the level of GSH, contributing to HIF-1 stabilization.

In conclusion, there are still large gaps that exist in our understanding of how astrocytes are affected during stroke. Given that the release of the Glu is considered to be the leading cause of brain damage following stroke, we determined the effect of HIF-1α expression and stabilization on how astrocytes respond to Glu toxicity during stroke. Our study has shown that HIF-1α expression protects astrocytes from Glu-induced damage. We also provide evidence that GSH plays a role in HIF-1α stabilization and promotes its protective effect. Taking this fact into consideration will lead to a better understanding of the protective mechanisms of astrocytes and provide a more effective approach not only to stroke therapy but also other pathological conditions that cause, or are exacerbated by, excitotoxicity.

ACKNOWLEDGEMENT

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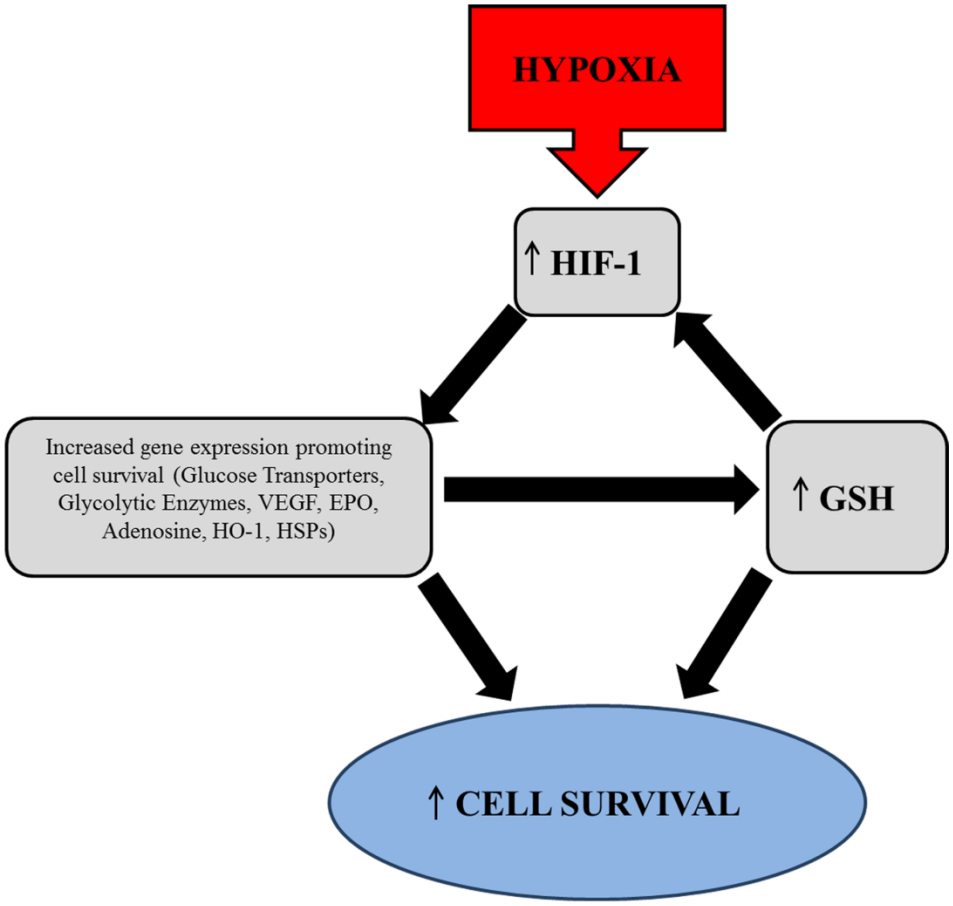

Figure 3.6. Schematic diagram of GSH-HIF-1 cross-talk in promoting cell survival during hypoxia. Hypoxia leads to HIF-1 stabilization which subsequently activates the expression of various pro-survival genes. These include erythropoietin (EPO), vascular endothelial growth factor (VEGF), heat shock proteins (HSPs), heme oxygenase 1(HO-1) and proteins that increase glucose metabolism such as glucose transporters and glycolytic enzymes. Genes such as those of glucose metabolism, HSPs, and HO-1 may increase the level of GSH causing it to exert its anti-oxidant effect and promote survival. In addition, the reducing environment created by GSH can in turn contribute to HIF-1 stabilization.

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Chapter 4: Ischemic Tolerance in an *in vivo* model of Glutamate "Preconditioning"

4.1 INTRODUCTION

Stroke results in a rapid cessation of blood flow that compromises energy metabolism in the affected brain tissue and leads to enhanced glutamate release (Benveniste *et al.*, 1984). Glutamate is ubiquitously distributed in the brain and is present in concentrations that are higher than any other amino acid (Fonnum, 1984), making it the principal excitatory neurotransmitter in the nervous system. Early studies demonstrated that blocking ionotropic glutamate receptors significantly reduced ischemic damage (Sims & Muyderman, 2010). This was attributed to its involvement in the excitotoxic cascade in which the increased glutamate release causes an excessive accumulation of calcium (Ca^{2+}) that triggers downstream pathways that cause cell death. Therefore, impeding excitotoxicity in experimental stroke models has been the most targeted mechanism in the development of neuroprotective treatments for stroke (O'Collins *et al.*, 2006; Minnerup *et al.*, 2012). In stroke patients, the effect of over 20 anti-excitatory drugs was evaluated in over 270 preclinical studies, none of which demonstrated a beneficial effect (O'Collins *et al.*, 2006). However, experimental studies on potential strategies to protect neurons have continued to increase over the last few years (Ginsberg, 2008; Minnerup *et al.*, 2012).

A current focus is on understanding the protective mechanisms that induce tolerance following ischemic preconditioning. Current knowledge suggests that preconditioning with a brief period of ischemia is an effective approach to decrease neuronal death after a more severe ischemic episode (Kirino, 2002). The same concept can be applied to excitotoxicity where preconditioning with a mild glutamate-induced stress can promote a tolerant state that reduces the injury caused by a subsequent, more severe glutamate exposure. Few studies have used glutamate preconditioning to evaluate the development of resistance to a subsequent ischemic insult. Lin et al. (2008) showed that neuronal death following *in vitro* ischemia was prevented by

glutamate preconditioning through the anti-apoptotic protein, Bcl-2 (Lin *et al.*, 2008). Other studies were also primarily focused on neuronal cultures, either hippocampal neurons (Mabuchi *et al.*, 2001) or in a neuronal-glial co-culture (Grabb & Choi, 1999). In this study, our main objective was to determine whether glutamate preconditioning would protect neurons from ischemia in an *in vivo* model. To test this, we used transgenic (Tg) mice that overexpress glutamate dehydrogenase 1 (Glud1) in the neurons of the nervous system. These Tg mice are characterized by a lifelong increase in Glud1 activity and increased synaptic glutamate release (Bao *et al.*, 2009). We hypothesized that chronic exposure of brain neurons to moderate levels of glutamate would activate protective pathways that reduce damage after a stroke. Our results demonstrate that the Glud1 Tg mice showed a decrease in infarct size and edema volume following middle cerebral artery occlusion (MCAO) when compared with wild type (Wt) mice. Furthermore, we explored the effect of preconditioning/ischemia on ubiquitin and Microtubule-associated proteins-2 (MAP2).

4.2 MATERIALS AND METHODS

4.2.1 Generation of Glud1 Tg mice

All experiments were conducted with the approval of the University of Kansas Institutional Animal Care and Use Committee. The transgenic Glud1 mice were generated as described in Bao et al. (2009). Briefly, linearized DNA containing the cDNA of mouse Glud1 was injected into fertilized C57BL6/SJL hybrid mouse oocytes. The cDNA was placed under the control of the Nse (neuron-specific enolase) promoter (Bao *et al.*, 2009).The animals used for the following experiments were 9- mos of age as that was when differences between the Tg and Wt

become more significant. Twenty two-month (mo) old mice were used for the studies on the effects of aging on ischemia-induced brain damage.

4.2.2 *In vivo* ischemia model

Reversible occlusion of the middle cerebral artery was induced using a well-established protocol (Clark *et al.*, 1997). The mice were anesthetized with 3% Isoflurane and oxygen and maintained with 1.5% Isoflurane, or to desired anesthetic effect, throughout the procedure. Buprenorphine was used as the analgesic and was injected pre-operatively at 0.05 mg/kg. Male mice were subjected to MCAO followed by a 24 hour (hr) period of reperfusion. TTC (2,3,5-triphenyltetrazolium chloride monohydrate) staining was used to assess brain damage (Ito *et al.*, 1997). Brain edema volume (V_{edema}) was also measured from the coronal sections that were stained by TTC by determining the volumes of both the ipsilateral (affected) hemisphere (V_{Ipsi}) and the contralateral hemisphere (V_{contra}) and using the equation: $V_{edema} = V_{contra} - V_{Ipsi}$ (Yan *et al.*, 2011).

4.2.3 Measurement of proteasomal activity

Brain tissues from 9-mo old Wt and Tg mice were homogenized and 20 µg of the cell homogenate were incubated with proteasome activity assay buffer in which their ability to cleave the flurogenic peptide substrate, Suc-LLVY-AMC was determined (Figueiredo-Pereira *et al.*, 1994). The first assay buffer for evaluation of the 26S proteasome function consisted of 50 mM Tris (pH 7.4), 5mM $MgCl_2$, 2 mM DTT, 2 mM ATP and the fluorogenic substrate Suc-LLVY-AMC (80 µM in 1% DMSO, Sigma-Aldrich). The second buffer for determining 20S proteasome function contained 20 mM HEPES (pH 7.8), 0.5 mM EDTA, 0.03% SDS and 80 µM

Suc-LLVY-AMC. The hydrolysis of Suc-LLVY-AMC into AMC (7-amino-4-methly-coumarin) was detected with a fluorescence plate reader at ex 380 nm and em 440 nm.

4.2.4 Immunohistochemistry

Immunostaining and statistical analysis were conducted by Dr. Ranu Pal and performed using the protocol described by Bao et al., (2009). In brief, following fixation and permeabilization of hemisected brain tissue (ipsilateral *vs.* contralateral hemispheres); 2 mm coronal sections were obtained and further sectioned into ~25 μm thick sections using a cryotome. The thin sections were incubated overnight in the following primary antibodies: anti-ubiquitin (polyclonal; rabbit; 1:250; Cell Signalling) and anti-MAP2A (monoclonal; mouse; 1:250; Millipore). The sections were then rinsed in PBS and incubated with fluorescent dye-labeled secondary antibodies (Alexa 488 goat anti-mouse and Alexa 568 rabbit anti-goat) at 37 °C for 2 hrs. Images were acquired on a Leica SPE2 laser confocal microscope. Pixel density counts were determined using the Leica Application Suite software. The graphs represent averages from the *stratum oriens* (SO), *stratum pyramidale* (SP) and *stratum radiatum* (SR) of the CA1 and CA3 regions of the hippocampus from 3 pairs of animals.

4.2.5 Statistical analysis

Student's t-test and three-way ANOVA analysis were used to determine statistical significance on SigmaPlot 12.5.

4.3 RESULTS

4.3.1 Decreased brain infarct and edema volume in the Glud1 Tg mice in the *in vivo* MCAO model of ischemic stroke

The 9-mo old Wt and Tg mice were subjected to MCAO followed by a 24 hr period of reperfusion. Using TTC staining, we were able to assess the extent of brain damage. The reduction of TTC by enzymes in living cells leads to the formation of water-insoluble formazan (red) and thus the area and volume of surviving tissue can be used to determine percent infarct size (Ito *et al.*, 1997). The results revealed that the Glud1 Tg mice had a 30% decrease in infarct volume compared to their Wt counterparts (Figure 4.1B). Brain edema volume was also measured using the 5 mm coronal sections by determining the difference between the volumes of both the ipsilateral hemisphere and the contralateral hemisphere. As shown in Figure 4.1C, the Glud1 Tg mice had a significantly lower edema volume following MCAO compared to the Wt mice. Together, these results support our hypothesis that chronic exposure to moderate levels of glutamate can protect against an ischemic insult.

4.3.2 Increased basal levels of proteasomal activity

Next, we aimed to elucidate the adaptive mechanisms that allowed the Glud1 Tg mice to be more resistant to ischemia. The ubiquitin-proteasome system (UPS) plays an essential role in ischemic tolerance (Meller *et al.*, 2008; Meller, 2009). To identify the role of the UPS in this observation, we first determined proteasome activities in the preconditioned Glud1 Tg mice and the Wt mice. Brain tissues from 9-mo old Wt and Tg mice were homogenized and proteasomal function was assessed. An ATP-free assay buffer was used to differentiate 20S proteasomal function from the 26S proteasomal function. We found that basal activities of the 26S and the 20S proteasome (without any prior treatment) were elevated in the Glud1 Tg mice (Figure 4.2). Although the significant increase in 26S proteasomal activity might suggest that this is due to increased protein ubiquitination as a result of the elevated glutamate levels, a previous study characterizing the Tg mice has concluded that accumulation of ubiquitin-

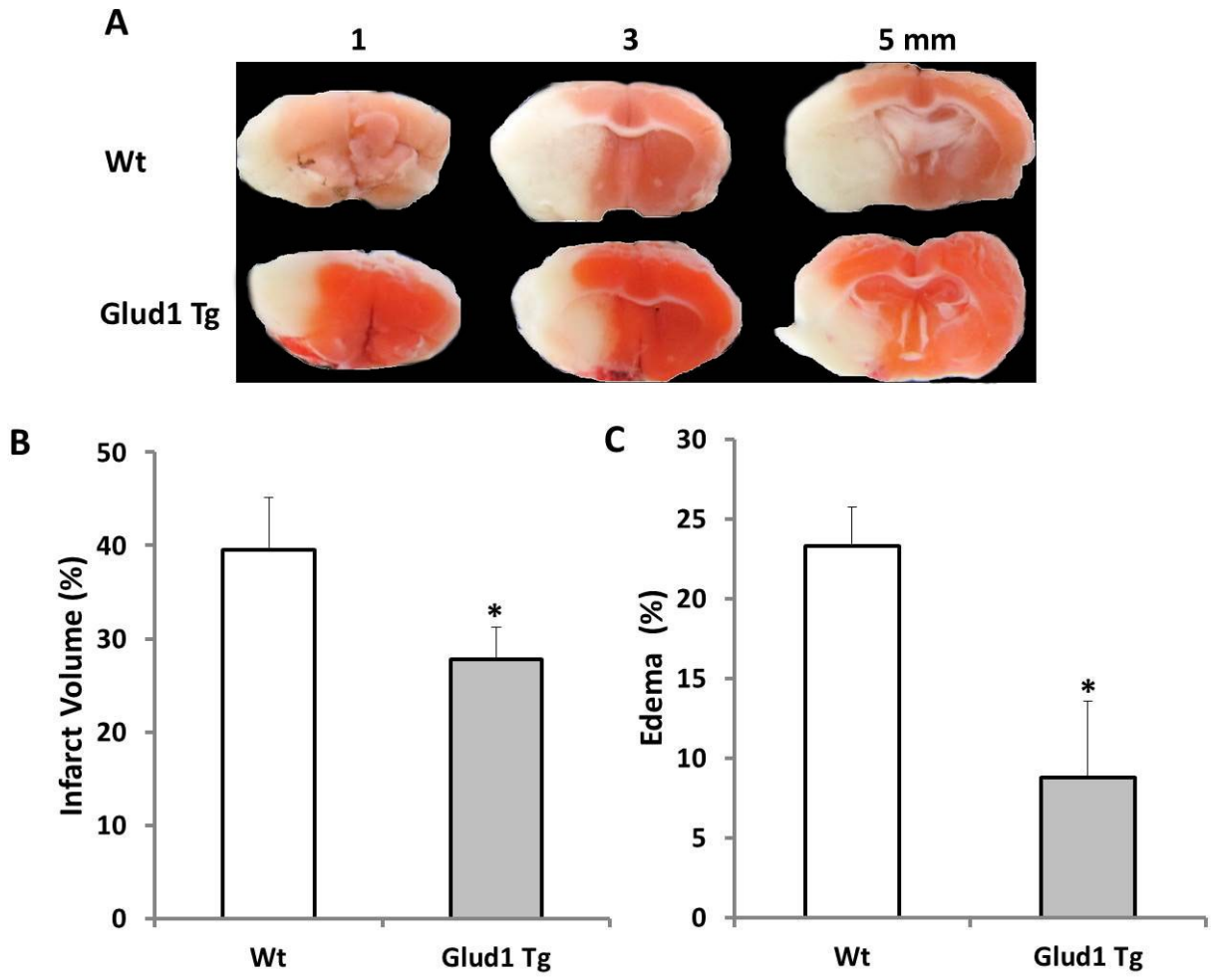

Figure 4.1 Effect of MCAO on brain tissue damage in Wt vs. Glud1 Tg mice. Brain damage was determined by TCC staining after mice were subjected to 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections proceeding from frontal to caudal. Sections were taken from the 1 mm position of the frontal pole and proceeded in 2 mm intervals to 5 mm. (B) Quantification of infarct volume was determined in TTC stained sections. (C) Quantification of brain edema volume estimated from TTC stained sections (n=5). Data presented as means ± SEM *P < 0.05, vs. Wt mice.

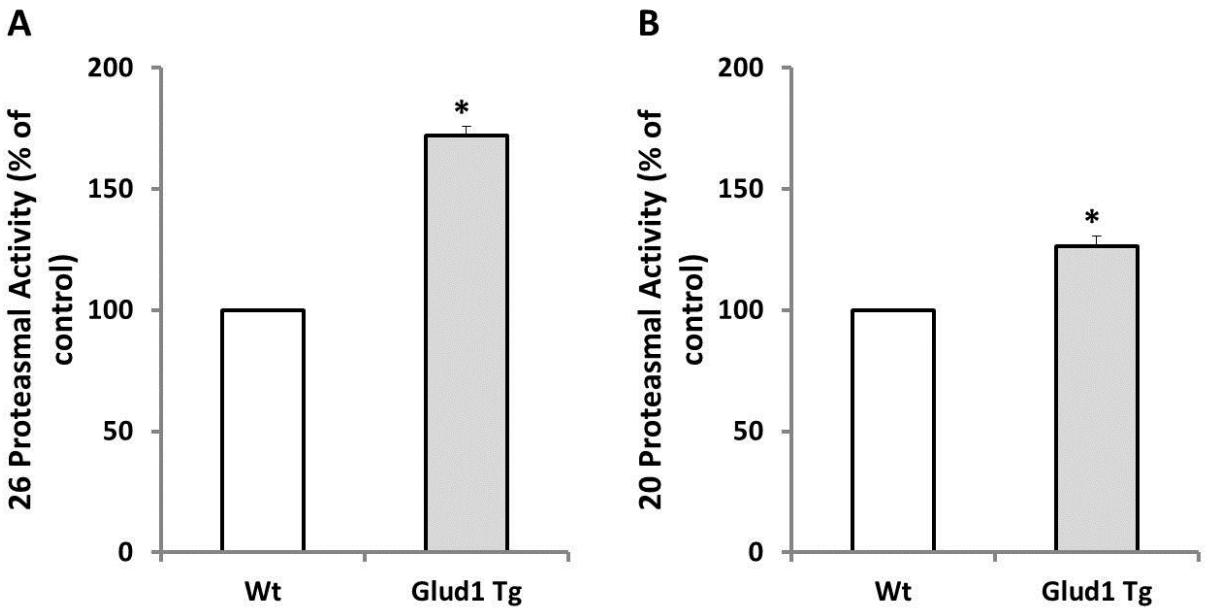

Figure 4.2. Brain proteasomal activity is increased in the Glud1 Tg mice. Activities of the (A) 26S and the (B) 20S proteasome were assayed by determining their ability to cleave Suc-LLVY-AMC (n=3). Data presented as means ±SEM *p<0.05, *vs*. Wt mice.

protein aggregates do not appear until the age of 16-20 mo (Bao *et al.*, 2009). This was confirmed in recent studies using 9 mo-old Tg and Wt mice as will be presented below. This suggests that at 9 mo, proteasome overload has not yet occurred.

4.3.3 Ubiquitin labeling in hippocampus following MCAO

To determine whether the increase in proteasome activity had an effect on ubiquitin and ubiquitinated proteins, we stained brain sections from Glud1 Tg and Wt mice after cerebral occlusion. Changes in the levels of ubiquitin and whether there is an even distribution of ubiquitin immunoreactivity or ubiquitin-positive aggregates present is a strong indicator of protection induced by glutamate preconditioning. In a review by Caldeira et al, it was noted that the formation of ubiquitin-protein aggregates is a protective response of cells against abnormal proteins and suggest a greater potential for cell survival in the immediate post-ischemic period (Caldeira *et al.*, 2014). As shown in figure 4.3A, ubiquitin levels seemed to increase in the CA1 region of the hippocampus in the Glud1 Tg mice with an even higher increase in the CA3. This change however was not statistically significant presumably due to the high variation in the mean pixel density (Figure 4.3B). Additional experiments to increase the sample size may provide more conclusive results. The ubiquitin labeling seemed to be evenly distributed in the areas with the high levels of ubiquitin with few ubi-protein aggregates. Damaged proteins can form toxic aggregates (Taylor *et al.*, 2002) and indicate a reduction in cell viability or the presence of vulnerable cells that are destined to die (Ge *et al.*, 2007). To confirm that the changes in ubiquitin levels were due to the effect of ischemia, levels of ubiquitin from the hippocampus of Glud1 Tg and Wt that were not subjected to MCAO were also determined (Figure 4.3C). The results showed no significant differences between the CA1 and CA3 of the Glud1 Tg and Wt mice (Figure 4.3D).

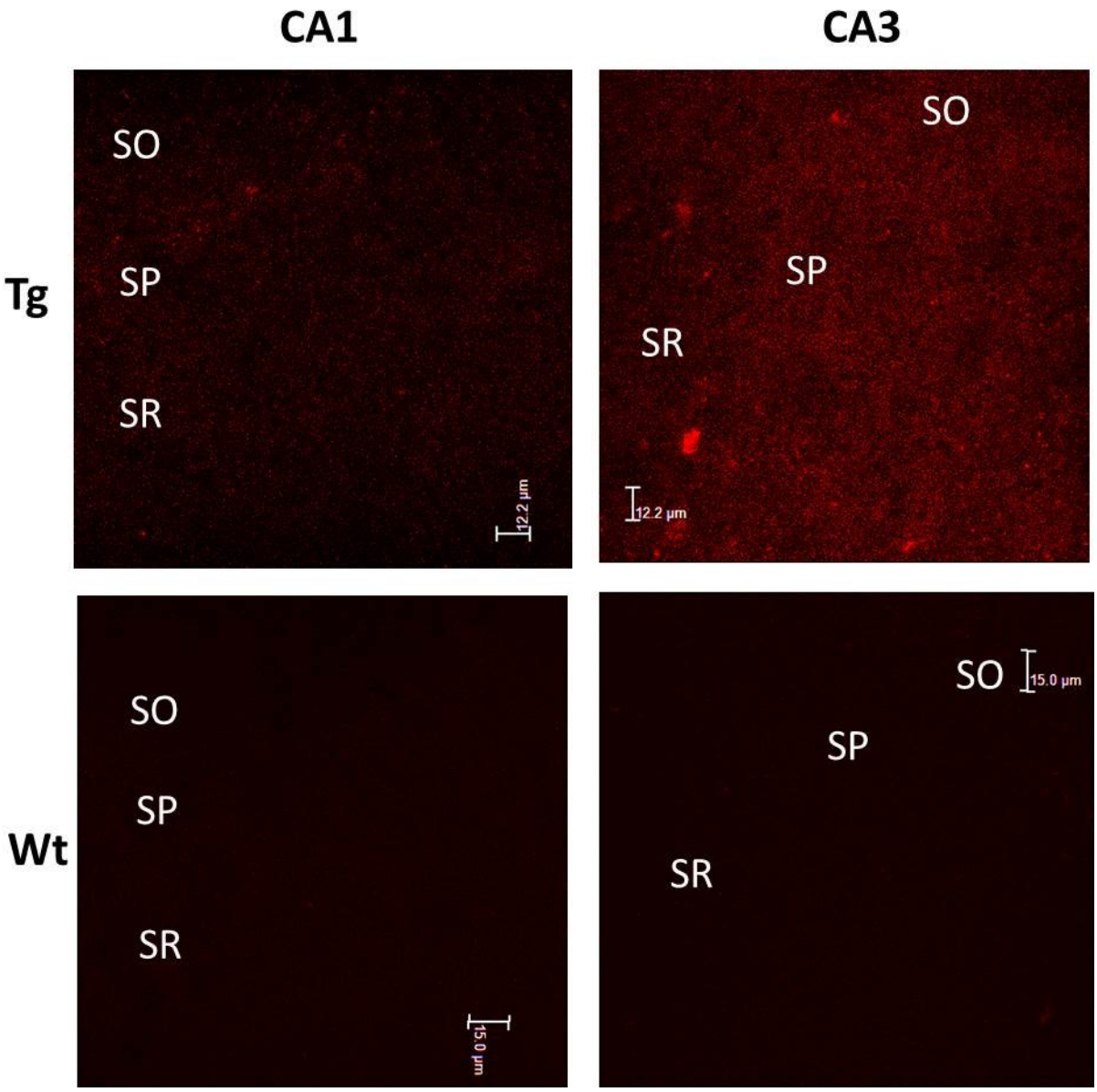

Figure 4.3A Ubiquitin expression in Glud1 Tg and Wt mice after MCAO. Representative immunofluorescent images showing labeling of ubiquitin in the ipsilateral CA1 and CA3 region of the hippocampus of a pair of 9-mo old Glud1 Tg and Wt mice that were subjected to *in vivo* ischemia/reperfusion and processed for immunohistochemical studies at the same time. SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

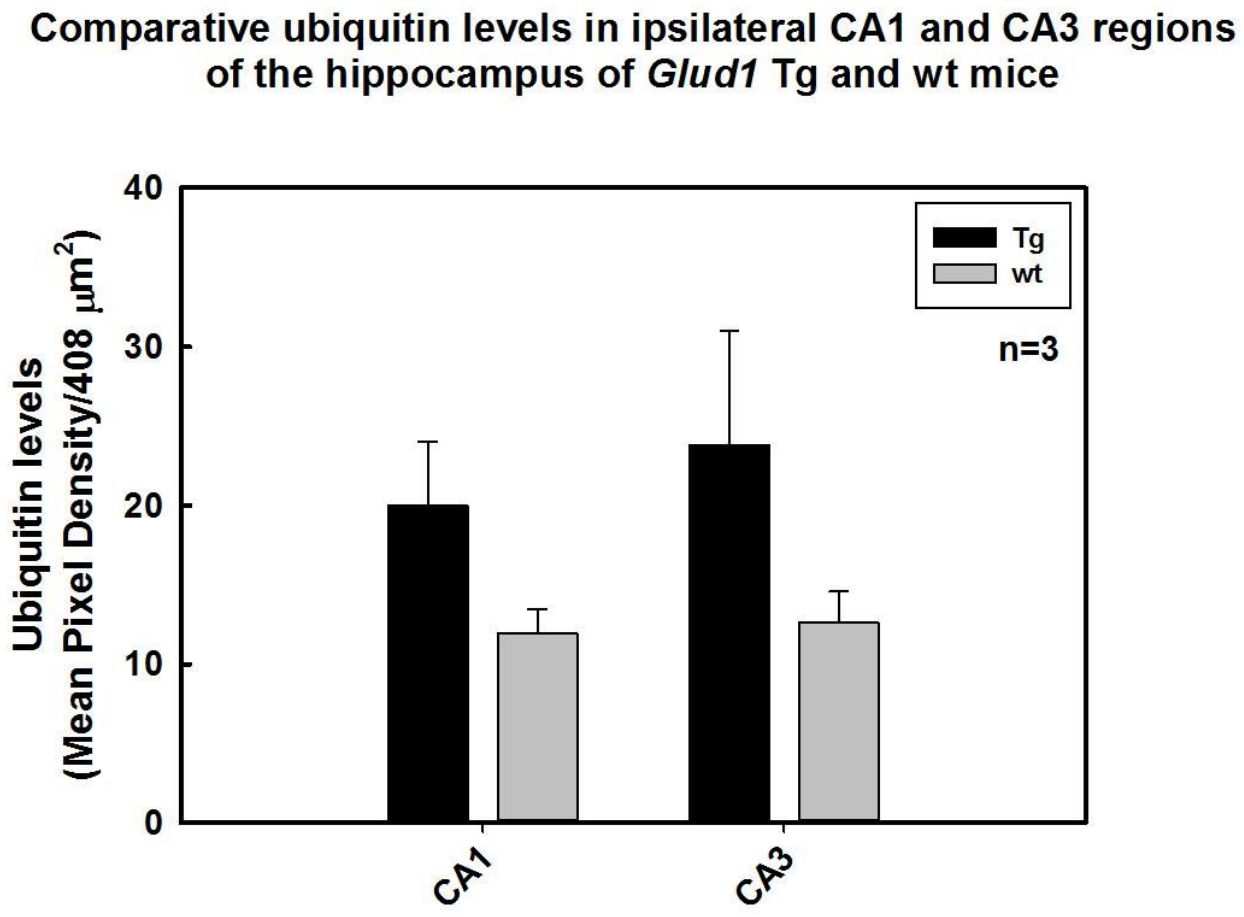

Figure 4.3B Quantification of ubiquitin expression in Glud1 Tg and Wt mice after MCAO. The mean pixel density of ubiquitin expression was determined from the multiple subfields of the ipsilateral CA1 and CA3region from 3 pairs (Glud1 Tg and Wt) of mice. All data are presented as means ±SEM (n=3).

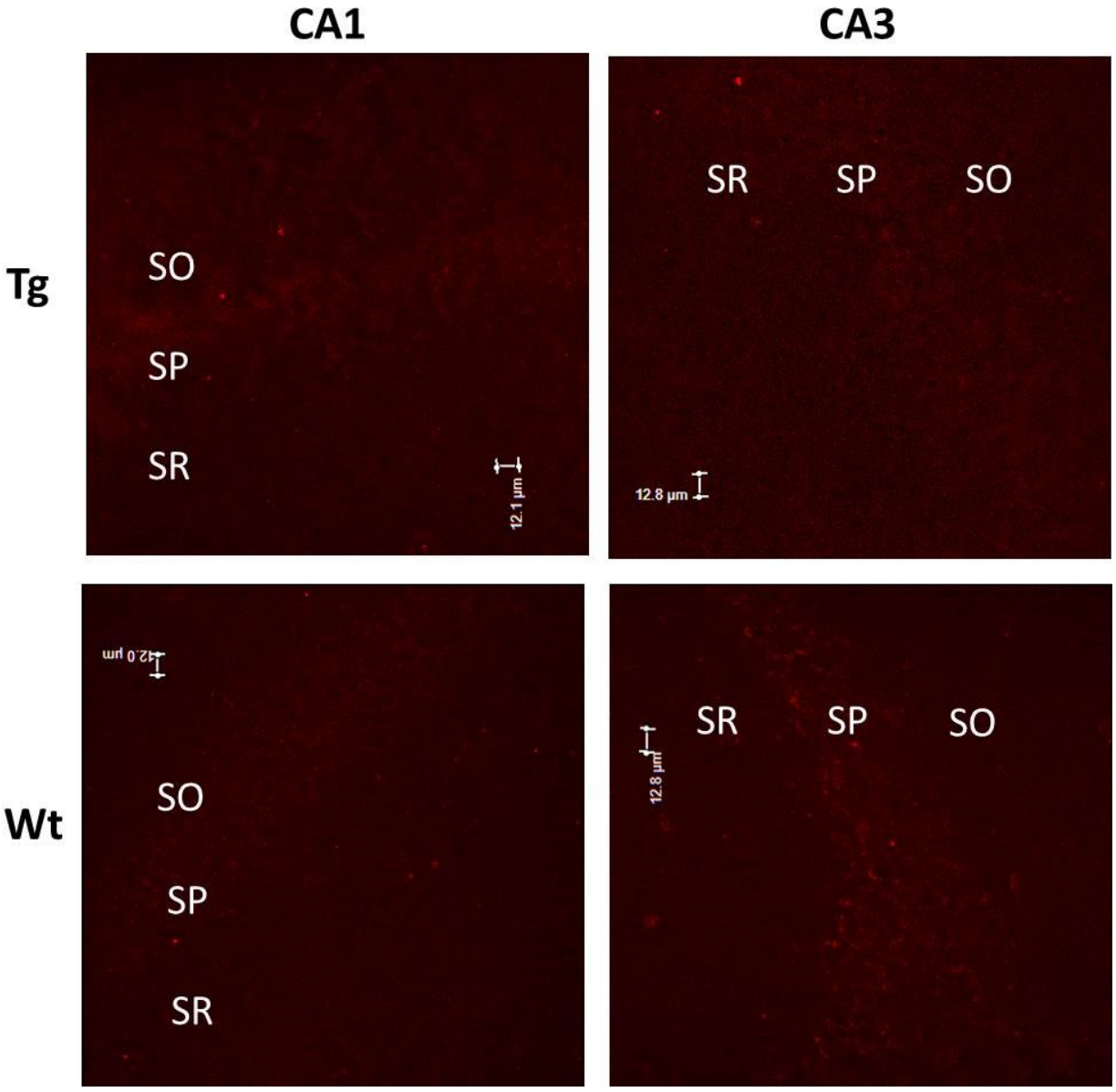

Figure 4.3C Baseline ubiquitin expression in Glud1 Tg and Wt mice. Representative immunofluorescent images showing labeling of ubiquitin in the CA1 and CA3 region of the hippocampus of a pair of 9-mo old Glud1 Tg and Wt mice that were processed for immunohistochemical studies at the same time. SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

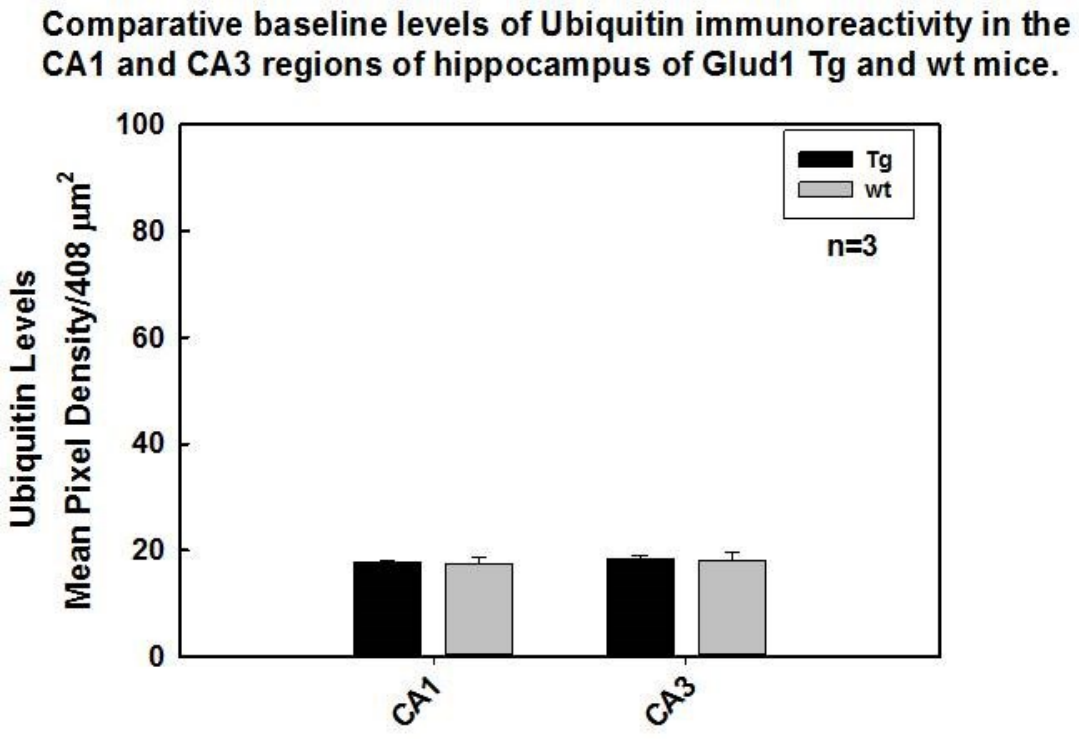

Figure 4.3D Quantification of baseline ubiquitin levels in Glud1 Tg and Wt mice. The mean pixel density of ubiquitin expression was determined from the multiple subfields of the CA1 and CA3region from 3 pairs (Glud1 Tg and Wt) of mice. All data are presented as means ±SEM (n=3). SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

4.3.4 MAP2 labeling is increased in the Glud1 Tg mouse hippocampus following MCAO

MAP2 is critical for the maintenance of normal cytoskeletal architecture and functions (Yan *et al.*, 2010). MAP2 is a useful marker of neuronal cell bodies and dendrites, not axons, and was previously used to demonstrate that the chronic elevations in glutamate release in the Tg mice were associated with a gradual deterioration of dendrites and cell death of particularly sensitive neurons, such as those in the CA1 region of the hippocampus (Bao *et al.*, 2009). Therefore, MAP2A immunoreactivity was used as a neuronal marker in the present study, as well. Notable differences were observed between the Glud1 Tg mice and the Wt mice. Unlike the baseline condition where MAP2A immune-labeling is significantly stronger in Wt than Tg mice (Figure 4.4), MAP2 levels were significantly increased ($p<0.003$) in the CA1 region of the hippocampus in the Glud1 Tg mice following ischemia. Although the CA3 region showed an increasing trend in MAP2 labeling, the differences were not statistically significant.

4.3.5 Effect of aging on the MCAO response of Wt and Tg animals

Focal ischemia has been shown to increase the infarct volume in aged animals (Davis *et al.*, 1995; Sutherland *et al.*, 1996) and the effectiveness of preconditioning-induced protection against severe ischemia has been reported to decrease with age (He *et al.*, 2005).To determine whether the tolerance induced by our model of i*n vivo* glutamate preconditioning was preserved in aged mice, we used TTC staining to calculate the infarct and edema volume in 22-mo old mice after MCAO and reperfusion. Figure 4.5 shows the Glud1 Tg mice at 22 mos were still protected in terms of a decrease in infarct and edema volume compared to their age-matched Wt counterparts. No significant differences were found between the 22-mo old and the 9-mo old mice of the same genotype.

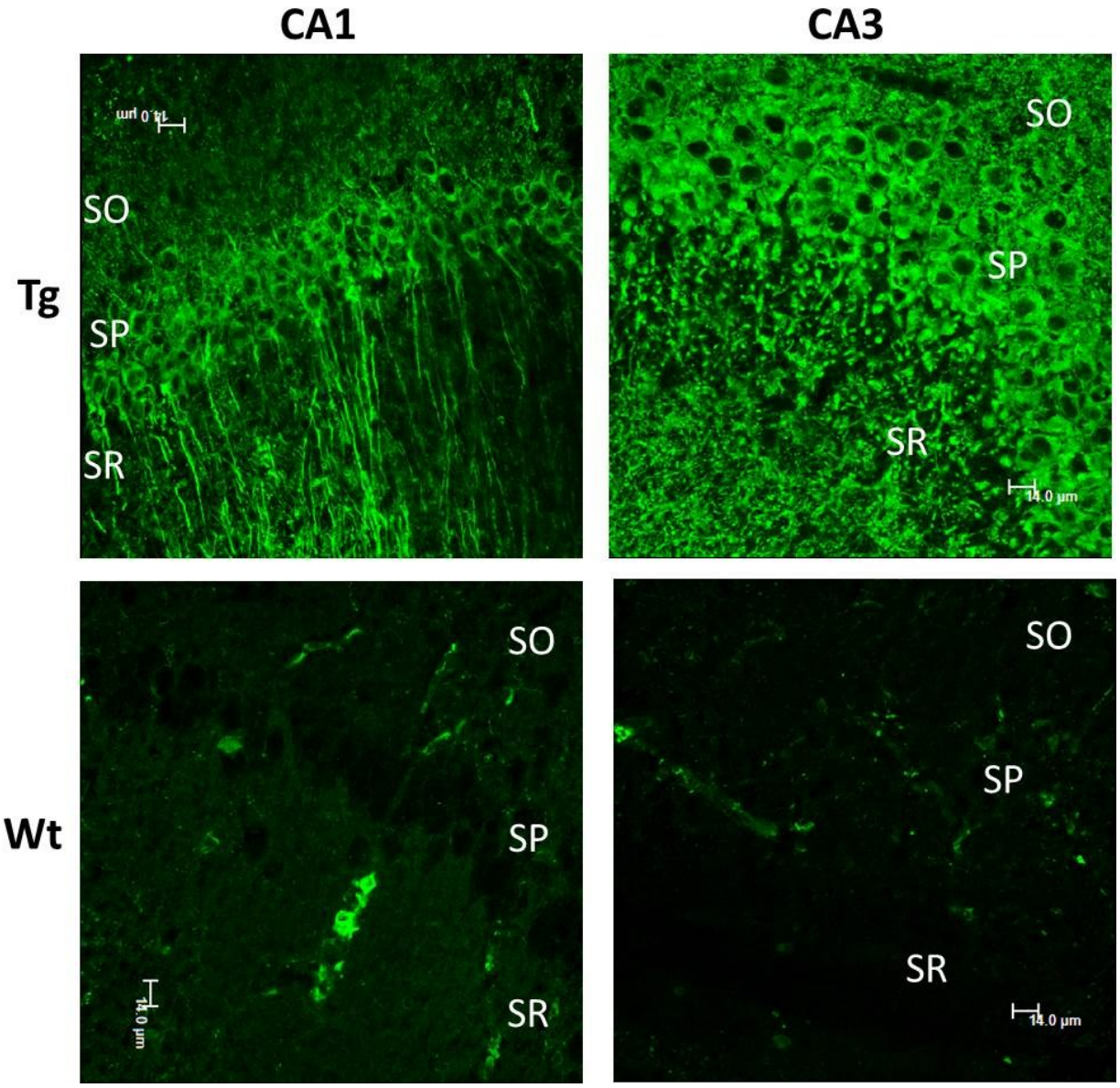

Figure 4.4A Immunolabeling of MAP2 in Glud1 Tg and Wt mice after MCAO.

Representative immunofluorescent images showing labeling of MAP2 in the ipsilateral CA1 and CA3 region of the hippocampus in a pair of 9-mo old Glud1 Tg and Wt mice that were subjected to *in vivo* ischemia/reperfusion and processed for immunohistochemical studies at the same time. SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

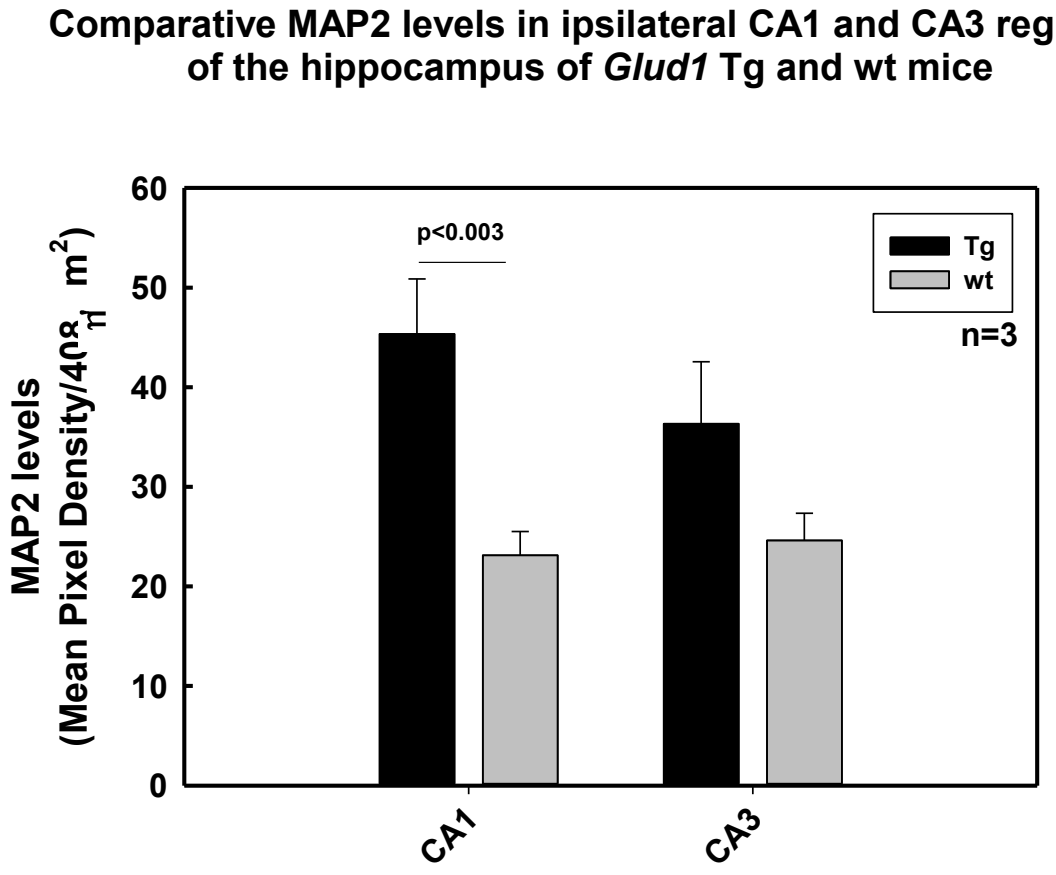

Figure 4.4B Quantification of MAP2 immunolabeling in Glud1 Tg and Wt mice after MCAO. The mean pixel density of MAP2expression was determined from the multiple subfields of the ipsilateral CA1 and CA3 region from 3 pairs (Glud1 Tg and Wt) of mice. All data are presented as means ±SEM (n=3).

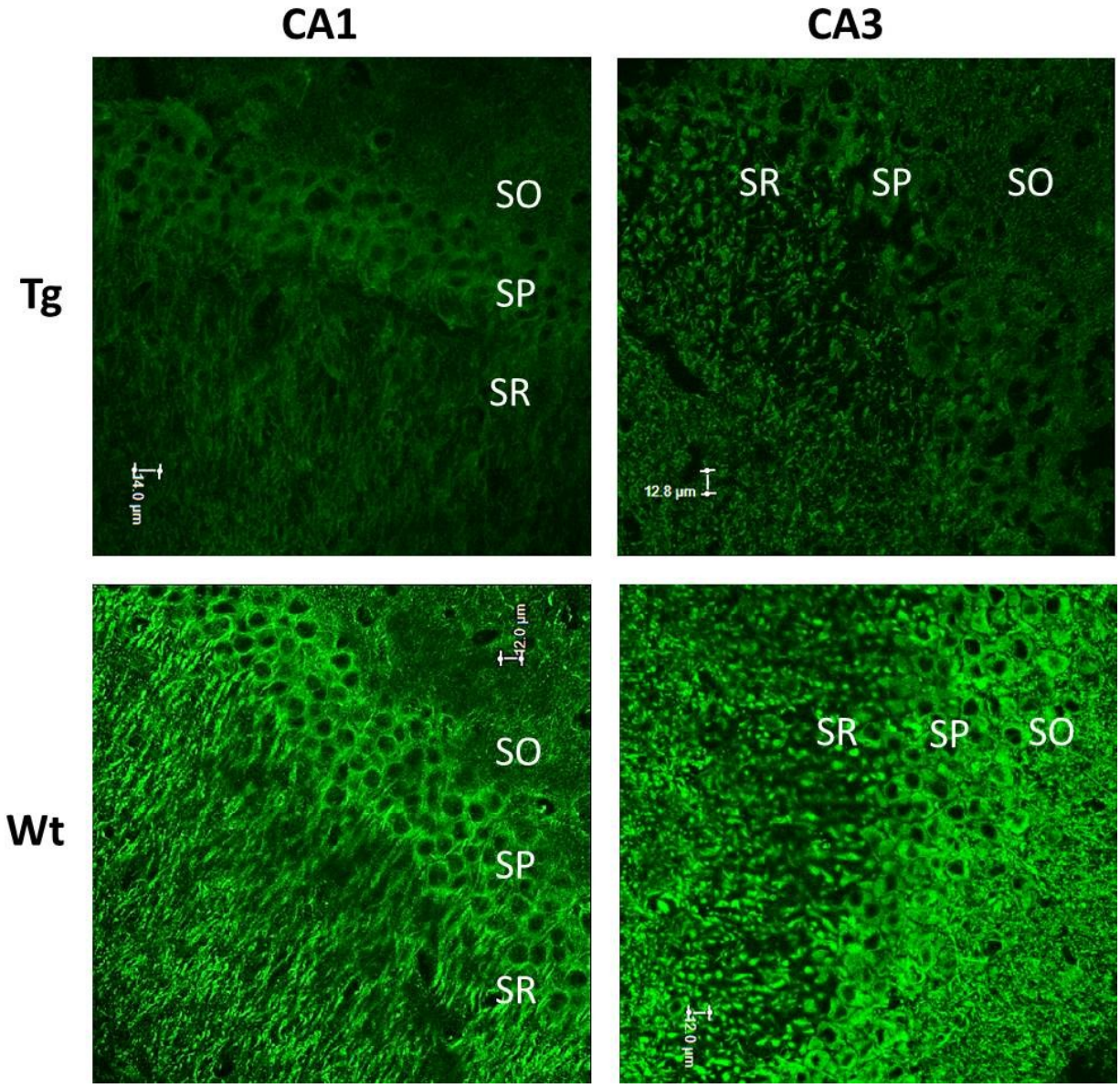

Figure 4.4C Baseline MAP2 expression in Glud1 Tg and Wt mice. Representative immunofluorescent images showing labeling of MAP2 in the ipsilateral CA1 and CA3 region of the hippocampus in a pair of 9-mo old Glud1 Tg and Wt mice that were processed for immunohistochemical studies at the same time. SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

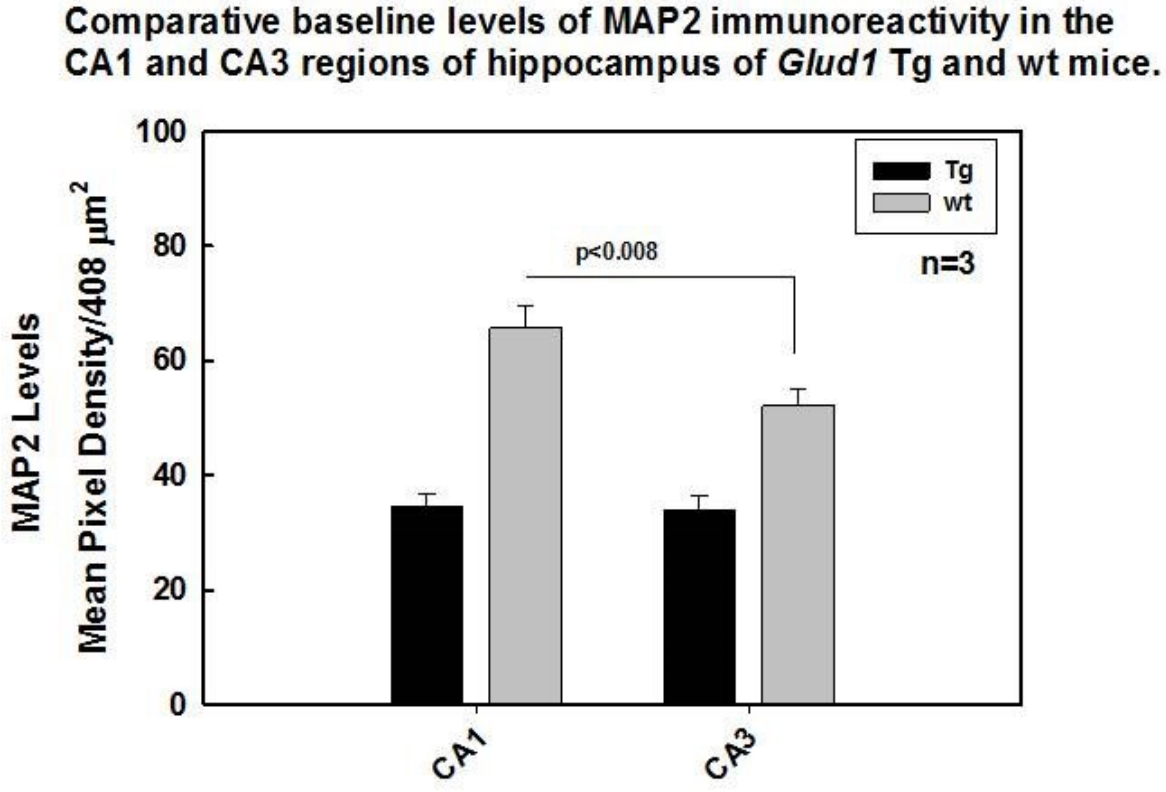

Figure 4.4D Quantification of baseline MAP2 levels in Glud1 Tg and Wt mice. The mean pixel density of MAP2expression was determined from the multiple subfields of the CA1 and CA3 region from 3 pairs (Glud1 Tg and Wt) of mice. All data are presented as means ±SEM (n=3). SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

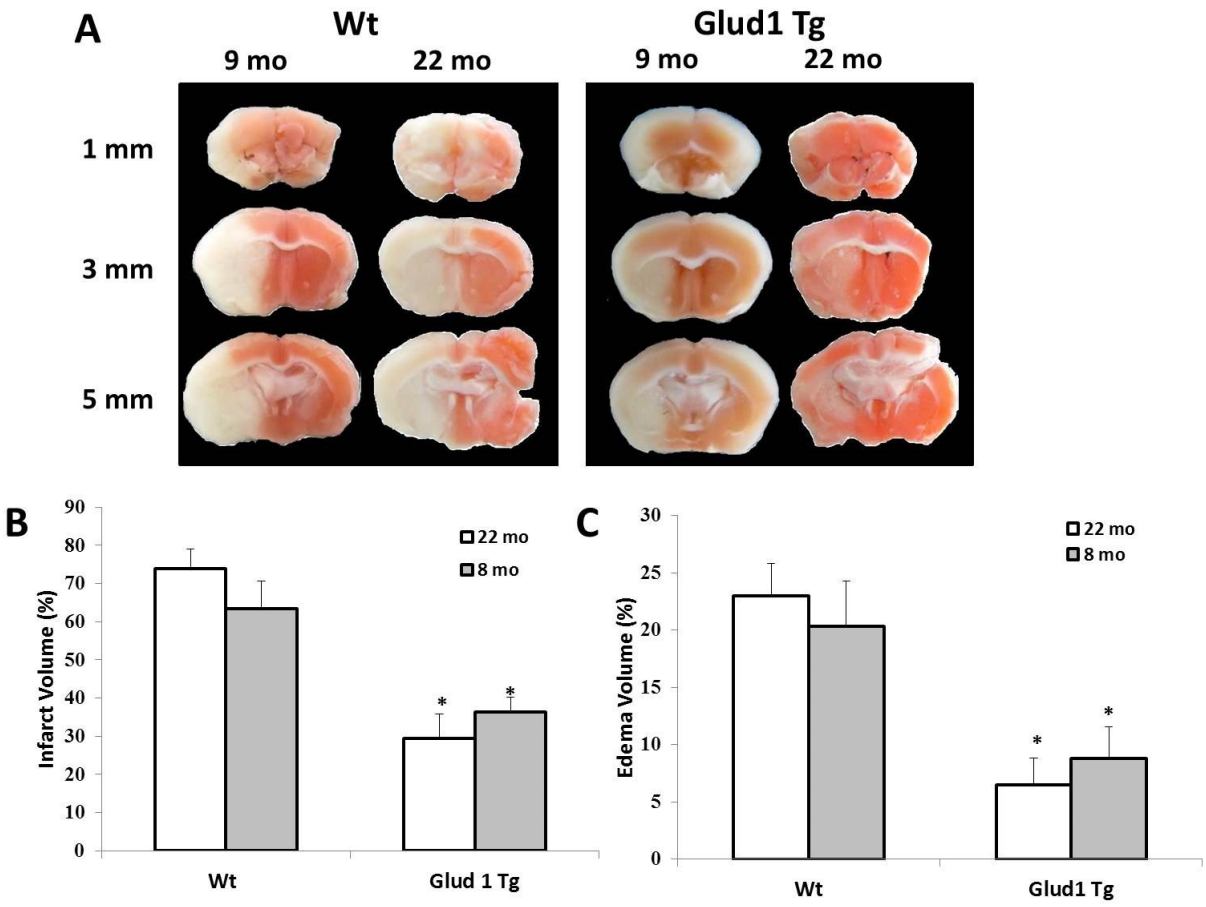

Figure 4.5. Effect of MCAO on brain tissue damage in 22 month old Wt and Glud1 Tg mice. Brain damage was determined by TCC staining in 9-mo and 22-mo old Wt and Glud1 Tg mice after 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections proceeding from frontal to caudal. Sections were taken from the 1 mM position of the frontal pole and proceeded in 2 mm intervals to 5 mm. (B) Quantification of infarct volume determined by TTC stained sections. (C) Quantification of brain edema volume estimated from TTC stained sections (n=3). Data presented as means ± SEM *P < 0.05, vs. Wt mice.

4.4 DISCUSSION

Stroke is a debilitating disorder with no effective neuroprotective therapy. Treatments for ischemic stroke are required to limit stroke induced morbidity and mortality as recombinant tissue plasminogen activator (rt-PA) is the only pharmacological agent that has been approved to treat acute ischemic stroke. Rt-PA is administered to only 2-5% of stroke patients due to the strict eligibility criteria (Donnan *et al.*, 2011). Since rt-PA is a thromolytic, it is considered an indirect neuroprotectant (Minnerup *et al.*, 2012) and strategies that directly target neuronal viability are still unavailable. A study that investigated the biochemical alterations in the peri-infarct tissue of stroke patients showed that extracellular glutamate was elevated after ischemia and remained elevated in patients that showed infarct progression over time (Woitzik *et al.*, 2014). This suggests that excitotoxicity is not only associated with the initial cell death following a stroke but also with delayed infarct progression.

In the current study, we showed as a proof-of-concept that the protective effect of ischemic tolerance can also be observed by substituting a short period of ischemia (preconditioning) with chronic, moderate hyperactivity of glutamate in brain. Glud1 Tg mice were used as our *in vivo* model of glutamate preconditioning. Glud1 is a mitochondrial enzyme that is involved in the glutamate biosynthesis pathway. It catalyzes the catabolism of glutamate to α-ketoglutarate (α-KG). Glud1 also catalyzes the reverse reaction to convert α-KG and ammonia back to glutamate (Stewart *et al.*, 1980). The Tg mice that were created over-express the Glud1 gene, have increased enzymatic activity and were shown to have moderate increases in Glu levels compared to their Wt littermates (Bao *et al.*, 2009). Unlike a previous mouse model that was a null mutant for the glutamate EAAT2 transporter (Rothstein *et al.*, 1996; Tanaka *et al.*, 1997) these Tg mice do not suffer from brain damage (until an older age) and have a

relatively longer life span (Bao *et al.*, 2009). We subjected 9-mo Glud1 Tg mice to MCAO to induce *in vivo* ischemia followed by a 24 hr period of reperfusion. Interestingly, the Glud1 Tg mice were more resistant to brain damage compared to the Wt, "non-preconditioned" mice. We then sought to determine possible mechanisms that are involved in the adaptive response to chronic glutamate exposure.

The proteasomal proteolytic pathways represent the main mechanisms responsible for the degradation of damaged or unwanted proteins. These pathways play a very significant role in maintaining normal, cellular homeostasis. Knockouts of proteasomal subunits have resulted in lethal phenotypes (Heinemeyer *et al.*, 1991; Orlowski, 1999), further indicating their importance. Protein unfolding and aggregation are dominant pathogenic events in the vulnerable ischemic neurons(Ge *et al.*, 2007). The irreversible accumulations of protein aggregates are often toxic to cells and can cause neuronal death. Protein ubiquitination involves a sequential process in which ubiquitin forms a thioester bond with an E1-ligase (requires ATP), then it is transferred to the E2-ligase and finally it is transferred from the E2-ligase to the target protein by an E3-ligase (Hershko, 1983). Ubiquitin is usually ligated to the exposed hydrophobic segments of unfolded proteins that need to be degraded. If ubi-proteins become too numerous to be degraded by the proteasome, they tend to aggregate. A study by Lui et al. (2005) showed that ischemic preconditioning was able to alleviate protein aggregation in neurons. Whether this reduction is due to a decrease in overall cell damage or because tolerant cells have an enhanced ability to eliminate the irreparably damaged proteins has yet to be elucidated. It is known that excess glutamate can induce the formation of ROS (Armstead *et al.*, 1989; Duchen, 2000; Kahlert *et al.*, 2005; Parfenova *et al.*, 2006) and that ROS can alter proteasomal activities as shown by us (Chapter 2) and previous reports (Ullrich *et al.*, 1999; Ding *et al.*, 2003; Grune *et al.*, 2004;

Aiken *et al.*, 2011). Therefore, we hypothesized that glutamate can indirectly lead to an increase in proteasomal activity that could be protective following ischemia and reperfusion. Initially we determined that glutamate preconditioning increased basal proteasomal activities in the Glud1 Tg mice. We then looked at the levels of ubiquitin to determine whether the proteasomal activity was preserved or compromised after the ischemic insult.

The increasing trend in the ubiquitin-immunoreactive proteins (Figure 4.3) suggests that an enhancement in 26S proteasomal activity may not play a role in this protective effect. A previous report characterizing the Glud1 Tg mice showed that although the 9-mo old Glud1 mice had only marginally higher ubiquitin levels compared to their Wt counterparts, examination of 16-20 mo old mice showed intense levels of ubiquitin staining in the Glud1mice compared to the almost non-existent staining in the Wt mice (Bao *et al.*, 2009). We confirmed this observation as shown in Figure 4.3C. It is reasonable to believe that the ischemic stress that occurred in our study at 9-mo was able to trigger whatever changes in the ubiquitin-proteasome system that would cause these observations at 16-20 mos (an additional 7-11 mos of glutamate exposure). Furthermore, analysis of the genome-wide gene expression in the hippocampus of the Glud1 mice showed that there were not only an upregulation in Ubiquitin-Specific Peptidases that degrade ubiquitinated proteins, but also increases in Ubiquitin-Conjugating Enzymes such as UBE2K and UBE3A, which covalently attach ubiquitin molecules to proteins that are destined for degradation (Wang *et al.*, 2010a). Whether a longer reperfusion period (>24 hrs) would ultimately result in an increase in delayed neuronal death in the Glud1 Tg mice is still unknown.

The increase in basal proteasomal activity (Figure 4.2) could be a compensatory mechanism to deal with the effects of the moderate increases in glutamate. Following a stroke, the loss of ATP can impair the activities of the 26S proteasome (Keller *et al.*, 2000). The 26S

proteasome, is an ATP-dependent protease composed of a core proteinase, the 20S proteasome, that is capped with two PA700 regulatory particles (the 19S complex) on both ends (Coux *et al.*, 1996). Tanahashi et al. demonstrated that *in vitro*, these subunits associate and disassociate in an ATP-dependent manner. ATP-rich conditions promote their association and the formation of the enzymatically active 26S proteasome and conditions lacking ATP causes their disassociation (Tanahashi *et al.*, 2000). Following transient forebrain ischemia, the ATP-dependent re-association of the 20S catalytic and PA700 regulatory subunits to form the 26S proteasome was severely impaired in the hippocampus (Asai *et al.*, 2002).

It is important to note that although the differences in ubiquitin immunostaining between the CA1 and CA3 regions of the hippocampus in the Glud1 Tg mice were not statistically significant it seems as though ubiquitin was present in higher levels in the CA3 (Figure 4.3). This is interesting because the CA1 is usually more sensitive than the CA3 when exposed to oxidative stress as shown by previous reports (Sarnowska, 2001; Wang *et al.*, 2005). More specifically, Bao et al. showed that the CA3 was more resistant to neuronal loss than the CA1 in the Glud1 Tg mice. The more vulnerable CA1 region also had decreased MAP2A labeling of dendrites and in synaptophysin labeling of presynaptic terminals (Bao *et al.*, 2009). The CA1 has higher superoxide levels and increases in both anti-oxidant genes and genes related to the formation of ROS (Wang *et al.*, 2005). Aiken et al. (2011) proposed a mechanism by which the proteasome can cope with oxidative stress through compositional and structural changes that modulate their activity. Oxidative stress triggers the disassembly of the 26S thus liberating the 19S particle and the 20S complex and increasing the ATP and ubiquitination- independent removal of oxidized proteins (Aiken *et al.*, 2011). At this point this disassembly is reversible (Wang *et al.*, 2010b), however with prolonged exposure *de novo* proteasome synthesis is activated (Ding *et al.*, 2003).

This upregulates both standard and inducible (immunoproteasomes) proteasomal components that are more functional (Pickering *et al.*, 2010) with amplified specific endopeptidase activities (Driscoll *et al.*, 1993) that are better able to degrade oxidized proteins than the standard 26S proteasome (Aiken *et al.*, 2011). Protection induced by ischemic preconditioning was lost in mice with immunoproteasome subunit deficiencies (Cai *et al.*, 2008).

Microtubule-associated proteins (MAPs) are key regulators of neural morphogenesis (Popa-Wagner *et al.*, 1999). During morphogenesis, early stage MAPs (MAP1B, juvenile tau, and MAP2c) are replaced with "late" MAPs which include MAP2B and MAP2B. These late MAPs have a profound effect on the organization of cellular microtubules (Lewis *et al.*, 1989) and provide structural stabilization and process outgrowth (Chen *et al.*, 1992), so that proper synaptic circuitry is maintained in the mature brain (Marsden *et al.*, 1996; Popa-Wagner *et al.*, 1999). MAP2A is preferentially associated with dendritic processes (Binder *et al.*, 1986) implicating a role in neuronal morphogenesis. Previous studies have reported that stress conditions can alter MAP2 levels in the brain cortex and hippocampus (Yan *et al.*, 2010). MAP2 localization and protein levels were disrupted in response to excitotoxicity (Arias *et al.*, 1997). Cerebral ischemia reduced MAP2 immunoreactivity in the hippocampus and cortex of neonatal rats (Malinak & Silverstein, 1996) and adult rats (Kitagawa *et al.*, 1989; Dawson & Hallenbeck, 1996). The most significant loss of MAP2 following ischemia was in the CA1 region in both rats (Inuzuka *et al.*, 1990) and gerbils (Yoshimi *et al.*, 1991). The susceptibility of MAP2 to ischemia and excitotoxicity was attributed to the elevation of intracellular Ca^{2+} concentrations cause rapid MAP2 proteolysis by calcium-activated proteinases (Kitagawa *et al.*, 1989). In Figure 4.4 we demonstrate that following MCAO, MAP2 was significantly increased in the CA1 region of the Glud1 Tg mice compared to their Wt littermates. This increase in immunolabeling

is more likely a result of increased protein levels and not an increase in the number of dendritic processes. This suggests that unlike the Wt mice, the Glud1 Tg may have a preserved ability to synthesize some of the components required for structural repair and maintenance (Popa-Wagner *et al.*, 1999). Since MAP2 is synthesized in the cell body and then transported to dendrites (Okabe & Hirokawa, 1989). This suggests that the Glud1 Tg mice could also have improved protein transport that enhances recovery after cerebral ischemia.

Previous studies have reported that the degree of preconditioning-induced protection is significantly diminished in aged rats (Fenton *et al.*, 2000; He *et al.*, 2005). In humans, transient ischemic attacks (TIA) were found to be neuroprotective against ischemic strokes. However, the protective mechanisms of TIAs were not present in elderly patients over the age of 65 as there were no significant differences in stroke severity between patients with and without TIAs (Della Morte *et al.*, 2008). To determine whether the tolerance induced by glutamate preconditioning was preserved in aged mice, we evaluated brain infarct size in 22-mo old Glud1 Tg mice after MCAO and reperfusion. We show that glutamate preconditioning was at least, if not more, effective in the aged (22-mo) versus the young adult (9-mo) mice. This was consistent with a study done by Dowden et al. (1999) in which they preconditioned gerbils with 1.5 min of ischemia before a subsequent 5-min occlusion of both carotid arteries (global ischemia). In young animals they reported 53-66.5% protection of CA1 neurons and approximately 75% protection in aged preconditioned gerbils (Dowden & Corbett, 1999). The authors attributed this difference to the decrease in density and sensitivity of *N*-methly-D-aspartate (NMDA) receptors that occur in the aged rodent brain (Gonzales *et al.*, 1991).

In conclusion, the phenomenon of ischemic preconditioning in the brain is well documented. However, for the first time we show that an *in vivo* model of glutamate

preconditioning was also able to induce tolerance and maintain cell viability in young adult mice that were subjected to cerebral occlusion. This protection was preserved in the aged 22-mo old mice. The results from the ubiquitin immunostaining remain inconclusive and additional studies will be required to further elucidate the role of the ubiquitin-proteasome system in the adaptive mechanisms that contribute to the induced tolerance. Even with the increasing trend of ubiquitin (lack of statistical significance) in the Glud1 Tg, the brain infarct was decreased .The increase in MAP2 levels may suggest an enhancement in protein synthesis and transport in the Glud1 Tg mice. In that case, the increase in ubiquitin may also be a result of increased overall protein synthesis. Ubiquitin is an indispensable protein that is required to maintain cellular repair and its depletion can have deleterious effects on cell viability (Hanna *et al.*, 2003). Yamashita et al. (1991) showed that mild hypothermia prevented delayed CA1 neuronal damage. They reported that increased ubiquitin synthesis and protein ubiquitination was an essential part of the protective mechanism (Yamashita *et al.*, 1991). Liu et al. show that ischemic preconditioning increased levels of free ubiquitin (Liu *et al.*, 2005). Future studies can be carried out to determine other molecular markers for growth, repair processes and protein synthesis.

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Chapter 5: Future directions and conclusion

5.1 FUTURE DIRECTIONS

5.1.1 Determining the mechanisms of HIF-1α stabilization in astrocytes

Although the endpoint for stroke therapy is to preserve neuronal function, it is important to study astrocytes and determine what adaptive mechanisms can improve their function and viability following ischemia. The importance of targeting astrocytes to ensure neuronal survival is described in detail in chapter 3. Therefore, future studies can be carried out to understand the potential mechanisms of HIF-1α degradation during ischemia in astrocytes. Preliminary studies showed that the activity of both the 26S and 20S proteasome was affected by *in vitro* ischemia (Figure 5-1). Proteasomal inhibition by MG-132 increased cell viability (Figure 5.2A) and this was correlated with increased HIF-1α protein stabilization (Figure 5.2B).

5.1.2 Antioxidant therapy in ischemic stroke

It has been well established that oxidative stress causes tissue damage following a stroke. During ischemia and reperfusion, there is a surge in the production of free radicals that lasts for at least 6 to 12 hrs (Flamm *et al.*, 1978; Amaro & Chamorro, 2011). This led to numerous studies that targeted the use of antioxidants to preserve neuronal function. Antioxidants such as Ebselen, Tirilazad, and NXY-059 were even tested in phase III clinical trials (Shi, 2009), however, they did not demonstrate beneficial effects in the trials despite the promising preclinical results. Those studies aimed at taking advantage of the antioxidant's ROS scavenging properties and their ability to reduce lipid peroxidation. In chapters 2 and 3 however, we show how ROS plays a critical role in regulating HIF-1α stabilization and degradation during ischemia. Using antioxidants to target this novel pathway can prove to be effective in providing neuronal and astrocytic protection.

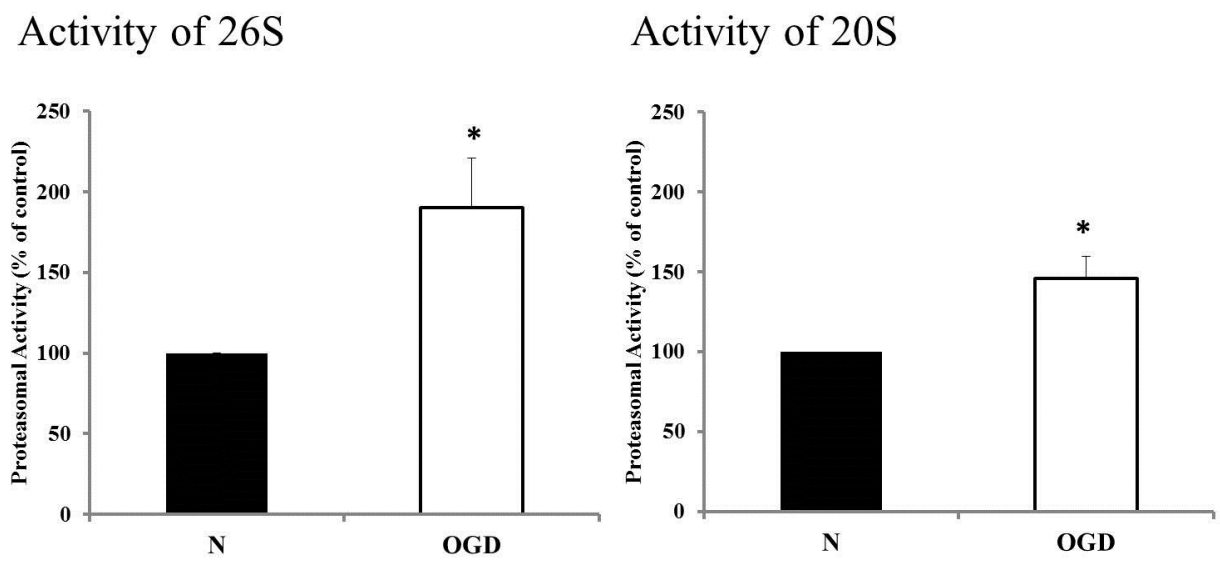

Figure 5.1 Oxygen/glucose deprivation (OGD) increases proteasomal activity in astrocytes. Primary cortical astrocytes were exposed to either normoxia (N) or OGD for 90 min and then the 26S and 20S proteasomal activity was determined (as described in section 2.3.2 Materials and Methods) from the lysates. The increase in both the 26S and 20S proteasome activity following OGD exposure was found to be significant. $*p < 0.05$ vs. normoxia. (n=3)

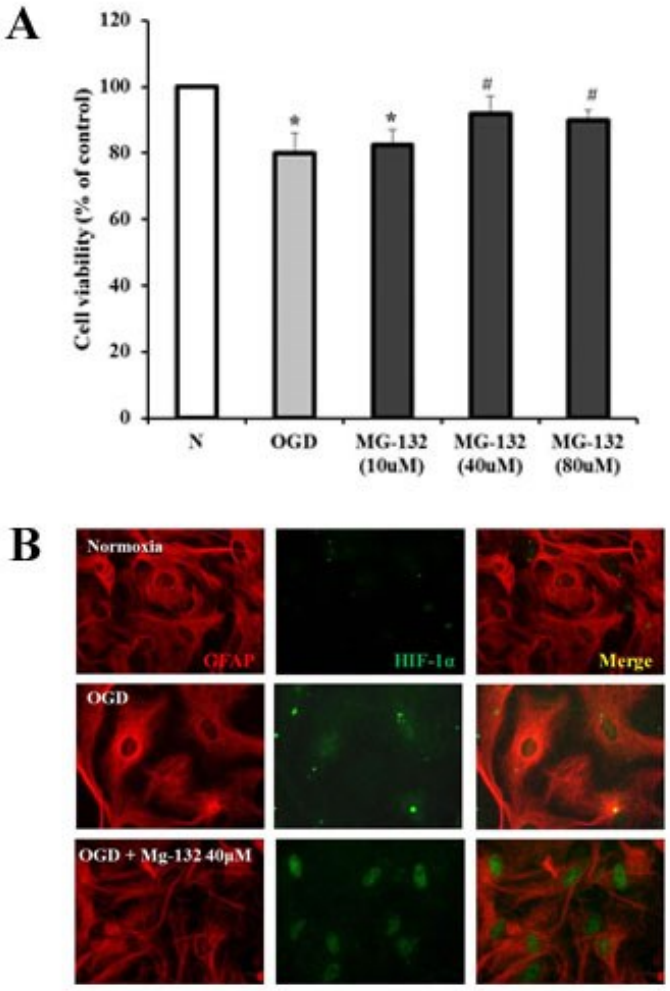

Figure 5.2 Proteasome inhibition by MG-132 increases astrocyte viability and stabilizes HIF-1α protein levels. (A) Astrocytes were pre-treated with a range of MG-132 concentrations (0, 10, 40 and 80 μM) and then exposed to OGD. Cell viability was assessed using the MTT assay. $*p < 0.05$ versus normoxia (N), $^{\#}p < 0.05$ versus OGD. (n = 3). (B) Representative immunofluorescent images showing MAP-2 (red) and HIF-1α (green) labeling in astrocytes exposed to normoxia or OGD for 90 min. Astrocytes were also pre-treated with 40 μM MG-132 and then subjected to OGD. (n=3)

Future studies will be done to determine the specific free radical species that contribute to HIF-1α degradation and which antioxidants can prevent the ROS-mediated increase in proteasomal activity. In our study we used proteasomal inhibitors to directly stabilize HIF-1α. However, we did not do any long-term studies (greater than 24 hrs) to determine any side-effects or neurotoxicity that can arise from inhibiting the proteasome. It is possible that an indirect approach of reducing the ROS responsible for the proteasome-mediated HIF-1α degradation versus the use of proteasome inhibitors could be more effective long-term. Therefore, future studies can also include a comparison between specific antioxidant therapy and proteasomal inhibition on preserving cell viability in both *in vitro* and *in vivo* models.

5.1.3 The effect of glutamate exposure on mitochondrial function in stroke

Mitochondria play a pivotal role in the regulation of neuronal survival and death. Their main function is energy production through respiration; however they are also involved in cellular apoptosis (Schon & Przedborski, 2011). During ischemia, the accumulation of Ca^{2+} through the over-activation of glutamate receptors (Liu *et al.*, 2009b) subsequently promotes mitochondrial dysfunction which is characterized by an increase in mitochondrial permeability, altered mitochondrial membrane potential ($\Delta\Psi_m$), activation of caspases through cytochrome C release and an increase in ROS formation (Brookes *et al.*, 2004; Liu *et al.*, 2009b). The uptake of Ca^{2+} into mitochondria is driven by the $\Delta\Psi_m$ (Brookes *et al.*, 2004) which is maintained by the mitochondrial enzymes of electron transport chain (ETS). Alterations in the enzymatic complexes of the ETS can have detrimental effects and has been shown to occur during brain ischemia. Complex I and complex III are the main contributors to the formation of ROS and increased oxidative stress (Liu *et al.*, 2002). Mitochondria are a major source of ROS; however they are also a target of ROS. For example, lipid peroxidation has been shown to modify

mitochondrial proteins and alter their function (Lucas & Szweda, 1998). Therefore, future work should study the effect of glutamate exposure on mitochondrial function and homeostasis and how that can affect the outcome of a stroke. Studies should focus both on the effect of glutamate toxicity (chapter 3) and glutamate as a preconditioning stimulus (chapter 4). Given that HIF-1α protected astrocytes during glutamate toxicity and that it has been shown to be a regulator of mitochondrial metabolism during ischemic preconditioning (Semenza, 2011), the role of HIF-1α under these conditions should also be determined.

During ischemia or ischemic preconditioning, cells will compensate by altering their metabolic pathways to adapt to the new anaerobic environment. In preliminary studies, we looked at these alterations by using the Seahorse XF96 Extracellular Flux analyzer (Figure 5.3). This method provides a real-time reading of pH which is indicative of changes in lactic acid levels and ultimately of glycolytic activity. It also measures oxygen consumption which relates the levels of oxidative phosphorylation, providing an effective assessment of mitochondrial function. We used a sublethal preconditioning stimulus of glutamate to induce an ischemic-tolerant state and determined the effect on astrocyte mitochondrial function after OGD exposure. Our results indicated that the moderate glutamate exposure provided mitochondrial protection against ischemic insults in astrocytes by increasing an adaptive mechanism that involves improved maximum mitochondrial respiration capacity (MRC) and spare respiratory capacity (SRC) (Brand & Nicholls, 2011). In addition, inhibiting HIF-1α with YC-1 or 2Me2 prevented the mitochondrial protection conferred by the glutamate preconditioning. Further studies should be conducted to confirm this in neurons and in the *in vivo* model of chronic glutamate exposure (Glud1 Tg mice). Other mitochondrial function parameters such as the mitochondrial membrane potential and activities of complexes I-IV of

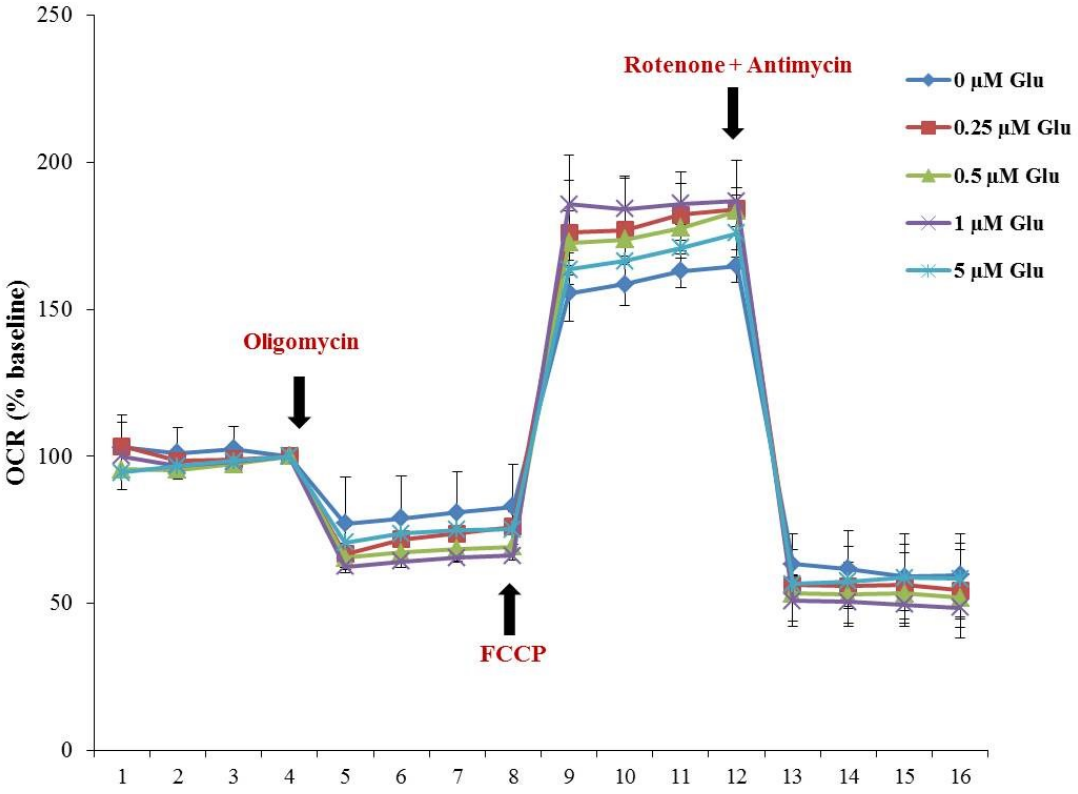

Figure 5.3 Assessment of mitochondrial function using the Seahorse XF96 Extracellular Flux analyzer. Measurement of oxygen consumption rate (OCR) in primary cortical astrocytes preconditioned with 0, 0.25, 0.1, 1 and 5 µM of glutamate (Glu) for 72 hrs then exposed to 90 min of oxygen and glucose deprivation (OGD). The XF96 Extracellular Flux analyzer sequentially exposed the cells to oligomycin (ATP synthase inhibitor), FCCP (mitochondrial uncoupler) and then a mix of rotenone and antimycin (inhibitors of Complex I and III respectively) (n = 3).

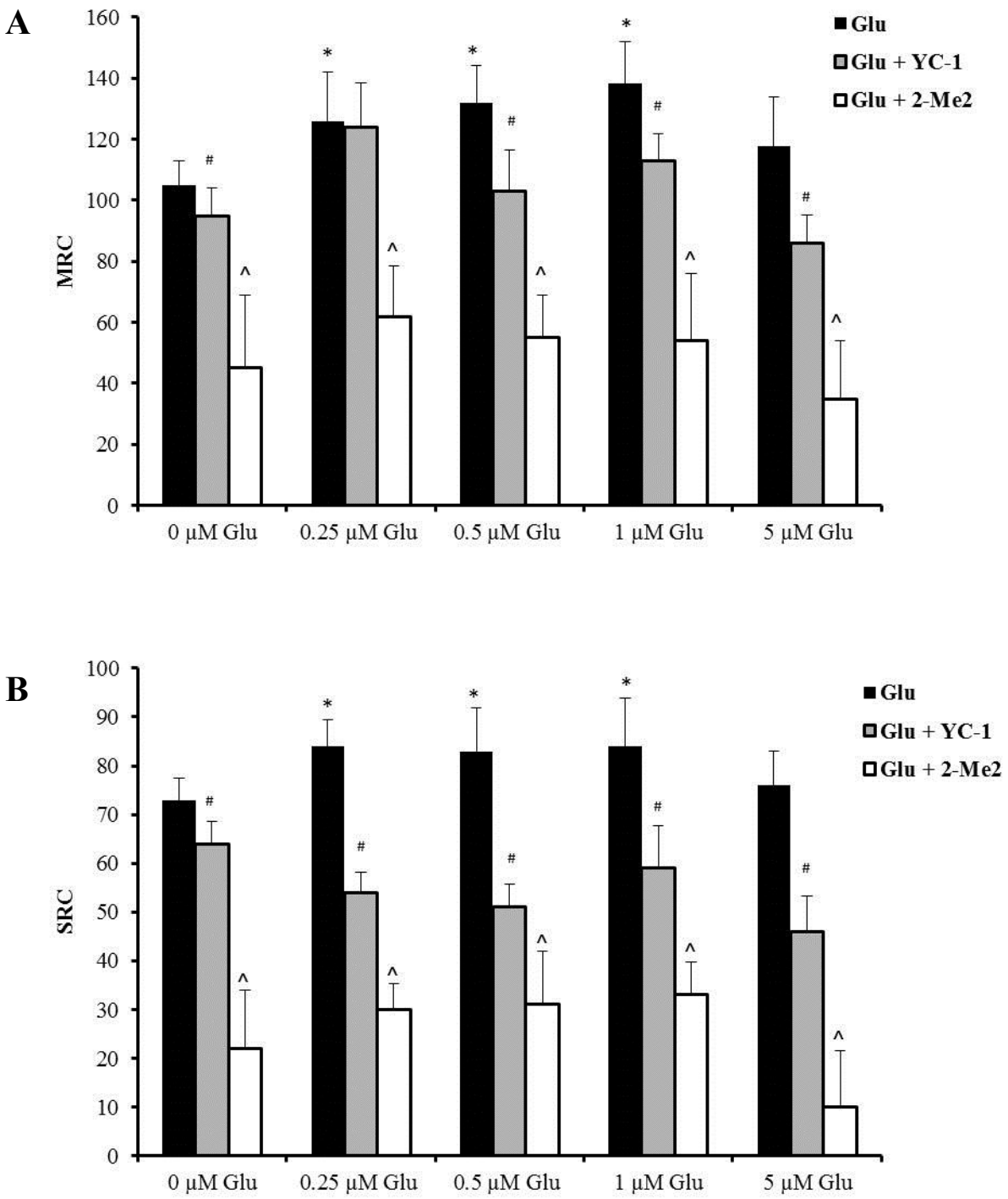
A
MRC
160
140
120
100
80
60
40
20
0
0 µM Glu
0.25 µM Glu
0.5 µM Glu
1 µM Glu
5 µM Glu
Glu
Glu + YC-1
Glu + 2-Me2
B
SRC
100
90
80
70
60
50
40
30
20
10
0
0 µM Glu
0.25 µM Glu
0.5 µM Glu
1 µM Glu
5 µM Glu
Glu
Glu + YC-1
Glu + 2-Me2

Figure 5.4 HIF-1 inhibition with YC-1 and 2-Me2 prevents the protective effect of preconditioning. Measurement of (A) maximum respiratory capacity (MRC) and (B) spare respiratory capacity (SRC) in primary cortical astrocytes preconditioned with 0, 0.25, 0.1, 1 and 5 μM of glutamate (Glu) for 72 hrs and then subjected to 90 min of oxygen and glucose deprivation (OGD). Astrocytes were pre-treated with the HIF-1 inhibitors, YC-1 and 2-Me2 (0.1mM) for 1 hr prior to OGD exposure. *$p < 0.05$ vs. control OGD + 0 μM Glu, #$p < 0.05$ YC-1 treated cells vs. OGD of same Glu concentration with no HIF-1 inhibitor, ^$p < 0.05$ 2Me2 treated cells vs. OGD of same Glu concentration with no HIF-1 inhibitor ($n = 3$).

of the electron transport system should also be evaluated. The mitochondrial potassium ATP channel (mK^+_{ATP}) on the inner membrane of the mitochondria has been suggested to play a more pronounced role in ischemic tolerance than the channels that reside on the plasma membrane (Schultz *et al.*, 1997; Obrenovitch, 2008; Liu *et al.*, 2009a). Activation and opening of the mK^+_{ATP} protected cortical cells from hypoxia (Domoki *et al.*, 1999; Liu *et al.*, 2003). mK^+_{ATP} antagonists abolished the effects of preconditioning (Hide & Thiemermann, 1996) and mK^+_{ATP} agonists leads to ischemic tolerance (Oldenburg *et al.*, 2002; Shimizu *et al.*, 2002; Kis *et al.*, 2004). Furthermore, opening the mK^+_{ATP} with diazoxide protected retinal cell cultures from glutamate exposure (Roth *et al.*, 2006). It has been suggested that this protection could be due to mK^+_{ATP} depolarizing the mitochondrial membrane potential and causing an increase in electron transport chain activity and thus more ATP production (Schultz *et al.*, 1997). We evaluated the levels of the mK^+_{ATP} channel in our *in vivo* model of glutamate preconditioning. The results showed that mK^+_{ATP} channel immunolabeling was increased in the CA1 and CA3 hippocampus of the Glud1 Tg mice compared to the Wt mice (Figure 5.5). Understanding the effect of mK^+_{ATP} channel upregulation on mitochondrial preservation would be a promising avenue to pursue in the future.

5.1.4 Glutamate preconditioning and antioxidant capacity

The level of antioxidant capacity in astrocytes and neurons would predict the level of brain ischemic tolerance induced by preconditioning. Therefore, an assessment of whether long-term mild glutamate exposure increases cellular antioxidant capacity in *in vitro* and *in vivo* models of ischemia should be considered. Activities of superoxide dismutase, glutathione peroxidase, and xanthine oxidase can be evaluated using colorimetric assay kits and levels of

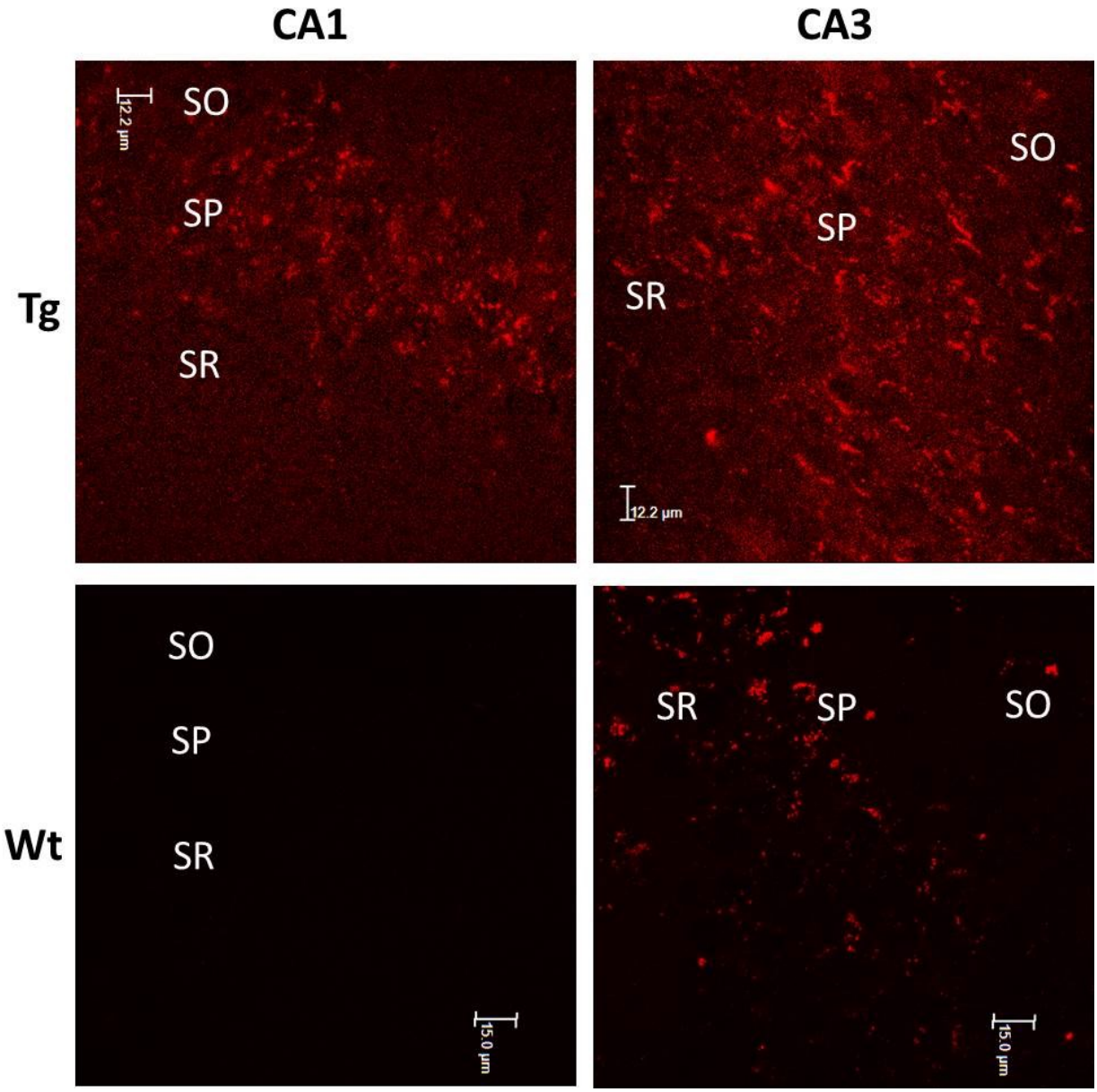

Figure 5.5A mK^+_{ATP} expression in Glud1 Tg and Wt mice after MCAO. Representative immunofluorescent images showing labeling (as described in section 4.2.4 Materials and Methods) of mK^+_{ATP} in the ipsilateral CA1 and CA3 region of the hippocampus in 9-mo old Glud1 Tg and Wt mice that were subjected to *in vivo* ischemia/reperfusion. SO, stratum oriens; SP, stratum pyramidale; SR stratum radiatum.

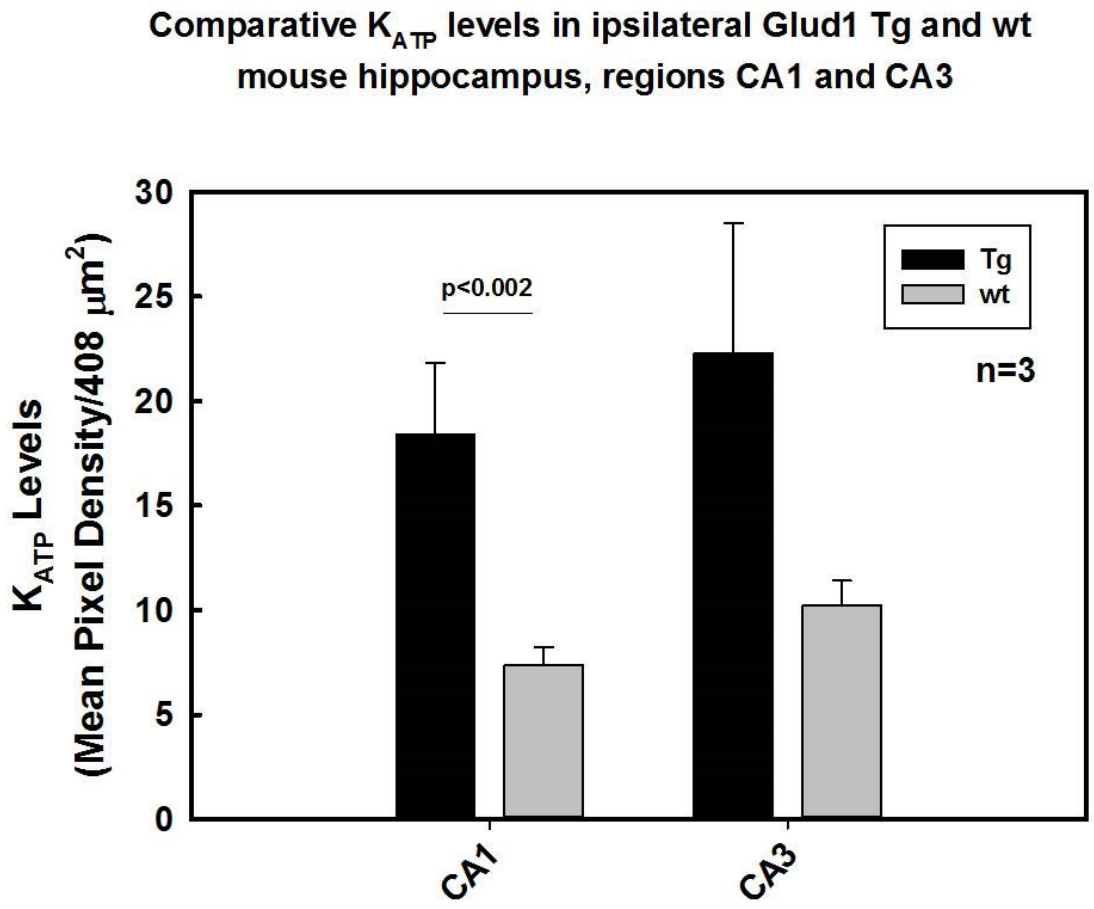

Figure 5.5B Quantification of mK+$_{ATP}$ immunolabeling in Glud1 Tg and Wt mice after MCAO. The mean pixel density of mK$^{+}_{ATP}$ expression was determined from the multiple subfields of the ipsilateral CA1 and CA3 region from 3 pairs (Glud1 Tg and Wt) of mice. All data are presented as means ±SEM (n=3).

different ROS species can be measured. Any relationships with HIF-1α stabilization or regulation can also be determined.

5.2 CONCLUSIONS

In summary, this dissertation aimed to investigate endogenous pathways by which the brain might protect itself from ischemia. The current therapy that is available following a stroke remains suboptimal and the need for the development of treatment options still exists. Understanding the mechanisms by which cells adapt in response to an ischemic insult is a very plausible approach to minimizing brain damage and improving stroke outcome. Even without preconditioning, brain cells naturally respond to hypoxia by triggering a host of defenses to counter cell injury and death (Sapolsky, 2001); the most critical of these responses being the stabilization of HIF-1α. Thus HIF-1 is part of the fundamental endogenous protective responses that occur after stroke that are innate but also adaptive following a preconditioning stimulus. In the first part of this work, we explored a different mechanism by which HIF-1α can be degraded during ischemia. We investigated the effects of ROS and ischemia on proteasomal activities in primary cortical neurons and showed that they resulted in increased 20S proteasomal activity. The 20S proteasome targets and degrades oxidized proteins and as we hypothesized, HIF-1α oxidation by ROS formed during ischemia made it susceptible to degradation by the 20S proteasome. The use of the proteasome inhibitors MG-132 (for *in vitro* experiments) and Epoxomicin (for *in vivo* experiments) increased HIF-1 accumulation and reduced ischemia-induced cytotoxicity.

The release of glutamate is the major cause of brain damage following a stroke; therefore, in the third chapter of this dissertation we determined HIF-1α expression and stabilization in

response to glutamate toxicity during hypoxia. We targeted astrocytes in this study given their role in glutamate uptake release and because they are critical determinants of both neuronal viability and functional recovery after stroke (Zhao & Rempe, 2010). We show that HIF-1α stabilization protects astrocytes from glutamate-induced damage while it's inhibition by 2-Me2 and YC-1 attenuated the recovery of astrocytes under hypoxia. Furthermore, GSH promotes HIF-1α protein stabilization which is consistent with our previous results that demonstrate how ROS can cause HIF-1α degradation. Inhibition of GSH with BSO reduced HIF-1α expression and abolished the HIF-1 mediated protection of astrocytes.

Finally, we investigated the effect that a life-long release of moderate levels of glutamate would have on the glutamate dehydrogenase 1 (Glud1) transgenic (Tg) that were subjected to middle cerebral artery occlusion (MCAO). Evaluation of the brain damage that occurred after MCAO revealed that the Glud1Tg mice had a reduced infarct and edema volume compared to their wild type counterparts. In addition, immunostaining of the CA1 and CA2 regions of the hippocampus showed that the Glud1 Tg mice had increased ubiquitin and MAP2 levels. While the ubiquitin results were inconclusive, the increase in MAP2 is indicative of an adaptive protective response that enhances protein synthesis and transport. Interestingly, although previous studies have reported that the ischemic tolerance phenomenon is not as effective in aged animals as it is in younger animals, our studies have shown that the significant decrease in infarct volume in the 9-month old Tg mice was also seen in the aged mice (22 months old). Therefore, it can be concluded that glutamate preconditioning is an effective approach for inducing ischemic tolerance.

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