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Glutamate Receptor-Mediated Taurine Release from the Hippocampus During Oxidative Stress

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**GLUTAMATE RECEPTOR-MEDIATED
TAURINE RELEASE FROM THE
HIPPOCAMPUS DURING OXIDATIVE STRESS**

**A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science**

By

**BRIAN C. TUCKER
B.A., MIAMI UNIVERSITY, 1999**

2012

Wright State University

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GRADUATE SCHOOL

September, 17th 2012

I HERBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Brian C. Tucker TITLED Glutamate receptor-mediated taurine release from the hippocampus during oxidative stress BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Tucker, Brian Christopher. M.S., Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2012. Glutamate receptor-mediated taurine release from the hippocampus during oxidative stress.

Oxidative stress is an important result of cerebral ischemia and has been directly linked to hippocampal swelling and cytotoxic brain edema *in vitro*. Swollen brain cells activate volume regulatory mechanisms including a significant efflux of the endogenous sulfonic amino acid taurine via volume-regulated anion channels (VRACs). Studies in brain slice preparations also suggest that the excitatory amino acid glutamate plays an important role in both brain tissue swelling and in cell volume regulation. We examined relationships between oxidative stress, glutamate receptor activation, cell swelling, and volume regulation in acutely prepared slices of rat hippocampus. Our results indicate that the release of taurine from intracellular stores is critical for isoosmotic volume regulation of the hippocampus exposed to oxidative stress. Further, taurine is lost from hippocampal cells during oxidative stress via the volume activated anion channel (VRAC). The glutamate AMPA receptors, and to a lesser extent NMDA receptors are coupled to hippocampal swelling during oxidative stress. Our results further suggest volume regulation of the hippocampus is regulated, in part, by glutamate signalling via NMDA and AMPA receptors. Establishing the importance of taurine mobilization during swelling induced by oxidative stress and presenting a significant role for glutamate receptors in swelling and volume regulation is an important step in understanding the cellular response to cytotoxic brain edema. Future work is needed to further our understanding of the response and adaptation of brain cells to edema leading to improved treatments and recovery from these devastating pathological conditions.

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CHAPTER I
INTRODUCTION

Brain edema is a common result of ischemic cardiovascular disease and is a major contributing factor to the morbidity and mortality in ischemic cerebrovascular insults including stroke and myocardial infarction [Klatzo et al. 1994; Plum et al. 1963]. However, current clinical treatments for brain edema address neither the cause of increased water content nor the reactions of brain cells to swelling under ischemic exposure. Further, the mechanisms used by brain tissue to regulate cell volume during brain swelling remain largely unclear. A deeper understanding of the mechanisms responsible for ischemia-induced brain edema will lead to improved treatment and recovery from this devastating pathological condition.

It is understood that brain edema in ischemic and hemorrhagic stroke is associated with the production of reactive oxygen species and consequent oxidative stress [Wagner et al. 2000]. It is further observed that oxidative stress induces swelling in brain slice preparations [Brahma et al. 2000], and that treatment with anti-oxidants can reduce edema formation and brain damage following ischemia and reperfusion [Cuzzocrea et al. 2000; Daneyemez et al. 1999]. In response to swelling induced by either hypotonic or oxidative stress conditions, brain slice preparations exhibit a significant efflux of the organic osmolyte taurine. The efflux of this molecule during cell swelling occurs via volume regulated anion channels (VRAC) [Saransaari et al. 2000; Pasentes-Morales et al, 2002]. Additional studies in brain slice preparations suggest that the excitatory amino acid glutamate plays an important role in both brain tissue swelling, and in cell volume regulation [MacGregor et al. 2003]. Glutamate also may modulate taurine efflux during ischemia, [Saransaari et al. 1997] or may have an inhibitory effect on swelling in certain cell types [Uckerman et al. 2006]. It has proven difficult to disentangle the related

processes of ischemia, oxidative stress, glutamate release, glutamate signaling, brain edema, and taurine efflux. It is the goal of this project to elucidate some of these complex relationships by examining glutamate signaling, tissue swelling and volume regulation in brain slices exposed to oxidative stress.

The background of this manuscript will serve as the foundation of the project, and will provide a detailed explanation of the previous research leading us to the hypothesis and specific aims of the project. Because the experimental model used in this study to induce brain edema is oxidative stress, the background will begin with a discussion of the cellular redox environment and sources of reactive oxygen species (ROS). I will then address both the pathological effects of oxidative stress and the physiological roles of ROS including its effect on cell volume regulation. The second section of this background will focus on the significant role taurine efflux plays for cell volume regulation as it relates to regulatory volume decrease. I will outline the mobilization of taurine during volumetric stress, and the significant role of volume regulated anion channels (VRAC) in this process. Finally, this background section will discuss recent studies that implicate glutamate as a significant mediator in ischemia-induced swelling and cell volume regulation. A comprehensive understanding of oxidative stress, the role of taurine efflux in cell volume regulation and the effects of glutamate on swelling and volume regulation will prove helpful as we try to disentangle the complex relationships between these inter-related processes.

CHAPTER II
BACKGROUND REVIEW

1. Reactive Oxygen Species

Reactive oxygen species (ROS) can have either beneficial or deleterious effects on the cellular environment. High concentrations of ROS elicit oxidative stress which can cause structural damage to nuclear DNA, cell membrane degradation, lipid peroxidation, protein dismutation and premature induction of apoptosis [Statdman et al. 1992; Evans et al. 2004; Barros et al. 2001]. Oxidative stress has been implicated in many pathological disease states ranging from Alzheimer's disease, amyotrophic lateral sclerosis, diabetes, heart attack, stroke and traumatic brain insult [Multhaup et al. 1997; Jackson et al. 1998; Milam et al. 1998; Halliwell et al. 1989]. In contrast, moderate concentrations of ROS have been found to play significant roles in many physiological signaling pathways and processes such as immune response mediation, control of vascular tonicity, oxygen sensing and induction of specific signal transduction pathways. This section will discuss: (i) the cellular redox environment; (ii) the physiological production of ROS; (iii) pathological ROS production and oxidative stress; (iv) the physiological role of ROS in cellular signaling, control and function; and (v) the role of ROS on cell volume regulation.

Physiological ROS Production- The Cellular Redox Environment

In mammalian physiology, energy is generated by the movement of electrons from oxidizable organic molecules to molecular oxygen. Oxygen's role in capturing energy from metabolic substrates during aerobic respiration makes it essential for survival. The efficient transfer of electrons to oxygen results in a chemically reducing intracellular environment in which the production of reactive free radical species often

occurs [Schafer et al. 2001]. The unpaired electron of the free radical molecule gives it an increased reactivity [Miller et al. 1990 Halliwell et al. 1989]. As a result, the term ROS applies to a variety of oxygen-containing molecules which have increased reactivity due to their unpaired electrons.

Traditionally, ROS have been regarded only as toxic by-products of metabolic origin, with deleterious effects on lipids, proteins and DNA [Statdman et al. 1992]. However, current research indicates that ROS are essential participants in cellular regulation and in specific intracellular signaling pathways [Valko et al. 2007]. Therefore, a proper redox balance becomes significant in the effective regulation of these signaling pathways. Redox balance is accomplished by a variety of antioxidant free radical scavenging molecules. Anti-oxidant species such as superoxide dismutase (SOD), catalase and glutathione neutralize potentially toxic ROS to remain within physiologic levels. The relationship between antioxidant content and ROS production will determine whether ROS is beneficial or deleterious within the cell [Thannickal et al. 2000].

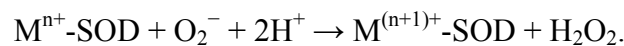
Physiological ROS Production - Oxygen

Oxygen is the primary acceptor of electrons during oxidative phosphorylation of aerobic respiration, and its ability to accept single electrons (see Equation 1) makes it the most important class of radical species [Miller et al. 1990]. In accepting single electrons, molecular oxygen is 'reduced,' making it a chemically strong oxidizing agent [Thannickal et al. 2000]. The result is a partially reduced oxygen molecule, known as superoxide radical (O_2^-) [Fruehauf et al. 2007].



The single-electron reduction of O₂ to its superoxide radical is created by both an enzyme catalyst, and by ‘electron leaks’ between complex I and complex III of the electron transport chain [Turrens et al. 2003, Kovacic et al. 2005]. The partially reduced superoxide radical plays a significant role in ROS concentration for two reasons; it is itself a free radical reactive oxygen species and it is the precursor to several other ROS including H₂O₂, hydroxyl radical (OH·), hydroperoxyl radicals (ROO·) and peroxynitrate radical (ONOO·) [Halliwell et al. 1999]. Thus, superoxide radical is an important component in the development of oxidative stress conditions.

Superoxide radicals are produced by several intracellular sources, most notably the electron transport chain, NADPH oxidase and xanthine oxidase. As a result of its constant physiological production, the concentration of superoxide radical requires constant regulation. The homeostatic regulation of superoxide radical is achieved by the scavenging enzyme super oxide dismutase (SOD, see Equation 2). SOD is extremely effective at catalyzing the reactive superoxide radical’s conversion to H₂O₂.



where, M = Cu(n=1) ; Mn(n=2) ; Fe (n=2) ; Ni(n=2) (2)

SOD is present in both the mitochondria and cytoplasm of cells. Mitochondrial ROS contains manganese (MnSOD), is transcribed in the nucleus, and is localized to the mitochondrial matrix. Cytoplasmic SOD is also transcribed in the nucleus but contains copper and zinc (Cu/ZnSOD). The enzymatic action of SOD on superoxide radical is diffusion limited, making it an extremely efficient antioxidant for this species.

SOD converts superoxide radical to H₂O₂, which is not a free radical species. However, the increased concentrations of H₂O₂ lead to further ROS production, as it is easily converted to the highly reactive and destructive hydroxyl radical. Hydrogen peroxide can result in the production of hydroxyl radical via two different mechanisms. The first mechanism is via the Fenton reaction, as H₂O₂ spontaneously reacts with Fe²⁺ (Equation 3).



Hydroxyl radical formation also occurs during the production of oxidized iron, and involves a two step process (Equations 4 and 5). It is initiated via Habor-Weiss reaction using an iron catalyst to reduce ferric ion to ferrous.



The second step utilizes the above outlined Fenton reaction, and results in the final production of the hydroxyl radical with the following net equation [Liochev et al. 2002].



Hydroxyl radicals are considered to be one of the most destructive forms of ROS, resulting in irreversible lipid peroxidation, DNA damage, and protein oxidation [Pastor et al. 2000]. Because of the toxicity of hydroxyl radical and the spontaneity of this energetically favorable reaction, it must be closely regulated. This regulation is achieved by two critical catalytic enzymes; catalase and glutathione. Catalase reacts with hydrogen peroxide to catalyze the formation of water and oxygen (Equation 6) [Chelikani et al.

2004]. Catalase is highly concentrated near peroxisomes located in close proximity to the mitochondria, a primary source for intracellular H₂O₂ production.



The second mechanism responsible for the decomposition of H₂O₂ involves glutathione (GSH). Similar to catalase, glutathione contributes to the breakdown of H₂O₂ into water, although it utilizes a different mechanism (Equation 7). Glutathione acts as a catalytic cofactor with other enzymes, such as glutathione peroxidase, as it detoxifies hydrogen peroxide into water [Valko et al. 2007].



According to Dröge et al. 2002 reduced glutathione is considered the major redox buffer within the cell and therefore acts as a representative indicator for the redox environment of the cell [Dröge et al. 2002; Schafer et al. 2001]. consequently, non-reduced glutathione is abundant within the cytoplasm, and acts as a significant anti-oxidant in both the prevention and regulation of oxidative stress.

In addition to the ROS discussed above, additional reactive oxygen species found in cells include organic hydroperoxides (ROOH), alkoxides (RO[•]) and peroxy radicals (ROO[•]) [Thannickal et al. 2007]. There is also a subclass of reactive radical species known as reactive nitrogen species (RNS). Like ROS, RNS contain one unpaired electron on their outer orbital. While the mechanisms may be different, RNS behave in a very similar manner as ROS, functioning in both physiological and pathological capacities. Because this research centers on oxygen radical species, the specific reactions involving

these reactive radical nitrogen species and their antioxidant buffers will not be discussed in detail. However, the importance of these radical species should be noted, as they play a significant role in the state of the overall intracellular redox environment. As significant contributors to total reactive radical (ROS/RNS) concentrations within the cell, RNS are an integral component in free-radical homeostasis and cellular pathology, especially in the development and regulation of oxidative stress.

Physiological ROS Production- Electron Transport Chain & ROS Production

There are numerous sources of intracellular ROS production. One major physiological source is via mitochondrial oxidative phosphorylation in the electron transport chain. Oxidative phosphorylation is the process by which ATP is produced by the transfer of electrons from NADH and FADH₂ to O₂ in the inner mitochondrial membrane. This occurs in a step-wise process as electrons are moved by electron transporters through a series of specialized protein complexes. The protein complexes within the inner mitochondrial membrane, known as Complex I, II, III and IV, have specific roles in electron transfer. The individual complexes within the electron transport chain make different contributions to the production of ROS.

The first protein complex in the electron transport chain is Complex I, the 'NADH ubiquinone oxidoreductase' pathway. Complex I is the largest of the respiratory chain components, consisting of a forty-six protein assembly, of which thirty-nine proteins are nuclear encoded and the remaining seven are mitochondrial encoded [Grivenikova et al. 2001]. The electron donor in Complex I is NADH, from which two electrons are transferred from NADH to ubiquinone within the mitochondrial membrane. As the two

electrons initially pass through Flavin Mononucleotide Phosphate (FMN), Iron-Sulfur clusters and quinone, four protons are pumped out of the mitochondrial matrix into the inter-membrane space [Ohnishi et al. 1985]. The final destination of the two donated electrons is ubiquinone (UQ), which also acquires two additional protons, forming ubiquinol (UQH₂). Ubiquinol accumulates within the mitochondrial membrane, where it then diffuses to Complex III, the next step in the electron transport chain [Metzler et al. 2001].

At complex II, the ‘Succinate-CoQ Reductase’ pathway, electrons are generated by the oxidation of succinate. The conversion of succinate to fumarate is a reaction of the tricarboxylic acid cycle and is catalyzed by succinate dehydrogenase. During the oxidation of succinate, electrons are delivered via FAD and Iron-Sulfur clusters into the inner membrane where they interact with the ubiquinol, which serves as an electron carrier. This accumulated reservoir of ubiquinol from Complex I and II within the inner mitochondrial membrane serves as the electron source for Complex III.

The bio-chemical and physiological complexity of electron transfer in Complex I and Complex II of oxidative phosphorylation have significant implications on the superfluous production of ROS. Because ubiquinone acts as the electron transporter from Complexes I and II to Complex III within the inner mitochondrial membrane, the stability of the electrons attached to ubiquinone is vitally important. As ubiquinone shuttles electrons from Complexes I and II to Complex III within the electron transport chain, the potential for free radical production exists [Berg et al. 2006]. An incomplete reduction of ubiquinone in Complex I generates ubisemiquinone radicals (UQH[•]), which are highly

reactive and quickly reduced by O₂ ubiquinol (UQH₂). If the highly reactive ubisemiquinone radical is not reduced, it will react to form the highly oxidizing superoxide radical, a predominant ROS contributing to oxidative stress. It is estimated that between 1-3% of all oxygen entering the electron transport chain becomes partially reduced to generate ROS as superoxide [Han et al. 2001; Lenaz et al. 2001].

The next step in the process involves Complex III, also called the 'cytochrome c' pathway. Complex III indirectly accepts the electrons from Complex I and Complex II by accessing the electron rich pool of ubiquinol created within the inner mitochondrial membrane. Due to its long hydrophobic tail, the reduced ubiquinol diffuses within the lipid of the inner mitochondrial membrane, where the electrons are then donated to Complex III. Within Complex III, electrons are transferred to various protein components, first via iron-sulfur clusters to cytochrome c, and then onto cytochrome b. While ubiquinol can carry two electrons, electron transfer occurs one electron at a time. This transfer occurs during the 'Q-cycle,' as electrons are transferred from the two-electron-carrying UQH₂ to the single-electron carrying cytochrome-c (cyt-c) [Metzler et al. 2001]. During this process, ubiquinol gives up its protons to the mitochondrial intermembrane space (IMS). One of its electrons is carried through Fe-S clusters and cyt-c₁ to cytochrome-c [Tian et al. 1998]. The second of its electrons is carried through two cyt-b proteins and is re-distributed onto another ubiquinone molecule to form a semiquinone radical. Similar to its production in Complex I, semiquinone radicals lead to the formation of superoxide radicals. As a result, both Complex I and Complex III are considered the primary contributors of ROS within the electron transport chain.

The final two components of the electron transport chain during mitochondrial oxidative phosphorylation involve Complex IV and Complex V. Complex IV, also known as cytochrome oxidase, acts as the final destination of the electrons transported from Complexes I through III to oxygen. Complex IV receives its electrons from cytochrome-c, which is a small, mobile protein that diffuses from complex III to complex IV. The electrons are passed through a number of cytochrome-a and copper ion centers. Cyt-a₃ actually performs the reduction of oxygen to water. Each NADH originally oxidized yields 2 electrons, enough to reduce half an O₂ molecule to H₂O. Therefore, two NADH are necessary to reduce the entire O₂ molecule to form H₂O molecules.

Complex V, commonly referred to as ATP Synthase, converts the H⁺ gradient created by the pumping of electrons during Complexes I thru IV, into ATP. ATP production is driven by the H⁺ gradient created on the inner mitochondrial membrane and the mitochondrial membrane potential. Collectively, these two driving gradient components make up the proton-motive force responsible for ATP production [Alberts et al. 1994]. Within Complex V, ATP synthase acts as a 'rotor' with its subunits rotating as protons flow through. This creates conformational changes of the sub-units resulting in the combination of ADP and inorganic phosphate to make ATP. However, in both Complexes IV and V, there is little potential for partially reduced free radicals. As a result, neither Complex IV nor Complex V are considered major contributors of ROS.

Physiological ROS Production- NADPH Oxidase

Another significant source of cellular ROS production is the six enzyme complex NADPH-Oxidase. NADPH-oxidase, also referred to as *NOX*, plays an important role in

host defense against microbes [Heyworth et al. 2003; Leto et al. 2006]. Phagocytes including neutrophils, eosinophils and macrophages produce large amounts of superoxide, with secondary production of other microbiocidal ROS [Knaus et al. 1991].

NOX consists of the catalytic subunit gp91*phox*, along with the regulatory subunits p22*phox*, p67*phox*, p47*phox*, p40*phox*, and the small Rac-GTPase [Vignais et al. 2002; Nauseef et al. 2004]. P47*phox*, p67*phox*, p40*phox* and GTPase are cytosolic while p22*phox* and the heme containing gp91*phox* are known to be membrane bound, and considered integral membrane proteins [Vignais et al. 2002].

In the inactive state, the sub-units associated with the NADPH-oxidase complex are dissociated. The individual sub-units of the NADPH-oxidase complex remain in an inactivated dormant state until they are activated, usually by inflammatory mediators or the presence of microorganisms. Interestingly, the protein sub-units of the NADPH-oxidase complex remain dormant through an intricate series of auto-inhibitory mechanisms [Kawahara et al. 2007]. In the resting cell, tandem domains on the cytosolic p47*phox* complex remain bound together at an auto-inhibitory region (AIR), inhibiting it from interacting with its membrane bound counterpart p22*phox* complex. Upon cell activation, phosphorylation at the AIR releases the tandem p47*phox* binding domain, exposing it so that it can then bind to p22*phox* [El Benna et al. 1994; Ago et al. 1999; Groemping et al. 2003]. Through a complicated series of protein-protein interactions, the binding domains of the remaining NOX components become exposed. These interactions facilitate binding of cytosolic and membrane bound protein subunits to each other, and to the membrane itself. As these subunits continue their integration, Rac-GTP translocates to the binding domain of the membrane bound p67*phox*, activating the enzymatic action

of NADPH oxidase [Groemping et al. 2003]. In active NADPH oxidase, electrons are transferred through two heme groups permitting reduction of molecular oxygen to the superoxide radical at the gp91*phox* sub-unit, thus contributing to increased ROS production.

Physiological ROS Production- Nitric Oxide Synthase

Nitric oxide synthase (NOS) is a highly regulated ROS producing enzyme with contributions to a multitude of body systems. NOS serves as a significant source of ROS production by catalyzing the synthesis of the free radical reactive nitrogen species nitric oxide. There are several different NOS species, each distinguished by their function and the unique manner in which they are regulated. NOS are classified as neuronal NOS (nNOS), inducible NOS (iNOS) or endothelial NOS (eNOS). Each type is encoded by a separate gene and although similar in structure, maintains uniquely specific functional roles [Tylor et al. 1997].

The first iso-form of NOS was found in neuronal tissue, and is thus classified nNOS, or NOS1. Bredt et al. 1999 established that the production of NO by nNOS in nervous tissue occurs in both central and peripheral nervous systems. Within the CNS, NO may mediate neuronal plasticity, implicating nNOS in the processes of learning and memory [Bredt et al. 1999]. In the PNS, nNOS has a far reaching presence, where it is found to contribute to NO production in the gut, macula densa in the kidneys, as well as the urogenital tract [Baranano et al. 2001; Beierwaltes et al. 1997; Tomita et al. 1997]. Another species of NOS is endothelial NOS (eNOS), a calcium dependent NOS that generates NO in blood vessels and thus regulates vascular function. In resting endothelial cells, Fleming et al. 1999 established eNOS enzyme catalyses a sustained release of NO.

Further, eNOS is upregulated by several factors including activation of acetylcholine receptors, or by shear stress [Staurt-Smith et al. 2002].

The final iso-form of NOS-generating enzyme, the calcium independent inducible NOS (iNOS), has functional roles in immunity and inflammation [Mungrue et al. 2003]. Due to its functional role in host immunity, the activation of iNOS usually occurs in an oxidative environment. In the presence of bacterial endotoxin, iNOS has been shown to play a significant role in increasing NO concentration in macrophages, vascular smooth muscle cells, and smooth muscle cells [Landry et al. 2001; Salkowski et al. 1997]. With these resultant increases in NO concentration, and NO's high affinity for ROS, there is increased opportunity for reaction with superoxide radical [Stuart-Smith et al. 2002]. This precipitates the formation of the reactive nitrogen species, peroxynitrite, one of the most toxic classes of free radical species [Kanai et al. 2001].

While the regulation and function of each class of NOS species is unique, the general role of all NOS enzymes is the synthesis of NO. At the molecular level, this occurs on the nitrogen atom of L-arginine in the presence of NADPH and molecular oxygen. NO is produced by carrying out a 5'-electron oxidation of the non-aromatic L-arginine with the aid of tetrahydro-bioterin [Chinje et al. 1997]. This occurs via two consecutive mono-oxygenation reactions forming N-hydroxy-L-arginine (NOHLA) as an intermediate. During this two step reaction, electrons are transferred from NADPH to O₂. The final oxidation step forms the NO and L-citrulline products.

Of the three iso-forms, nNOS and eNOS are constitutive (cNOS), while iNOS is considered to be inducible [Majano et al. 2001]. The nNOS and eNOS iso-forms are both calcium dependent, as the intracellular Ca²⁺ concentration regulates the binding of

calmodulin, which initiates the transfer of electrons from the flavins to the heme [Bredt et al. 1999]. The iNOS, in contrast, acts independently of Ca^{2+} , as calmodulin remains attached to the iNOS iso-form regardless of Ca^{2+} concentration.

Physiological ROS Production – Peroxisomes

Peroxisomes act as another source of ROS production, particularly in the contribution to total cellular H_2O_2 . Peroxisomes are membrane bound organelles important for intracellular metabolism, detoxification and protein transport [Bovaris et al. 1972]. Peroxisomes include a multitude of H_2O_2 -generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-a-hydroxyacid oxidase and fatty acyl-CoA oxidase [Thannickal et al. 2000]. The H_2O_2 produced by these peroxisomal oxidases has been found to oxidize a variety of substrates in additional “peroxisomal” reactions [Tolbert et al. 1981]. These “peroxisomal” oxidative reactions play a significant role in detoxification processes of ethanol in the kidney and liver cells, as well as in the β -oxidation of fatty acids [Alberts et al. 1994]. Due to the downstream breakdown by peroxisomal catalase, peroxisomal H_2O_2 concentrations usually remain within physiological levels [Bovaris et al. 1972; Poole et al. 1975]. However, peroxisomal H_2O_2 production contributes to total cellular H_2O_2 concentration, implicating a potential role in pathological ROS levels and oxidative stress. The effects of pathological ROS will be discussed in more detail later in this background.

Physiological ROS Production- Xanthine Oxidase

Another intracellular source of ROS is xanthine oxidase. Xanthine oxidase is an intracellular, membrane associated enzyme, responsible for catalyzing the oxidation of hypoxanthine to xanthine. Xanthine oxidase further catalyzes xanthine into uric acid, and is

known to play a significant role in the catabolism of purines in humans [Harrison et al. 2002]. Xanthine oxidase is not considered to be a major source of ROS under physiological conditions; however it becomes a contributor during periods of hypoxia, hypoxia/reperfusion and oxidative stress [Dröge et al. 2002; Kayyali et al. 2001].

One of the unique characteristics of xanthine oxidase is the dynamic relationship with its sister enzyme, xanthine dehydrogenase. Hille et al. 1995 established that, depending on the method of purification, either dehydrogenase or oxidase can be obtained from most intracellular sources. Further, for most mammalian systems, these two enzymes can be reversibly converted by oxidation-reduction of cysteine residues [Hille et al. 1995]. Xanthine oxidase can be converted to xanthine dehydrogenase by reversible sulfhydryl oxidation which may occur in the presence of reducing agents like dithiothreitol [Battelli et al. 1973]. The primary difference between the dehydrogenase and the oxidase is the presence of an NAD^+ binding site in the dehydrogenase indicating xanthine dehydrogenase can reduce NAD^+ [Hille et al. 2006]. Conversely, xanthine oxidase does not have the NAD^+ binding site, and instead appears to reduce molecular oxygen. The reduction of molecular oxygen with xanthine oxidase results in the formation of two types of ROS; H_2O_2 , and superoxide radical [Harris et al. 1997]. The production of superoxide radical by xanthine oxidase plays a role in several pathological conditions, most notably hypoxia-reperfusion injury and the development of oxidative stress [Parks et al. 1988].

Physiological ROS Production- Additional Sources

Most of the physiological production of ROS occurs during mitochondrial oxidative phosphorylation in the electron transport chain by means of the catalytic

enzymes NADPH, nitric oxide synthase and xanthine oxidase or within organelles such as peroxisomes [Inoue et al. 2003]. Other sources of physiological ROS production include processes in the endoplasmic reticulum and the nuclear membrane [Freeman et al. 1982]. The endoplasmic reticulum plays an important role in lipid and protein biosynthesis. Further, smooth ER contains enzymes that catalyze the detoxification of lipid soluble drugs and other harmful metabolic by-products. Smooth ER contains p-450 and b-5 families of enzymes capable of oxidizing unsaturated fatty acids and further reducing molecular oxygen to superoxide and H₂O₂ [Aust et al. 1972; Capdevila et al. 1981]. The nuclear membrane contains cytochrome oxidases with electron transport systems similar to that of the ER, making it a potential site of ROS production [Thannikal et al. 2000]. While their specific function is unknown, it has been postulated that these enzyme systems exhibit “electron leaks” that precipitate ROS which are particularly damaging to cellular DNA *in vivo* [Halliwell et al. 1989]. An additional, yet poorly understood source of ROS is the auto-oxidation of catecholamines including epinephrine and dopamine. The usual byproduct of such reactions is superoxide radical, which will affect the overall cellular redox state, as well as the level of oxidative stress. Auto-oxidation of dopamine also may play a significant role in dopamine-induced apoptosis [Ziv et al. 1997; Offen et al. 1998].

The discovery and understanding of the physiological contributors to ROS production and redox regulation have developed greatly in the last four decades. However, it is now increasingly evident that the physiological production of ROS reaches farther than initially predicted, with even more significant physiological and pathological consequences. Due to the large number of unique ROS species, and the complexity of the

mechanisms that produce them, continued research on physiological ROS production is essential to our understanding of oxidative stress.

Physiological Role of ROS- Cellular Signaling, Control and Function

ROS play a significant role in cellular communication, acting as a major component in cell signaling and in signal transduction pathways [Poli et al. 2004]. Signal transduction can be triggered by hormones, growth factors, cytokines and neurotransmitters. In response to these stimuli, gene expression pathways are activated through modulation of transcription factors [Fruehauf et al. 2007]. Activation of these signaling pathways can induce many physiological processes including muscle contraction, gene expression, cell proliferation/differentiation, apoptosis and nerve transmission [Thannickal et al. 2000]. Further, exogenously applied H₂O₂ or increased intracellular generation of H₂O₂ influences the function of various pathways, including protein-tyrosine phosphatase/kinase signal transduction and activation of transcription factors [Varela et al. 2004].

Over the past two decades, ROS have been discovered to play a significant role in signal transduction pathways. Valko et al. 2006 explain that in response to cytokines, growth factor and hormones most cell types elicit a “small oxidative burst” generating increased concentration of ROS within the cell. This observation directly implicates ROS as a secondary messenger in the signal transduction pathway and affecting the resulting signal response cascade [Lowenstein et al. 1994].

A multitude of receptor ligands have been found to induce secondary ROS production in non-phagocytic cells, including cytokines, growth factors and hormones. Three of the first described and most widely recognized cytokines to induce secondary

ROS production are TNF- α , IL-1 and IFN- γ [Sundaresan et al. 1996]. As a result of the secondary ROS production, TNF- α receptor may activate nuclear factor- $\alpha\beta$ (NF- $\alpha\beta$) via a ROS-dependent mechanism [Janssen-Heininger et al. 1999]. Further, according to work by Tolbert et al. 1981, TNF- $\alpha\beta$ -induced production of mitochondrial ROS has been directly implicated in induction of apoptotic cell death. There also appears to be ROS dependent TNF- α mechanisms involved in cell adhesion, production of chemokines and IL-8 expression [Lakshminarayanan et al. 1997; Nakamura et al. 1998].

Interleukin-1 (IL-1) leads to secondary ROS production as a result of ligand activation. In redox regulation, IL-1 functions in a manner similar to that of TNF- α [Terry et al. 1999]. Studies by Lang et al. [1999] have shown that IL-1, TNF- α , and H₂O₂ induce insulin-like growth factor binding protein-1 synthesis in Hep G2 cells. Bonizzi et al. 1999 also demonstrated several ROS-dependent pathways leading to NF- $\alpha\beta$ activation by IL-1 β and Kheradmand et al. 1998 found that expression of collagenase by IL-1 may be mediated by an autocrine loop involving Rac1, ROS and NF- $\alpha\beta$ in fibroblasts [Thannickal et al, 2000]. These studies indicate IL-1-induced ROS production plays a significant role in signal transduction. In these and other experiments [Lo et al. 1998], the activation of these pathways appears to be ROS dependent, implicating the essential role of ROS as a signal transduction regulator in a multitude of cell processes, over a wide range of cell types.

Another large class of cell surface receptors implicated in ROS-mediated signaling is the receptor tyrosine-kinase (RTK) binding growth factors. Specific mechanisms of growth factor induced ROS production and the consequent role of ROS

(specifically H₂O₂) in secondary signal transduction are well documented in contemporary reviews [Thannickal et al. 2000; Valko et al. 2007; Dröge et al. 2002]. However, EGF and PDGF serve as good examples of RTK receptors implicated in downstream ROS/H₂O₂ dependent signal transduction. EGF increases intracellular Ca²⁺ concentrations in keratinocytes followed by ROS increase and PLA₂ activation [Goldman et al. 1997]. Further, EGF-generated ROS mediate ‘inhibitory cross talk’ with another signal pathway activated by G-protein-coupled receptor [Zhang et al. 1999]. PDGF also increases intracellular concentrations of H₂O₂, which is required for PDGF-induced tyrosine phosphorylation, MAPK activation, DNA synthesis and chemotaxis [Sundaresan et al. 1996]. Both EGF and PDGF also induce H₂O₂-dependent activation of p70(s6k) that, through the up-regulated translation of mRNA transcripts encoding for components of the protein synthetic machinery, is critical for cell cycle progression from G₀/G₁ to the S phase [Bae et al. 1999]. Finally, exogenous H₂O₂ induces tyrosine phosphorylation and activation of PDGF-a, PDGF-b and EGF [Gamou et al. 1995; Goldkorn et al. 1998]. These studies indicate that both endogenous and exogenous ROS, and more specifically H₂O₂, are both capable of mediating specific components in signal transduction and transcription pathways.

Additional ROS-generating kinase receptors include the TGF- β super-group of serine/threonine kinase receptors. TGF- β 1 stimulates ROS/H₂O₂ production in many cell types [Islam et al. 1997; Kayanoki et al. 1994; Sanchez et al. 1996]. Shibanuma et al. 1994 demonstrated that TGF- β 1-stimulated H₂O₂ production has a direct role in the induction of early growth response-1 (egr-1) gene. In addition to these studies, work by Shibanuma and others, indicates that TGF- β 1 typically inhibits growth of most target

cells [Das et al, 1992; Thannickal et al. 1995]. These studies establish the relationship between TGF- β and growth inhibition, and outline a novel mechanism behind TGF- β 1 induced H_2O_2 production. Extracellular catalase blocked both the TGF- β 1-induced increase in H_2O_2 , and *egr-1* growth gene expression. Thus, TGF- β 1-stimulated H_2O_2 production may be generated predominantly extracellularly [Ohba et al. 1994]. Barcellos-Hoff et al. 1996 suggest that TGF- β 1 may induce intracellular H_2O_2 production, which is then released into the extracellular space before diffusing back into the cell. This concept has implications on the potential effects of an increased extracellular H_2O_2 concentration, specifically during periods of systemic oxidative stress. The results imply that if TGF- β 1 stimulated pathways are up-regulated as a by-product of H_2O_2 diffusing into the cell, then increased extracellular ROS via oxidative stress could also diffuse through the cell membrane, pathologically affecting these downstream pathways.

G protein-coupled receptors are another class of cell surface receptors shown to generate ROS in many mammalian cell systems. Some of the ligands responsible for G protein-coupled-R stimulated ROS production are ANG-II, serotonin (5-HT), thrombin and bradykinin [Alberts et al. 1994]. The physiological roles of ANG-II mediated by secondary ROS include vasopressor activity [Rajagopalin et al. 1996], smooth muscle cell hypertrophy [Zafari et al. 1998], induction of insulin-like growth factor-1 receptor [Du et al. 1999], and IL-6 production [Kranzhofer et al. 1999]. Further studies suggest that ANG-II stimulated H_2O_2 production elicits downstream consequences on the p38 family of MAPK pathways [Usio-Fukai et al. 1998]. 5-HT stimulates production of O_2^- via several mechanisms, including activation of NADPH oxidase [Lee et al. 1999]. It

appears, at least in part, that the 5-HT-induced superoxide radical exerts downstream effects on the p42/p44 MAPK pathway, leading to cell proliferation [Lee et al. 1999].

Finally, ion channel-linked receptors induce ROS production. Ion channel-linked receptors play an integral part in the nervous system, acting as ligand-gated channels that mediate the synaptic transmission of information. Many ion channel receptor ligands are neurotransmitters such as acetylcholine, 5-HT, glutamate and glycine. The relationship between ion channel-linked receptors and the role of ROS in signal transduction is not as well described as for other ROS-mediated receptor pathways. However, due to the distinctive role of ion channel-linked receptors in the nervous system, there is interest in the effects of neurotransmitter-induced ROS production, and its' downstream role in physiological cellular processes. For example, glutamate has been found to induce ROS production in neuronal cells, and has been implicated as a key mediating factor in several neuro-physiological processes including cell volume regulation [Morales et al. 2007].

The ability of ROS to function in specific cellular signaling pathways lies in their unique properties of chemical reactivity. Superoxide radical ($O_2^{\cdot-}$) and H_2O_2 have specific biological targets, whereas hydroxyl radical (OH^{\cdot}) exhibits more indiscriminate reactivity. This observation implies that ROS signaling molecules during normal physiological conditions are more likely to utilize superoxide and H_2O_2 rather than hydroxyl radical as secondary messenger molecules if the signaling pathway is specific in nature.

Physiological Role of ROS- Mechanisms of ROS Signaling

ROS signals typically do not act as the primary agonist within signal transduction pathways, but rather, as co-signals that allow integration and regulation of cellular

activities. Acting as co-signals, or secondary messengers, ROS have been found to target specific receptor pathways within the cell, as well as many of the MAPK pathways. For example, MAPK pathways ROS (specifically H₂O₂) activates each signaling pathway in a type and stimulus-specific manner [Valko et al. 2007].

Iles et al. [2002] found that endogenous production of H₂O₂ by a respiratory burst specifically induces the MAPK-ERK (Extracellular-Regulated Kinase) pathways, but not the MAPK-p38 kinase pathway. Conversely, exogenous H₂O₂ activates the MAPK-p38 pathway, while not inducing the MAPK-ERK activity in rat macrophages [Iles et al. 2002, Valko et al. 2007]. The significance of these observations lies in the difference between the activated pathways. MAPK-ERK is a pathway typically associated with cell proliferation, whereas MAPK-p38 pathway is associated more with cell differentiation and apoptosis [Kyriakis et al. 2001]. Thus, the source of the ROS (i.e. intracellular vs. extracellular) acting on the pathway is a significant determinant of which specific receptor pathway becomes activated. This characteristic has significant implications in physiological cell signaling processes, as well as in the differentiating role of ROS signaling during periods of pathological oxidative stress.

As previously discussed, ROS have also been implicated as secondary messengers mediating downstream activation of NF- $\alpha\beta$ [Baud et al. 2001]. NF- $\alpha\beta$ is one of the most recognized classes of transcription factors associated with MAPK/ROS dependent pathway activation [Thannickal et al. 2000; Valko et al. 2007; Dröge et al. 2002]. NF- $\alpha\beta$ is a transcription factor long considered to be oxidant responsive and regulates the expression of a multitude of genes involved in cell survival, differentiation,

inflammation, growth and immune response [Pande et al. 2005; Meyer et al. 1993; Schreck et al. 1992].

Activator protein-1 (AP-1) is another class of transcription factor affected by upstream ROS mediated signaling pathways. AP-1 is unique in that exogenous oxidants, ligand-induced ROS and anti-oxidants have been implicated in AP-1 activation [Lo et al. 1995; Puri et al. 1995]. Further, according to Valko et al. [2007] AP-1 activity appears to be induced in response to certain metals in the presence of H₂O₂, as well as other cytokines, chemical and physical stresses. AP-1 appears to be activated as a stress response in the regulation of cellular proliferation and apoptosis [Gabitta et al. 2000].

Physiological Role of ROS- Antioxidant Signaling

In addition to ROS, anti-oxidants play a physiological role in cellular signaling. Because of the complex and dynamic nature of the redox environment within the cell, any alteration of antioxidant activity would directly alter the efficacy of ROS-signaling messengers. Therefore, the intracellular concentration of glutathione (GSH), the major H₂O₂ redox buffer within the cell, has considerable implications on redox pathway activation. GSH can play a role in blocking apoptosis and GSH depletion is observed contemporaneously with the onset of apoptosis. A more reducing environment (regulated in part by high levels of anti-oxidants like GSH) tends to stimulate cell proliferation, while a slight shift towards oxidation initiates cell differentiation. Further intracellular oxidation leads to apoptosis and necrosis with mild oxidizing stimuli leading to apoptosis, while an intense oxidizing effect induces necrosis [Cai et al. 1998; Evans et al. 2004; Voehringer et al. 2000; Valko et al. 2007]. These observations not only indicate the significance of the redox status within the intracellular signaling environment, but also

implicate a potential role for GSH and other anti-oxidants as potential regulators in the redox signaling environment.

In summary, ROS play important roles as secondary messengers, specifically in the downstream regulation of cell proliferation, differentiation and apoptosis.

Concurrently, there is a burgeoning body of evidence implicating ROS in numerous other cellular signaling pathways including: the mediation of immunological response of lymphocytes [Bonizzi et al. 2000]; regulation of vascular tone [Lander et al. 1997]; sensing changes in oxygen concentration in the carotid body [Prabhakar et al. 2000]; regulation of erythropoietin production [Rancourt et al. 2010]; regulation of cell adhesion properties [Roy et al. 1999]; and in the regulation of swelling-induced taurine efflux in many cell lines [Lambert et al. 2004]. These processes are well summarized in contemporary literature [Dröge et al. 2002; Valko et al. 2007; Thannickal et al. 2000]. Section two will discuss the role of ROS for the regulation of cell volume and its effect on swelling-induced taurine efflux.

Pathological ROS Production - Oxidative Stress

Over-accumulation of ROS within the cellular environment may lead to the toxic metabolic pathology of oxidative stress, an imbalance in pro-oxidant/antioxidant concentrations. Elevated ROS concentrations can damage mitochondrial and nuclear DNA [Evans et al, 2004] and cause lipid peroxidation, [Barros et al. 2001] inflammation, [Lorgis et al, 2010], premature induction of apoptosis, [Malik et al. 2007] and aberrant organismal aging [Khansari et al, 2009]. The effects of oxidative stress have been implicated in diabetes, cancer, emphysema, arthritis, cataracts, atherosclerosis and various other components of cardiovascular disease [Fruehauf et al. 2007]. Further,

oxidative stress has more recently been implicated as a major contributor in neurodegenerative disorders including Alzheimer's disease [Multhaup et al. 1997], Parkinson's disease [Radunovic et al. 1997], Huntington's disease [Mukherjee et al. 1997], ALS [Jackson et al. 1998], cytotoxic brain edema, and ischemia/re-perfusion neuronal injuries [Milam et al. 1998]. The specific effects of oxidative stress on these disease states are outlined comprehensively in contemporary literature [Thannickal et al. 2000]. For the purposes of this review, we will briefly discuss three major consequences of oxidative stress following ischemia/reperfusion on cellular function including; DNA damage and lipid peroxidation. The major point of discussion, however, will focus on the pathology of oxidative stress as it relates to neuronal ischemia/reperfusion injuries and cytotoxic brain edema.

Oxidative Stress – DNA Damage

Oxidative damage to DNA is a far reaching consequence of oxidative stress, affecting both nuclear and mitochondrial DNA. Mitochondrial DNA is more susceptible to oxidative damage due to its proximity to one of the primary sources of ROS, the mitochondrial electron transport chain. Mitochondrial DNA also exhibits diminished oxidative repair capacity compared to its nuclear counterpart [Finkel et al. 2000; Kregel et al. 2007]. Some of the deleterious effects of DNA oxidation include nucleotide base deletion and mutation, DNA-DNA cross linking, DNA-protein cross bridges, direct oxidation of base pairs, and DNA strand breaks [Markesbery et al. 2007; Cadet et al. 1999]. The resulting modifications lead to aberrant physiological functioning of the cell, including alterations in transcriptional and translational processes affecting cell survival, apoptosis and senescence [Greer et al. 2005; Grishko et al. 2003; Hazra et al. 2007].

The biochemistry of oxidative damage to DNA is unique, resulting from direct interaction of ROS with the DNA strand structure. Unlike other damaging oxidative stress mechanisms which are predominantly caused by peroxide and superoxide radicals, oxidative DNA damage is caused primarily by hydroxyl radical. The reaction with DNA occurs as the hydroxyl radical attacks the deoxyribose component of the DNA structure, resulting in release of free bases from DNA and strand breaks [Hazra et al. 2007].

Oxidative Stress – Lipid Peroxidation

Because cell membranes contain a high concentration of poly-unsaturated fatty acids, they are particularly susceptible to lipid peroxidation during oxidative stress [Barros et al. 2001]. Poly-unsaturated fats contain multiple double bonds, thereby creating an abundance of unstable and highly reactive hydrogens [Marnett et al. 1999]. During lipid peroxidation, free radicals abstract a hydrogen atom from the polyunsaturated fatty acid moiety of membrane phospholipids, initiating a radical-mediated chain reaction [Sodergren et al. 2001]. The initial product is a new free radical species on the fatty acid which then can propagate to an adjacent unsaturated moiety. As the phospholipids of the cell membrane become compromised, the integrity of the cell membrane is reduced and membrane fluidity is increased. The reaction continues until termination, which can occur when two radicals react with each other [Dix et al. 1993]. The termination process is expedited by antioxidants like vitamin E, SOD, and catalase, which can neutralize the reactive radicals and thereby protect the cell membrane from further degradation [Porter et al. 2005; Cerutti et al. 1994]. Oxidation of cell membranes is a significant mechanism leading to growth inhibition and cell death [Cerutti et al.

1994]. Further, lipid peroxidation often triggers apoptosis, activating the intrinsic cell death pathway present within cells [Sodergren et al. 2001].

Oxidative Stress – Ischemia-Reperfusion Injury

Oxidative Stress is a significant sequela of ischemia-reperfusion injuries [Alexandrova et al. 2008]. Ischemia-reperfusion injuries occur from a prolonged period of inadequate oxygen and metabolic substrate supply causing tissue ischemia, followed by a period of reperfusion. The result of an ischemia-reperfusion episode is the initiation of a wide array of inflammatory and metabolic disturbances that aggravate local injury and impair healthy physiological organ function [Dorweiler et al. 2007]. Conditions associated with ischemia-reperfusion injury include acute vascular occlusions like stroke, and myocardial infarction; surgical procedures like organ transplantation, coronary bypass and vascular surgery; and as a consequence of major metabolic trauma like cerebral hemorrhage, near drowning and carbon monoxide poisoning [Choi et al. 1990]. Further, ischemia-reperfusion commonly results in increased ROS production, leading to deleterious consequences associated with oxidative stress. Ischemia dramatically alters oxygen metabolism within the cell and causes increased accumulation of ROS [Dorweiler et al. 2007]. Under normal conditions, 95% of oxygen in mitochondrial respiration is reduced to H₂O without any free radical intermediates. The remaining 5% is reduced by a univalent pathway in which free radicals like superoxide anion and H₂O₂ are produced [Becker et al. 2004; Dorweiler et al. 2007; Han et al. 2001]. Under physiological circumstances, these mitochondrial radical intermediates are safely metabolized to H₂O by dismutase, catalase and the glutathione peroxidase system. During periods of ischemia, anti-oxidant defenses are compromised, allowing increased concentrations of

H₂O₂ and the highly reactive hydroxyl radical [Becker et al. 2004] which damages proteins and initiates lipid peroxidation [Granger et al. 1988].

In addition to a breakdown in anti-oxidant defenses during ischemia, there is also a paradoxical increase in ROS production during ischemic hypoxia. Guzy et al. [2006] found that the primary source of ROS production during hypoxia is complex III of the electron transport chain. As a result of ischemia, when oxygen supply is limited, the electron transport chain of the inner mitochondrial membrane becomes reduced, resulting in oxygen radical production [Traystman et al. 1991]. Guzy et al. [2006], proposes that the increased ROS production during hypoxia is the result of the lifetime of the reactive ubisemiquinone radical in complex III, the tendency for mitochondrial ROS to be released towards the matrix compartment as opposed to the inner-membrane space, and the ability of O₂ to access the ubisemiquinone radical in complex III [Guzy et al. 2006]. Further, Guzy et al. [2006] suggests that during hypoxia the electron transport chain acts as an O₂ sensor by releasing ROS into the intracellular environment. The ROS release triggers several responses including activation of the hypoxia-inducible factor (HIF-1 α) [Turrens et al, 2003]. Because complex III is suggested to be a significant source of ROS production during hypoxia, this implicates complex III as a possible source of oxidative stress in ischemia/reperfusion injuries.

While hypoxia is established to cause an increase in ROS production, restoration of oxygen during reperfusion also has been found to cause a significant increase in ROS production [Dorweiler et al. 2007; Guzy et al. 2006; Traystman et al. 1991; Maxwell et al. 1997]. During the reperfusion stage of ischemia/reperfusion injuries, oxygen deprived tissue becomes flooded with O₂, causing downstream mitochondrial dysfunction and

increased ROS production. In addition nitric oxide in the tissue reacts with the increasingly prevalent superoxide radical to form peroxynitrite (ONOO^-), which dissociates into the extremely cytotoxic NO_2 and OH^- radicals. Further, there is increased ROS production by xanthine oxidase following reperfusion. The specific mechanisms of increased ROS production during reperfusion from these and other sources are discussed in detail in contemporary literature [Chrissobolis et al. 2011]. These studies establish that both ischemia and ischemia/reperfusion injuries cause a significant increase in ROS production leading to oxidative stress.

Oxidative Stress- Brain Edema

Brain edema is a devastating pathological consequence of cerebral ischemia-reperfusion injury [Dorweiler et al. 2007]. Consequent to ischemia-reperfusion, brain edema is mediated, at least in part, by increased levels of ROS and oxidative stress [MacGregor et al. 2003]. Because brain edema is the major contributing factor to morbidity and mortality in stroke, understanding the interactions between ischemia, oxidative stress and brain edema could have far reaching implications [Kasner et al. 2001; Steiner et al. 2001].

Fundamental to understanding the relationships between ischemia, oxidative stress and brain edema is an understanding of post-ischemic brain edema formation. The content of ATP in the brain is dependent on a continuous supply of energy and approaches zero within approximately four minutes of complete ischemia [White et al. 1993]. As a result of ATP depletion, essential energy-dependent cellular mechanisms begin to fail. As ion pumps cease to function, membrane ion gradients deteriorate, selective and non-selective ion channels open, and intracellular and extracellular ions

move towards their equilibrium causing membrane depolarization. During this anoxic depolarization, potassium ions leave the cell while sodium, chloride, and calcium ions enter. The loss of membrane ion gradients also decreases neuronal glutamate uptake leading to exaggerated neuronal excitation and resulting additional sodium ion influx. Consequently, intracellular osmolarity increases, causing neuronal swelling and the development of cytotoxic brain edema. Cytotoxic brain edema is the first stage of ischemic edema and occurs in the first five minutes of ischemia [Ozben et al. 1998]. During acute ischemic stroke, this initial edema is reversible and the blood-brain barrier remains intact. However, after prolonged ischemia of several hours, the blood-brain barrier becomes compromised and vasogenic brain edema develops. Vasogenic edema causes death in one-third of insults and three-quarters of hemorrhagic brain insults [Ozben et al. 1998]. The high mortality rate from vasogenic edema underscores the importance of understanding and controlling brain edema in its early cytotoxic stage, before the condition becomes irreversible and fatal.

The destruction of ion gradients, however, is not the only mechanism involved in the development of post-ischemic brain edema. In addition, the increased ROS production that occurs during ischemia and reperfusion can lead to brain edema both *in vitro*, [Chan et al. 1982; Brahma et al. 2000] and *in vivo* [Oh et al. 1991; MacGregor et al. 2003] and has been associated with brain edema in ischemic and hemorrhagic stroke [Deng et al. 2000; Wagner et al. 2000]. Studies have also shown that endogenous and exogenous anti-oxidants reduce edema formation following ischemia-reperfusion, and intracerebral hemorrhage [Deng et al. 2000; Nakamura et al. 2004]. Because tissue ischemia has been found to increase ROS production [Deng et al. 2000], and increased

ROS production has been found to precipitate brain edema [Chan et al. 1982], it can be hypothesized that ischemia induced brain edema is mediated, at least in part, via a ROS dependent mechanism.

Early studies by Chan et al. [1982] suggested that oxidative stress could induce brain edema in brain tissue slices. Brahma et al. [2000] showed that brain slices incubated in ascorbate, a prominent anti-oxidant in the brain, have diminished water accumulation *in vitro* [Brahma et al. 2000]. We have observed brain tissue swelling induced by oxidative stress in mouse hippocampal slices using 3-D microscopy similar to that shown in swelling induced by hypoosmotic exposure [Figures 1 and Figure 2]. Further studies show ROS scavenging agents (ascorbate, Trolox, dimethylthiourea, Tempol) significantly attenuated ischemia induced water gain [Brahma et al. 2000]. These studies suggest ROS play a significant role in brain edema formation.

Cell Volume Regulation

Cell volume regulation is essential for proper cell function and survival. To avoid excessive alterations in cell volume, cells utilize several regulatory mechanisms. Because mammalian cell membranes are highly permeable to water, the greatest factor affecting cell volume is the osmotic gradient between the intracellular and extracellular environments. Any alteration in either intracellular, or extracellular osmolarity will alter cell volume. Cells exposed to a hypertonic extracellular environment will shrink, as water flows down its chemical potential gradient from an environment of high chemical potential inside the cell, to lower chemical potential outside the cell. Inversely, cells exposed to a hypotonic environment will swell as water moves from the higher chemical potential outside the cell, to the lower chemical potential inside the cell.

Figure 1

Swelling induced by hypoosmotic exposure in hippocampal mice neurons Three-dimensional confocal microscopy illustrates the effect of hypoosmotic (200 mOsm) exposure on stratum pyramidal cell bodies in the CA1 area of mouse hippocampus *in vitro*. Three GFP-expressing pyramidal cells in the CA1 layer show increases in cell volume within 15 min of exposure to hypoosmotic aCSF. Confocal images were obtained at 1.5 μ M intervals

Figure 1

After 15 min

Baseline

Hypoosmotic Exposure

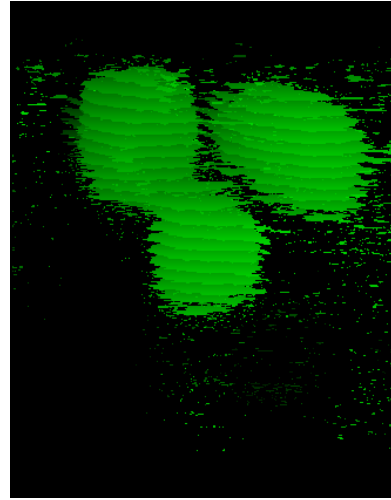
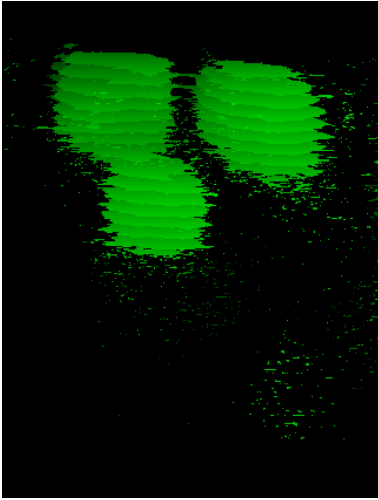
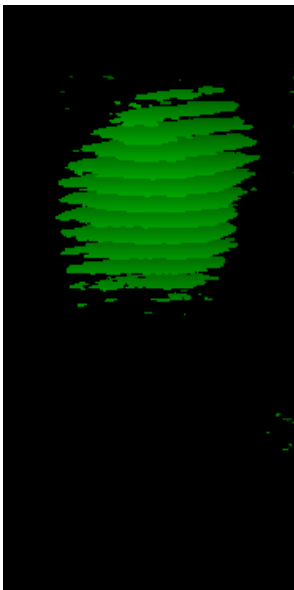


Figure 2

Swelling induced by H₂O₂ exposure in hippocampal mice neurons Three-Dimensional confocal microscopy illustrates the effect of oxidative stress on an individual GFP-expressing pyramidal cell body in a mouse hippocampus. A pyramidal cell in the CA1 layer shows significant increases in cell volume within 15 min of exposure to aCSF containing 2 mM H₂O₂. Confocal images were obtained at 1.5 μM intervals.

Figure 2

Baseline After 15 min
H₂O₂ Exposure



Cells generally regulate the direction of water flow across their plasma membrane by manipulating intracellular or extracellular osmolarity. This occurs through metabolism and transport of both inorganic (K, Na, Cl), and organic (taurine, alanine, glycine, sorbitol, betaine, etc.) osmolytes into and out of the cell. There are two prominent classes of volume regulatory mechanisms; regulatory volume increase (RVI), which compensates for cell shrinkage; and regulatory volume decrease (RVD), which compensates for cell swelling. RVI is well documented in contemporary reviews [Lang et al. 1998; Wehner et al. 2003; Hoffman et al. 2009]. Recent studies have suggested that ROS may play a significant role as an intracellular messenger in the mediation of RVD [Lambert et al. 2003]. This background will focus primarily on RVD and the ROS mediating signaling mechanisms involved in controlling RVD.

Cell Volume Regulation- The Role of ROS

ROS are produced in response to osmotic stress in several cell types [Varela et al, 2004] and are a mediating factor in swelling-induced activation and inactivation of the volume-sensitive pathway for the release of the organic osmolyte taurine [Lambert et al. 2003, Lambert et al. 2008]. These experiments suggest ROS is a significant intracellular signaling messenger during periods of osmotic stress, and more specifically as a mediator of regulatory volume decrease.

Cellular swelling activates RVD mechanisms including the volume sensitive outwardly rectifying Cl⁻ channels (VSOR, also referred to as volume regulated anion channel or VRAC in this background) in many cell types [Jackson et al. 1995]. While one role of VRAC channels seems to be RVD, they are also involved in other physiological processes including cell proliferation and apoptosis [Shen et al. 2000]. Recent studies

have suggested ROS, specifically H₂O₂, can play a role in the activation of VRAC channels [Varela et al. 2004]. In HTC cells, H₂O₂ (200 μM) in isotonic solution reversibly activated outwardly rectifying Cl⁻ currents. Thus, H₂O₂ can activate a Cl⁻ current, even in the absence of cell swelling. Further when HTC cells were exposed to hypotonic solutions, a transient 15 fold increase in H₂O₂ concentration was observed within the first 30 seconds of exposure. This increase in H₂O₂ production was reduced in the presence of an NADPH-oxidase inhibitor. These results suggest that hypotonic exposure induces H₂O₂ production, and that H₂O₂ production is generated, at least in part, through an NADPH-oxidase dependent mechanism.

Similar studies by Lambert et al. [2002] also suggested that ROS play a mediating role in regulatory volume decrease. H₂O₂ exposure potentiated swelling-induced taurine efflux in NIH 3T3 fibroblasts during exposure to hypotonic medium, and the observed taurine efflux was mediated via iPLA2 and 5-lipoxygenase (5-LO) signaling pathways. Further studies by Lambert et al. [2008] indicate that the source of ROS involved in iPLA2 mediated volume-sensitive taurine efflux pathway is NADPH-oxidase.

More recent studies by Friis et al. [2008] clarified the specific subtypes of NADPH-oxidase involved in swelling-induced taurine efflux regulation. The unique characteristics of the NADPH-oxidase complex of NIH 3T3 fibroblasts provide a system for elucidating the specific components involved in osmotically induced NADPH-oxidase ROS production, and related taurine efflux. NIH 3T3 fibroblasts express three NADPH-oxidase components; p22 (a nox-4 iso-type), p47, and p67 [Friis et al. 2008], and appear to release superoxide radicals from the NADPH-oxidase complex into the intracellular compartment. Using this cell culture model, Lambert et al. [2003] and Friis et al. [2008]

found that iPLA₂ and 5-LO activity is required for swelling induced activation of taurine efflux, ROS are produced by the NADPH-oxidase complex subsequent to PLA₂ activation, the NOX-4 iso-type account for the swelling-induced increase in ROS production in NIH3T3 cells, and ROS inhibit a protein tyrosine phosphatase (PTP1B) causing a potentiation of swelling induced taurine efflux. Additional studies have further established the role of ROS in RVD of several other cell types including HTC cells, [Varela et al. 2004] and skeletal muscle cells [Ortenblad et al, 2003].

There is also new evidence that suggests ROS may regulate VRAC which facilitate excitatory amino acid release in microglial cells [Harrigan et al. 2008]. Exogenously applied H₂O₂ increased swelling-activated glutamate release in microglia. In addition, stimulation of microglia with zymosan, a treatment used to stimulate NOX immune function, triggered production of endogenous ROS and increased glutamate release via VRAC in swollen cells. Further, a significant efflux of D-aspartate (a non-metabolizable analogue of glutamate) was observed upon H₂O₂ exposure, suggesting that rat microglial VRAC are permeable to glutamate [Harrigan et al. 2008]. In addition to establishing the role of NADPH-oxidase in mediating these microglial VRAC and the subsequent effect on glutamate efflux, Harrigan et al. established that NOX-4 was the probable source of endogenous ROS mediating VRAC activation and glutamate release. In the present study, I will examine specific roles of both ROS and glutamate signalling on cell volume regulation.

ROS Summary

ROS production and oxidative stress occur as a result of ischemia and ischemic-reperfusion injuries and may cause brain tissue swelling *in vivo*. ROS also has a

prominent role in cellular signaling, through a variety of mechanisms. More specifically, ROS is a mediator of cell volume regulation via activation of VRAC in many cell types, leading to taurine efflux during RVD. Because ROS can both initiate tissue swelling and mediate cell volume regulation it plays an important role for brain edema development consequent to oxidative stress. The present study will examine the role of ROS for brain tissue swelling mediated by glutamate release as well as its role as a mediator of cell volume regulation during oxidative stress.

2. Taurine

Taurine (2-aminoethanesulfonic acid) is a sulfonic amino-acid that functions in many capacities including: neuromodulation of synaptic activity in the brain, induction of neuronal hyper-polarization, inhibition of neuron firing, membrane stabilization and neuro-development [Choe et al. 2012, Saransaari et al, 1992; Huxtable et al. 1992; Kontro et al, 1983]. Taurine has been found to protect neurons against excitotoxicity and prevent deleterious metabolic cascades induced by ischemic hypoxia [Schurr et al. 1987]. Taurine is one of the most abundant free amino acids in the brain, and has a concentration higher than that of glutamate during certain developmental periods [Kontro et al. 1983; Saransaari et al. 2006]. Dating back to Lange et al. [1963] taurine was introduced as a component of osmo-regulation in marine animals. At present, taurine's role in the central nervous system is best understood as an osmolyte [Pasantes-Morales et al. 2002]. The many roles of taurine in physiological and pathological function are well discussed in published reviews and are out of the scope of this discussion [Wright et al. 1986; Huxtable et al. 1992]. Rather, this discussion will focus on: (i) defining taurine structure

and synthesis; (ii) examining mechanisms of taurine accumulation and release; (iii) describing the role of taurine as an osmolyte for RVD; and (iv) evaluating neuro-protective effects of taurine related to glutamate toxicity. This discussion will unify the major themes of the present project; oxidative stress, cell volume regulation, and the role of glutamate on taurine mobilization during oxidative stress.

Taurine – Structure and Synthesis

Taurine is one of a handful of known naturally occurring sulfonic acids, and is derived from the sulfhydryl amino acid, cysteine. Taurine is both a sulfonic acid and a beta-amino acid, and unlike other amino acids which contain a carboxyl group, it contains a sulfonate group. Some types of human cells can synthesize taurine from cysteine via the cysteine sulfonic acid pathway. Via cysteine dioxygenase, the sulfhydryl moiety of cysteine is oxidized to form cysteine sulfonic acid. Cysteine sulfonic acid is then decarboxylated by sulfinoalanine decarboxylase forming hypotaurine. Finally, hypotaurine is either spontaneously or enzymatically oxidized to yield taurine. Because humans have some capacity to synthesize taurine, it is considered a conditional essential amino acid. However, the majority of taurine in humans is acquired directly from food sources [Wright et al. 1986].

Taurine – Cellular Accumulation

As a primary organic osmolyte involved in neuronal RVD, regulation of intracellular taurine concentrations is of particular importance. Although several cell types, including astrocytes, can synthesize taurine from cysteine, intracellular taurine accumulation is primarily achieved through uptake by a specific membrane transporter [Lambert et al. 2011]. Intracellular taurine content can reach millimolar concentrations

[Beetsch et al. 1996]. The transporter responsible for mediating taurine uptake is a high-affinity, sodium and chloride ion dependent co-transport system. With this system, up to three sodium ions are translocated with one chloride ion and one taurine molecule [Beetsch et al. 1996; Wehner et al. 2003]. In the rodent brain, two distinct high affinity carrier transporters have been identified, with each showing distinct regional and cellular distribution characteristics [Pow et al. 2002]. Functionally, during periods of hyperosmotic exposure, an increase in transporter mRNA and a concomitant increase in the Vmax of taurine transporter contributes to increased intracellular taurine content [Cammarata et al, 2002].

In addition to carrier mediated taurine transport, a diffusional transport component can contribute to intracellular taurine accumulation [Takahshi et al. 2003]. This diffusion component may be mediated via passive taurine movement through anion, or volume-sensitive organic osmolyte channels [Jackson et al. 1993]. The mechanisms of taurine uptake indicate that maintaining proper intracellular taurine concentration is an efficient and well regulated process. Further, these mechanisms underscore the importance of taurine accumulation as a vital component in effective cell volume regulation.

Taurine – Cellular Release

While accumulation of taurine seems to occur primarily by carrier-mediated transport, taurine release appears to be mediated by several mechanisms including; simple passive diffusion, carrier mediated transport, and exocytosis [Pases-Morales et al. 2002]. Taurine diffusion through cell membranes is strictly driven by concentration gradients, and exhibits non-saturable transport kinetics. The rate of taurine release via

diffusion will depend on intracellular and extracellular taurine concentrations. In contrast, carrier mediated release of taurine occurs via reversal of the uptake transporter. It is sodium-dependent, exhibits saturation kinetics, and is subject to competitive inhibition by structural analogs, such as alanine [Saransaari et al. 2000].

Taurine release via VRAC is regulated by volumetric stress [Saransaari et al. 2000]. The Cl⁻ channels activated during hypo-osmotic swelling are typically outwardly rectifying with an intermediate unitary conductance of 40-78 pS, with inactivating potentials at +60 mV and above [Pasantes-Morales et al. 2002]. This volume sensitive Cl⁻ channel has been characterized in many brain cell types including astrocytes, C6 glioma cells, and in cerebellar and hippocampal neurons [Jackson et al. 1995; Li and Olson 2008; Patel et al. 1998]. VRAC exhibit high selectivity of anions over cations, permeability to most monovalent anions, and a broad anion selectivity [Pasantes-Morales et al. 2002]. Enhanced activation of VRAC channels has been shown in the presence of ATP, indicating purinergic receptors may be involved in the mediation of VRAC activity during volume regulation [Mongin et al. 2002; Li et al. 2004]. Li et al. [2004] demonstrated that volume regulatory efflux of amino acids, including taurine via VRAC, is also dependent upon an elevated or permissive level of intracellular calcium and calmodulin activation in astrocytes [Li et al. 2004]. Experimentally, VRAC mediated taurine release can be used to indicate when mechanisms of RVD have been activated.

Specific mechanisms of VRAC activation during oxidative stress remain poorly defined. As previously discussed, VRAC have been shown to be activated by ROS in swollen microglia leading to increased release of excitatory amino acids [Harrigan et al. 2008]. However, because oxidative stress has been found to cause swelling in both neural

tissues and cells, it is less clear if ROS can regulate VRAC in the absence of swelling. In the present project, I will examine potential mechanisms linking oxidative stress and cell swelling to VRAC activation and taurine release.

Taurine – Mobilization During Cell Volume Regulation

In brain tissue, *in situ* and *in vitro* studies indicate that taurine is the most important osmotically active organic molecule involved in volume regulation during both hypo-osmotic hyponatremia, and hyperosmotic dehydration [Verbalis et al. 1991; Bedford et al. 1993]. Organic osmolytes, including taurine, are especially important for brain volume regulation for two reasons; first, the brain is especially vulnerable to cell volume alterations as a result of its rigid surrounding skull and dura structures and second, unlike ions, organic osmolytes have limited affect on neuronal excitability and are compatible with normal macromolecular function [Law et al. 1994]. Release of inorganic electrolyte osmolytes such as K, Na, and Cl during cell volume regulation in the CNS, would alter ion gradients across the cell membranes and thus affect ion transporters, membrane polarization, and excitability [Law et al. 1994; Verbalis et al. 1991]. However, K, Na and Cl are lost from the brain during the first three hours of hyponatremia, indicating these electrolytes play a significant role in the immediate volume decrease response in the intact brain [Berl et al. 1990; Pasantes-Morales et al. 2002]. However, during periods of chronic hyponatremia, release of electrolytes decreases over time [Verbalis et al. 1991] while cells activate release of osmotically active organic solutes including polyalcohols, methyl amines and amino acids such as taurine, glutamate, glutamine and glycine. According to Huxtable et al. [1992] taurine may be the ‘perfect osmolyte’ in the CNS because it is metabolically inert and exhibits

only weak synaptic interaction [Huxtable et al. 1992]. Further, taurine as well as other organic osmolytes are often referred to as ‘compatible osmolytes’ based on the concept that they do not perturb cellular macromolecules even when the solutes are at high concentrations [Yancey et al. 2005; Brown et al. 1972]. Thus, the use of taurine and other organic osmolytes for volume regulation, allows cells to circumvent the deleterious effects of altered ion concentration disturbances, without compromising other vital cell functions [Bagnasco et al. 1993; Burg et al. 1994, Lang et al. 1998].

The average intracellular taurine concentration in adult human cells is between 2-6 mM and even higher for many neurons, including cerebellar Purkinje cells and hippocampal pyramidal cells [Banay-schwartz et al. 1993; Nagelhus et al. 1993; Pow et al. 2002]. Exposure of brain tissue to hypoosmotic conditions causes a reduction in brain taurine content by as much as 85% [Kimelberg et al. 1995; Verbalis et al. 1992]. Taurine efflux is enhanced in both cerebral cortical slices, and hippocampal tissue slices exposed to hypoosmotic conditions [Franco et al. 2000; Law et al. 1994]. Taurine also enhances volume regulation in hippocampal slices swollen osmotically and during exposure to oxidative stress in isoosmotic conditions [Kreisman et al. 2003]. These results indicate that brain tissues exposed to hypoosmotic conditions utilize a release of intracellular taurine, followed by the osmotically obliged water to facilitate regulatory volume decrease [Haussinger et al. 1994; Kimelberg et al. 1995]. As a result, tissue cellular swelling is reduced or eliminated and vulnerability to potential pathological consequences of prolonged alterations in cellular volume are thereby reduced [Kimelberg et al. 1995].

While the above studies indicate taurine is released from cells in response to hypoosmotic conditions, the redistribution of the taurine released during these conditions is not well understood. Lehmann et al. [1991] suggested that some of the increase in extracellular taurine in hippocampal tissue under hypoosmotic conditions may come across the blood brain barrier. Solis et al. [1988] illustrated that during the early stages of hypoosmotic hyponatremia, cells mobilize intracellular taurine contents into the extracellular space [Solis et al. 1988]. Nagelhus et al. [1993] showed during the first hour of hypo-osmotic hyponatremia, taurine contents of cerebellar Purkinje cells are reduced, while the taurine contents of neighboring astroglial cells are increased. Further, early studies dating back to the 60's have observed a greater swelling of glial cells relative to that of neurons during pathological conditions characterized by cytotoxic edema [Plum et al. 1963; Klatzo et al. 1967; Betz et al. 1989]. More recent studies have demonstrated that, in cell culture, taurine is preferentially lost from osmotically swollen neurons compared with astroglial cells, and that swollen neurons rapidly down-regulate the taurine transporter [Olson et al. 2000; Olson et al. 2006]. These studies suggest that during pathological conditions characterized by brain edema, taurine is mobilized from neurons, and into surrounding glia [Olson et al. 2006]. These observations may represent a neuroprotective mechanism by which neurons and glial cells transport taurine to minimize the effects of neuronal swelling on brain function, and potential downstream consequences of chronic brain edema.

Taurine- Other neuroprotective mechanisms

The most well documented role of taurine in neuroprotection is its role in volume regulation. However, studies indicate additional mechanisms by which taurine may act in

a neuroprotective capacity. Intracellular taurine protects neurons against glutamate-induced neuronal damage in primary neuronal culture. The neuroprotective effect of taurine has been largely attributed to its functions in maintaining intracellular calcium homeostasis, membrane integrity (Pasantes-Morales et al. 1984] and as an antioxidant [Wu et al. 2009]. Further, Leon et al. [2009] proposed that taurine protects neurons against glutamate-induced neurotoxicity in part, by preventing glutamate induced membrane depolarization through inhibition of intracellular Ca^{2+} accumulation and thereby inhibiting glutamate-induced apoptosis.

Taurine-Summary

The research discussed here indicates that taurine plays an important role in many aspects of neuroprotection. Studies indicate taurine as a significant effector in intracellular signaling pathways and illuminate an important neuroprotective role of taurine in glutamate-induced neuronal damage. Further studies indicate that taurine plays an integral role in cell volume regulation during both osmotically-induced and oxidatively induced swelling [Kreisman et al. 2003; Li et al. 2004]. While research has helped establish taurine as a significant factor in volume regulation in the CNS, the mechanisms behind these effects remain largely unclear. In this work we will examine taurine release from hippocampal tissue slices during exposure to H_2O_2 . With this model of exposure to oxidative stress, taurine release and volume will be measured under different conditions including; during exposure to VRAC channel blockers, during exposure to glutamate receptor antagonist and in conditions which inhibit tissue swelling. By examining taurine release during these conditions we will clarify specific

neuroprotective mechanisms of taurine as they relate to volume regulation during oxidative stress.

3. Glutamate

Glutamate receptor activation has been suggested to be a factor in ischemia-induced brain edema [MacGregor et al. 2003]. Because glutamate plays a multi-faceted role in the central nervous system, this review will include only a brief physiological review of the role of glutamate as a neurotransmitter in the CNS, and the receptor subtypes involved in glutamate signaling in neurons and glial cells. After discussing the physiological foundation of glutamate signaling in the CNS, this review will focus on three central topics relevant to our study, (i) the increase in glutamate concentrations in the CNS during tissue ischemia; (ii) the role of glutamate in the development of post-ischemic brain edema; and (iii) the role of glutamate receptors in cell volume regulation. This background on glutamate will elucidate associations between oxidative stress, tissue edema, glutamate receptor activation and cell volume regulation. This interaction is central to the project hypothesis relating the role of glutamate receptors during oxidatively induced brain edema.

Glutamate – Glutamate Signaling

Glutamate is the primary excitatory neurotransmitter in the central nervous system. Nutritionally, it is classified as a non-essential amino acid, as it can be synthesized from other molecules, primarily α -ketoglutarate and glutamine [Nicholis et al. 1994]. The generation of glutamate from α -ketoglutarate occurs in the mitochondria in an efficient one-step process. Glutamine is used in the synthesis of neuronal glutamate

and is provided primarily by glial cells. An elementary physiological overview of the synthesis, release, uptake and metabolism of glutamate in neurons and glial cells is outlined below. Further, this review provides the foundation for understanding glutamate metabolism during pathological conditions such as ischemia and oxidative stress.

Neurons use glutamate in many processes, most notably as an excitatory neurotransmitter in the CNS. Glutamate is present in two metabolic pools within neurons. Neurotransmitter glutamate is stored within synaptic vesicles and is compartmentalized from non-neurotransmitter glutamate [Gonzales et al. 1997]. Glutamate signaling begins as glutamate-containing vesicles fuse with the cell membrane, releasing their content into the synaptic cleft. The next step in the signal transduction process involves the activation of glutamate-specific receptors, inducing excitation of the post-synaptic neuron. The process is terminated as the glutamate is transported via specific carrier proteins back to the pre-synaptic neuron and to surrounding glial cells. Glutamate returning to the pre-synaptic neuron is transported and packaged directly into synaptic vesicles. Glutamate transported into glial cells is rapidly converted by glutamine synthetase into glutamine, where it then can be transported back into the pre-synaptic cell for conversion back to glutamate. As a result, glial cells play two essential roles in glutamate signaling in the CNS: terminating the glutamatergic synaptic signal and ensuring a sufficient supply of pre-cursor glutamine for neuronal glutamate synthesis. The glutamate re-uptake system of glial cells is indispensable for preventing excessive glutamate excitation of the post-synaptic neuron.

Glutamate – Glutamate Receptor Sub-types in Neurons and Glial Cells

Glutamate receptors are the primary molecules involved in excitatory signal transmission [Nakanishi et al. 1992]. The post-synaptic responses to glutamate are mediated by two separate classes of receptors: ionotropic (iGlu) receptors and metabotropic (mGlu). The iGlu classes of receptors are cation-specific ion channels responsible for mediating fast excitatory transmission. There are three different families of iGlu, which are defined by their respective pharmacological antagonist profile; α -amino-3-hydroxyl-5-methyl-4-isoazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA).

The second class of glutamate receptors is a family of G-protein-coupled receptors called mGlu. mGlu are found in both neurons and glia where they exert a multitude of effects on glia function and provide a mechanism for glial-neuronal interactions. The mGlu family is a seven transmembrane domain receptor that exerts its effects on both ion channels and on secondary messenger systems. There are eight different sub-types of mGlu receptors (mGlu1-8), differentiated by agonist pharmacology, signal transduction pathways, and sequence homology [D'antonio et al. 2008]. mGlu receptors modulate synaptic transmission, including the processes responsible for long-term potentiation and long-term depression. In neurons, mGlu1 and mGlu5 receptors modulate post-synaptic efficacy, whereas mGlu2, mGlu3, mGlu4, mGlu7 and mGlu8 are mainly pre-synaptic and regulate neurotransmitter release [Ferragutti et al. 2006]. The specific structures and functions of mGlu receptor sub-types are well discussed in contemporary reviews [D'antonio et al. 2008; Pin et al. 1995;

Ferragutti et al. 2006]. For the purposes of this review, the remainder of the discussion will emphasize the iGlu receptor sub-type, including NMDA and AMPA receptors.

NMDA receptor (NMDA-R) is an ionotropic glutamate receptor selectively activated by the glutamate agonist NMDA. NMDA-R activation is unique in two capacities: first, it is a ligand-gated channel requiring two ligand agonists for activation; and second, its activation is voltage dependent. The ligand component of NMDA-R activation requires binding to the agonist-binding site which has affinity for both glutamate and aspartate (although aspartate does not stimulate receptors as strongly as glutamate). An additional ligand required for activation of NMDA-R is the 'co-agonist', glycine. Finally, NMDA-R activation requires membrane depolarization. At resting membrane potential (approx. -65 mV) extracellular Mg^{2+} blocks NMDA-R ion channels. Depolarization of the cell membrane by 20-30 mV causes Mg^{2+} ions to be released from the channel, thus opening the ion channel [Ozben et al. 1998]. The final result of NMDA-R activation is flow of Na^+ and small amounts of Ca^{2+} ions into the cell, and K^+ out of the cell [Dingledine et al. 1999; Liu et al. 2000].

AMPA receptor (AMPA-R), also known as quisqualate receptor, is a non-NMDA type ionotropic glutamate receptor activated by both quisqualate and the glutamate analog AMPA. As an ionotropic receptor, AMPA-R mediates fast synaptic transmission within the CNS, and generates fast excitatory postsynaptic potentials (EPSP) [Purves et al. 2004]. AMPA receptors are glutamate gated cation-selective channels permeable to Na^+ and K^+ , but are impermeable to Ca^{2+} [Stern-Bach et al. 2004]. Although impermeable to Ca^{2+} , AMPA receptors help activate mechanisms that result in the entry of Ca^{2+} into neurons. The Ca^{2+} entry results from the AMPA-induced membrane depolarization

which, by alleviating the Mg^{2+} block of the NMDA receptor, activate the NMDA receptor with a subsequent influx of Ca^{2+} . Further, the AMPA induced membrane depolarization is large enough to activate voltage operated Ca^{2+} channels [Ozben et al. 1998]. Because AMPA-R activation requires only the presence of glutamate and no membrane depolarization, AMPA receptors are more suited for relaying the faster “on/off” excitatory signaling within the CNS [Gonzales et al. 1997].

Glutamate – Neurotoxicity in Cerebral Ischemia

Extracellular glutamate concentrations are significantly increased in ischemic brain tissue [Park et al, 1988]. The increased extracellular concentration is a result of enhanced release of the amino acid from neurons [Scatton et al. 1994] and decreased re-uptake by surrounding neurons and glial cells [Dallas et al. 2007, Ozben et al. 1998]. During ischemia, oxygen-glucose deprivation depletes energy formation, resulting in failure of essential energy-dependent membrane ion gradients. As ion pumps fail, membrane ion gradients deteriorate, ion channels open, and most intracellular and extracellular ions begin to equilibrate causing anoxic depolarization [Ozben et al. 1998]. Pre-synaptic depolarization opens voltage-sensitive calcium channels (VSCC) allowing Ca^{+} to enter [Pulsinelli et al. 1992]. This increased calcium triggers release of excitatory amino acids (EAA) including glutamate. Additionally, the loss of membrane ion gradients also decreases glutamate uptake. The resulting uncontrolled increase in extracellular glutamate concentration over-activates post synaptic NMDA and AMPA receptors in a condition known as ‘excitotoxicity’. Excitotoxicity causes many deleterious effects following ischemic injury including oxidative stress and brain edema.

Glutamate is an underlying cause in ischemic injury and in the formation of brain edema [Juurlink et al. 1997; Love et al. 1999; Brahma et al. 2000]. Glutamate-receptor activation has been shown to cause edema in normoxic brain slices *in vitro* [Espanol et al. 1994; Brahma et al. 2000]. Glutamate receptor activation also has been found to contribute to the initial swelling that accompanies anoxic depolarization in slices [Werth et al. 1998]. Prior studies by Oh et al. 1991 indicate that NMDA antagonists can decrease brain swelling *in vivo* [Oh et al. 1991]. More recent *in vitro* work by MacGregor et al. [2003] found that blocking glutamate receptors with NMDA and AMPA/kainate antagonists greatly attenuated tissue water gain after ischemic insult [MacGregor et al. 2003]. Although the exact mechanism remains unclear, these studies suggest glutamate receptor activation is a significant factor in the formation of brain edema.

MacGregor et al. 2003 also established that both glutamate and ROS are underlying factors in the formation of brain edema in an oxygen and glucose deprivation model of cerebral tissue ischemia. Their study indicated that glutamate receptor antagonists and antioxidants result in similar attenuation of tissue edema in slices. An additional study indicated that a treatment cocktail containing both glutamate receptor antagonist and antioxidant showed a greater attenuation of tissue edema than any single treatment component [MacGregor et al. 2003].

Glutamate- Cell Volume Regulation

Glutamate is a significant factor mediating cell volume regulation. Release of taurine is enhanced by glutamate receptor agonists under hypoosmotic conditions [Menendez et al. 1990]. Saransaari et al. [1997] observed that ionotropic glutamate receptor agonists NMDA, kainate and AMPA potentiated taurine release from

hippocampal tissue. This observed enhancement of taurine release was inhibited by treatment with glutamate receptor antagonists of each ionotropic receptor subtype (NMDA, kainate, and AMPA). These results indicate that the effect of glutamate on taurine efflux is receptor-mediated in this hippocampal model. NMDA is generally considered the most powerful agonist of taurine release but AMPA may be the most effective at lower glutamate concentrations [Magnusson et al. 1991; Saransaari et al. 1991, 1994, 1997]. Finally, Saransaari et al. [1997] also established that both NMDA and AMPA receptors were involved in taurine release throughout the life span, while kainite receptor-mediated release does not appear to function in adults. These early works of Saransaari et al. in 1991, 1994 and 1997 were seminal studies in that they linked glutamate receptor activation with taurine release in hippocampal tissue.

More recent studies have established a significant role for glutamate receptors in osmo-regulation [Morales et al. 2007; Uckerman et al. 2009]. A significant increase in extra-synaptic glutamate concentrations is observed in response to hypoosmotic treatments. Tissues exposed to combined AMPA and kainate receptor antagonist CNQX show significantly decreased taurine release in response to hypoosmotic treatment [Morales et al. 2007]. Uckerman et al. [2009] observed inhibition of osmotic glial cell swelling that is mediated by glutamate receptor activation. These studies suggest that hypoosmolarity facilitates glutamate release, which, acting on ionotropic glutamate receptors, contributes to the osmo-regulatory response [Morales et al. 2007; Uckerman et al. 2009].

Glutamate-Summary

Glutamate plays multiple roles in the processes of ischemic injury and oxidative stress, neuronal and glial swelling, and in the mediation of cell volume regulating mechanisms in the CNS. Glutamate concentrations greatly increase as a result of tissue ischemia and these elevated glutamate levels contribute to both tissue injury and the formation of brain edema. In the presence of tissue swelling, glutamate has also been observed to mediate cell volume regulating mechanisms. Contemporary studies have uncovered some of the effects of glutamate in cell swelling and volume regulation in the CNS. However, the specific role of different ionotropic glutamate receptors during brain tissue swelling induced by oxidative stress remains largely unclear. In this present study I will examine the role of ionotropic glutamate receptors, NMDA and AMPA, for hippocampal tissue swelling and volume regulation during oxidative stress. These experiments will elucidate the specific role of glutamate in cell volume regulation during oxidatively-induced brain edema.

4. Summary of Background

The research presented in this background describes the tightly entangled relationships between the processes of oxidative stress, cellular swelling, cell volume regulation and glutamate signaling in the CNS.

- Reactive Oxygen Species (ROS) - Brain edema following ischemia or ischemia-reperfusion is associated with the production of reactive oxygen species and consequent oxidative stress to brain tissue. Oxidative stress is a causative factor in brain tissue swelling, although the specific mechanisms are not well understood.

The specific aims of this project will elucidate a potential mechanism explaining ROS-induced brain tissue edema *in vivo*.

- Taurine - In response to swelling, brain slice preparations activate volume regulating mechanisms which include a significant efflux of the endogenous amino acid taurine. Acting as an organic osmolyte, taurine mobilization is an essential component in regulatory volume decrease following cell swelling of both neuronal and glial cells. Taurine efflux is facilitated by volume regulating anion channels (VRAC) and ROS may directly or indirectly affect this membrane pathway. This project will examine the effects of ROS on taurine efflux via VRAC during volume regulation.
- Glutamate - Extracellular glutamate concentrations increase greatly as a result of tissue ischemia and elevated glutamate levels contribute to both tissue injury and in the formation of brain edema. In the presence of tissue swelling, glutamate plays a role as a signal for cell volume control mechanisms. However, the role of specific ionotropic glutamate receptors during brain tissue swelling induced by oxidative stress remains largely unclear. This project will examine the role of ionotropic NMDA and AMPA receptors during hippocampal tissue swelling and volume regulation induced by oxidative stress.

To gain a deeper understanding of the interactions between reactive oxygen species, taurine efflux and glutamate action, this project will address the following issues: the effect of oxidative stress on cell volume regulation mechanisms in brain tissue slices, the role of VRAC channels for volume regulation during oxidatively induced swelling, and finally, the role of glutamate on both brain tissue swelling and volume regulation during

oxidative stress. In addressing these specific aims, our project will examine critical components of the relationships between oxidative stress, glutamate signaling, tissue swelling and cell volume regulation. Further, it will provide deeper insight into the mechanisms responsible for ischemia induced brain edema leading to improved treatment and recovery from these devastating pathological conditions.

CHAPTER III
HYPOTHESIS AND SPECIFIC AIMS

1. Rationale

Oxidative stress induces hippocampal tissue cell swelling via a glutamate signaling pathway and increases taurine efflux via VRAC channels. Further, glutamate plays a role in the signaling of volume regulation pathways in the CNS. As a result, we have proposed the following mechanism for taurine efflux initiated by oxidative stress in the hippocampus.

Ox. Stress ⇔ *Activates Glut-R* ⇔ *Hippocampal Swelling* ⇔ *VRAC Activation* ⇔
Taurine Efflux ⇔ *Volume Regulation*

Specific Aim I, will establish the role of taurine efflux for volume regulation during oxidative stress. I will then examine the above proposed mechanism in two additional specific aims. In Specific Aim II, importance of tissue swelling (IIa) and the role of VRAC channels (IIb) for taurine release and volume regulation will be examined. In Specific Aim III I will evaluate the function of NMDA and AMPA glutamate receptors for oxidative stress-induced tissue swelling and volume regulation.

2. Specific Aim I

Specific Aim I establishes that taurine efflux enhances volume regulation during oxidative stress in hippocampal tissue slices.

Specific Aim I: Experiments

Experiment I: Taurine efflux from hippocampal slices will be measured during exposure to hydrogen peroxide. Taurine contents of some slices will be modified by incubation and perfusion of some slices with aCSF containing taurine. This treatment will restore taurine lost during slice preparation to physiological levels. Other slices will be incubated and perfused with aCSF without taurine.

3. Specific Aim II

In Specific Aim II we hypothesize that taurine efflux initiated by oxidative stress:

- a. is mediated by volume-regulated anion channels (VRAC)
- b. is activated by cellular swelling.

Specific Aim II: Experiments

Experiment II.A. Hippocampal slices with physiological taurine content will be exposed to hydrogen peroxide in the presence or absence of DCPIB, a specific inhibitor of VRAC channels. Hippocampal tissue swelling and taurine release will be monitored in this experiment.

Experiment II.B. Hippocampal slices with physiological taurine content will be exposed to hydrogen peroxide in isoosmotic aCSF (290 mOsm) or in aCSF containing mannitol to raise osmolality to 350 mOsm. Hippocampal tissue swelling and taurine release will be monitored in this experiment.

4. Specific Aim III

In Specific Aim III we hypothesize that taurine efflux initiated by oxidative stress is mediated by a glutamate receptor pathway.

Specific Aim III: Experiments

Experiment III: Hippocampal tissue slices prepared to have physiological or low taurine contents will be exposed to hydrogen peroxide. For some slices, NMDA or AMPA receptors will be inhibited with MK-801 or CNQX, respectively. Hippocampal tissue swelling and taurine release (in slices with physiological taurine contents) will be monitored in this experiment.

CHAPTER IV
MATERIAL AND METHODS

1. Ethical Approval

All experiments described in the present study were performed in accordance with protocols approved by the Wright State University Institutional Laboratory Animal Care and Use Committee (IACUC). Anesthesia protocols approved for these experiments conform to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Protocols are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications N0. 80-23) revised 1996.

2. Slice Preparation

Experimental studies were performed using an acute preparation of hippocampal slices prepared from male Sprague-Dawley rats (250-400 g). Prior to experimentation, animals were housed in a controlled environment facility located on the Wright State University Campus, (25° C, 12-h light/dark cycle) with food and water available *ad libitum*. Animals were transported to our facility on the day of use.

Animals were rapidly asphyxiated to apnea with halothane, and placed in a supine position on a dissection table. The thoracic cavity was exposed and the animal was perfused via the left cardiac ventricle using artificial cerebrospinal fluid (aCSF) bubbled in 95% O₂ consisting of: 124 mM NaCl, 3.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM Na₂HPO₄, 26 mM NaHCO₃, and 10 mM glucose. After perfusion of approximately 50 ml of aCSF over 1 min, animals were decapitated using a guillotine and the scalp was cut along midline. The skull was opened along midline working from the caudal to rostral direction. Using surgical rongeurs, skull fragments were removed exposing first the

cerebellum, followed by the cerebral cortex. Upon full brain exposure, underlying cranial nerves were severed and the cortex, midbrain and cerebellum were transferred rapidly to a chilling solution consisting of frozen aCSF slush.

After 5-7 minutes, the cerebellum was removed from the midbrain, and the brain hemisected into right and left hemispheres. Hippocampi then were dissected from the brain hemispheres, and the middle one-third of each was sectioned into 400 μm slices using a McIlwain tissue slicer. Slices were cut transversely in a plane that preserved Schaeffer collateral connections [Teyler et al, 1980]. Slices were incubated at room temperature for at least 90 min in chamber bubbled with 95% O_2 + 5% CO_2 . Solutions were made on the day of the experiment. The aCSF solution used for incubation and initial treatments was isoosmotic (290 mOsm). Osmolarity was adjusted by adding small volumes of 3 M NaCl and confirmed using a vapor pressure osmometer (Wescor, Salt Lake City, Utah).

3. Tissue Treatment and Perfusate Sampling

Tissue taurine content is lost during slice preparation [Kreisman et al, 2003]. To examine the role taurine plays for volume regulation, our experimental protocol examined hippocampal tissue slice preparations that were either taurine-repleted during incubation and perfusion or were left depleted of taurine following preparation. Taurine-repleted slices were incubated at room temperature in the bubbler chamber with aCSF containing 1 mM taurine, while taurine-depleted slices were incubated in aCSF without added taurine. After incubation, brain tissue slices were transferred to an interface-type imaging chamber with oxygenated aCSF (2 ml/min) perfused at $35^\circ \pm 0.5$ C.

Generally the experimental time course started with 90 min perfusion in isosmotic aCSF followed by 25 min perfusion with aCSF containing 2 mM H₂O₂. When drug treatments were used, they were added to the perfusate beginning 15 min prior to H₂O₂ exposure. For experiments with taurine repleted slices, 1 mM taurine was added to the aCSF during the initial 30 min of perfusion. The following 60 min period in taurine free aCSF allowed complete efflux of exogenous taurine from the extracellular compartment. Effluent perfusate samples were collected in 2 min fractions for analysis of amino acid efflux. In these experiments, control and drug studies were performed on the same day with slices from the same animal.

4. Slice Imaging

An intrinsic optical signal (IOS) was used as an indirect measure of tissue volume. IOS has been used to measure brain tissue volume changes in both osmotically and excitotoxically stressed brain tissue slices [Andrew et al. 1999]. Hippocampal tissue slices were trans-illuminated with white light from a quartz-halogen source with a voltage-regulated power supply, and observed with a stereo microscope (model SMZ-2, Nikon, Melville, NY) During perfusion, images of the entire hippocampal slice were recorded every 60 sec using a standard video camera with fixed gain. Using the Image J program, we designated the striatum radiatum of the CA1 area of the hippocampus as our selected region of interest for measuring changes in tissue IOS throughout our experiments. The mean intensity for the region of interest was recorded into a text file, and then transferred to Windows Excel (Microsoft) software for graphing and analysis.

5. Taurine Concentration Analysis

Perfusate solution was collected in 2 min fractions throughout incubation of the slices on the imaging stage. Solutions were frozen at -70 °C, and 1 ml aliquots were lyophilized and then resuspended in 0.1 ml of 0.6 M HClO₄. Upon termination of the experiment, hippocampal tissue slices were fixed in 1 ml of 0.6 M HClO₄ and sonicated. This suspension was then centrifuged at 10,000 x g for 1 min. Amino acids were determined in the concentrated perfusate samples and in the supernatant from the hippocampal slices by HPLC [Olson and Li 2000].

Taurine concentrations were determined on a high-performance liquid chromatograph (HPLC) system with a 5 µm C18 reverse phase column (250 x 4.6 mm) and fluorimetric detection with 340 nm excitation and emission cut-off at 420 nm. Pre-column derivitization was performed with o-phthalaldehyde-2-mercaptoethonal-borate reagent. After a 120 sec reaction period, the reaction was terminated with iodoacetamide and the solution injected into the HPLC column 30 sec later. The mobile phase was composed of 50 mM sodium phosphate and 38% methanol (pH = 5.65).

6. Statistics

Analyses were performed using one-way repeated measure ANOVA followed by Dunnett's test for *post hoc* comparisons. Time dependent changes in IOS were evaluated with linear and non-linear regression. Data are given as means ±SE. Analysis was performed using GBStat program software (Dynamic Microsystems, 1997). Curve fits

were performed using pClamp 8 software (Axon Instruments, Sunnyvale, California). A level of $p < 0.05$ was considered critical for assigning statistical significance.

CHAPTER V
RESULTS

1. Specific Aim I

In Specific Aim I we examined the role of taurine in volume regulation during oxidative stress by exposing hippocampal slice preparations to H₂O₂ and measuring both volume change and taurine concentrations in the perfusate. We measured the effects of H₂O₂ exposure on slices incubated in taurine supplemented aCSF (previously defined as “taurine repleted”), and on slices incubated in aCSF alone (previously defined as “taurine depleted”).

Taurine enhances volume regulation in hippocampal slices during oxidative stress

Hippocampal slices prepared by incubation in normal aCSF without taurine have reduced taurine contents compared with the hippocampus *in situ* [Kreisman et al, 2003]. Slices prepared in this manner demonstrated a significant increase in IOS ($3.27 \pm 1.25\%$ at $t = 25$ min, $n = 5$) during exposure to 2 mM H₂O₂, indicating a statistically significant increase in volume. In contrast, slices incubated with aCSF supplemented with 1 mM taurine have normal taurine contents during experimentation and showed little swelling throughout the period of oxidative stress ($1.08 \pm 0.61\%$ at $t = 25$ min, $n = 12$) [Figure 3] and [Table 1]. By multiple linear regression, the rate of change in IOS (\pm SE) was 0.064 ± 0.012 percent/min in taurine repleted slices while the rate in taurine depleted slices was 0.137 ± 0.019 percent/min. The difference between these rates (0.073 ± 0.022 percent/min) was statistically significant. Taurine depleted slices exhibited non-detectable levels of taurine in the perfusate prior to and during H₂O₂ exposure (data not shown). In contrast, slices incubated in 1 mM taurine had measurable taurine in the

Figure 3

Volume change in the hippocampus during oxidative stress. Hippocampal slices were perfused with aCSF containing 2 mM H₂O₂ from t = 0 min to the end of the data sampling period shown. Values are the mean ± SEM for 5-12 independent experiments. The intrinsic optical signal (IOS) was calculated for areas of the stratum radiatum in the CA1 region of the hippocampus. Slices incubated with normal aCSF during preparation (solid symbols, n = 5) showed a significant increase in IOS during exposure to H₂O₂ while slices incubated with 1 mM taurine (hollow symbols, n = 12) showed little change in IOS. Regression lines were calculated from the relative intensity values between 0 min and 25 min. * indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to H₂O₂ treatment.

Figure 3

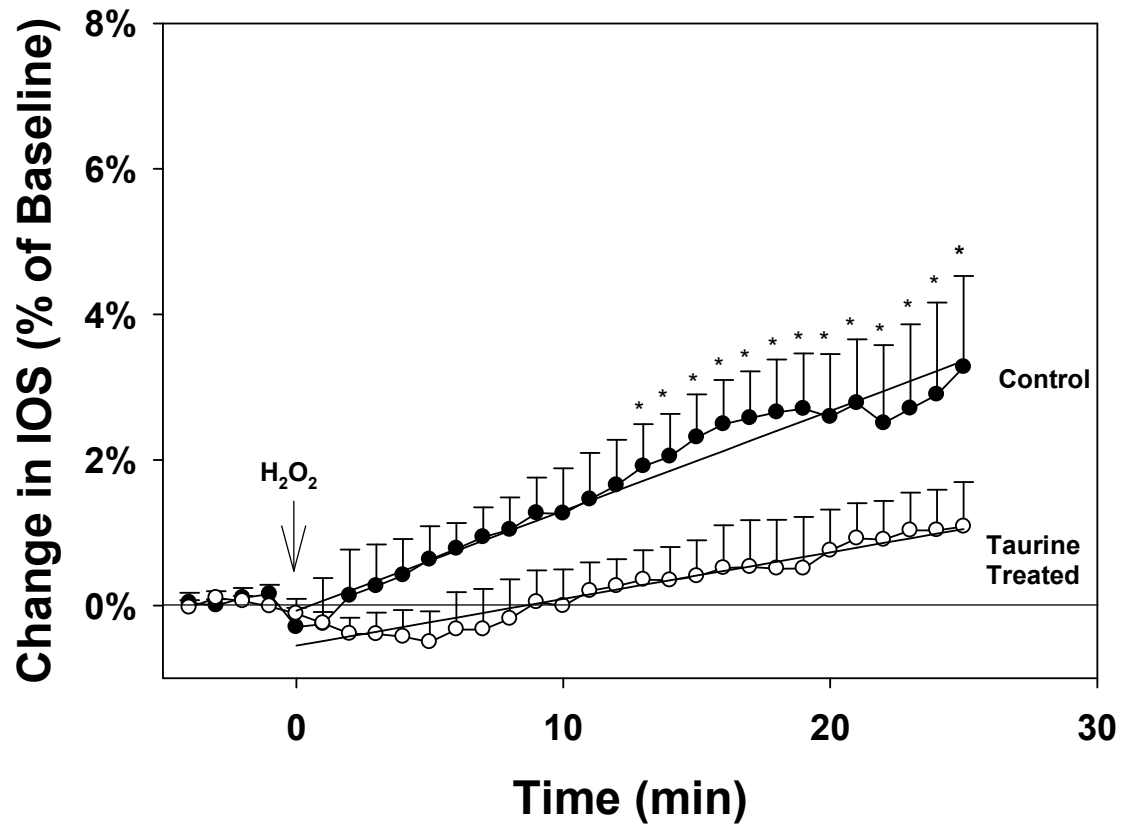


Table 1

Summary of the effect of H₂O₂ on volume of taurine depleted and taurine repleted hippocampal slices. Changes in the intrinsic optical signal (IOS) were measured in the stratum radiatum of the CA1 region and determined after 25 min of H₂O₂ exposure. These represent time points which resulted in peak responses observed in control slices as shown in Figure 3. Values are the mean ± SEM. * Indicates values which are significantly different from baseline measurements determined prior to H₂O₂ treatment.

Table 1

	Volume Change: H ₂ O ₂ effect on taurine depleted hippocampal slices (% change in IOS at 25 min)	Volume Change: H ₂ O ₂ effect on taurine repleted hippocampal slices (% change in IOS at 25 min)
Control	3.27 ± 1.25%* (n = 5)	1.08 ± 0.61% (n = 12)
CNQX	1.19 ± 0.39% (n = 6)	2.54 ± 0.78%* (n = 5)
MK-801	1.93 ± 0.26%* (n = 6)	5.01 ± 2.36%* (n = 5)
DCPIB	N/A	3.05 ± 1.12%* (n = 4)

perfusate and demonstrated an increase in taurine perfusate concentration of $335 \pm 178\%$ ($n = 3$) during the period of H_2O_2 exposure [Figure 4].

2. Specific Aim II

In Specific Aim II we investigated whether taurine efflux during oxidative stress is regulated via VRAC in this hippocampal tissue model by monitoring both volume and taurine efflux in the presence and absence of a VRAC inhibitor. We further examined whether H_2O_2 could elicit taurine efflux from the tissue in the absence of net swelling.

Taurine efflux in hippocampal slices requires VRAC activity

To measure the role of VRAC during H_2O_2 exposure, hippocampal tissues were treated with the VRAC inhibitor, 20 μ M DCPIB [Abdullaev et al, 2006]. We observed that taurine repleted slices, treated with DCPIB demonstrated a significant increase in IOS of $+3.05 \pm 1.12\%$ ($n = 4$) upon exposure to H_2O_2 over 25 min [Figure 5]. Further, by multiple linear regression we calculated a rate of IOS change during H_2O_2 exposure of 0.064 (± 0.012) percent/min in taurine repleted slices without drug treatment and a slope of 0.129 (± 0.004) percent/min in taurine repleted slices treated with DCPIB. The difference in these rates was 0.065 (± 0.005) percent/min) indicating a statistically larger rate of increase IOS in DCPIB treated slices. The observed increase in IOS indicates hippocampal tissue swelling occurs during H_2O_2 exposure with VRAC inhibition.

We observed a transient increase in taurine efflux upon exposure to DCPIB in normal aCSF. However, the rate of taurine efflux returned to a new stable baseline prior to H_2O_2 exposure [Figure 6]. After returning to this baseline, no increase in taurine efflux

Figure 4

Taurine efflux from hippocampal slices during oxidative stress. Hippocampal slices were perfused with aCSF containing 2 mM H₂O₂ from t = 0 min to the end of the data sampling period shown. Values are the mean ± SEM. Taurine concentrations were measured in the effluent of the aCSF that perfused hippocampal slices prepared to maintain normal taurine contents. The taurine concentration in the aCSF perfusate increased significantly ($335 \pm 178\%$, n = 3) at the start of H₂O₂ exposure and remained elevated for 10 min. * indicates values which are significantly different from the baseline measurements determined prior to H₂O₂ treatment.

Figure 4

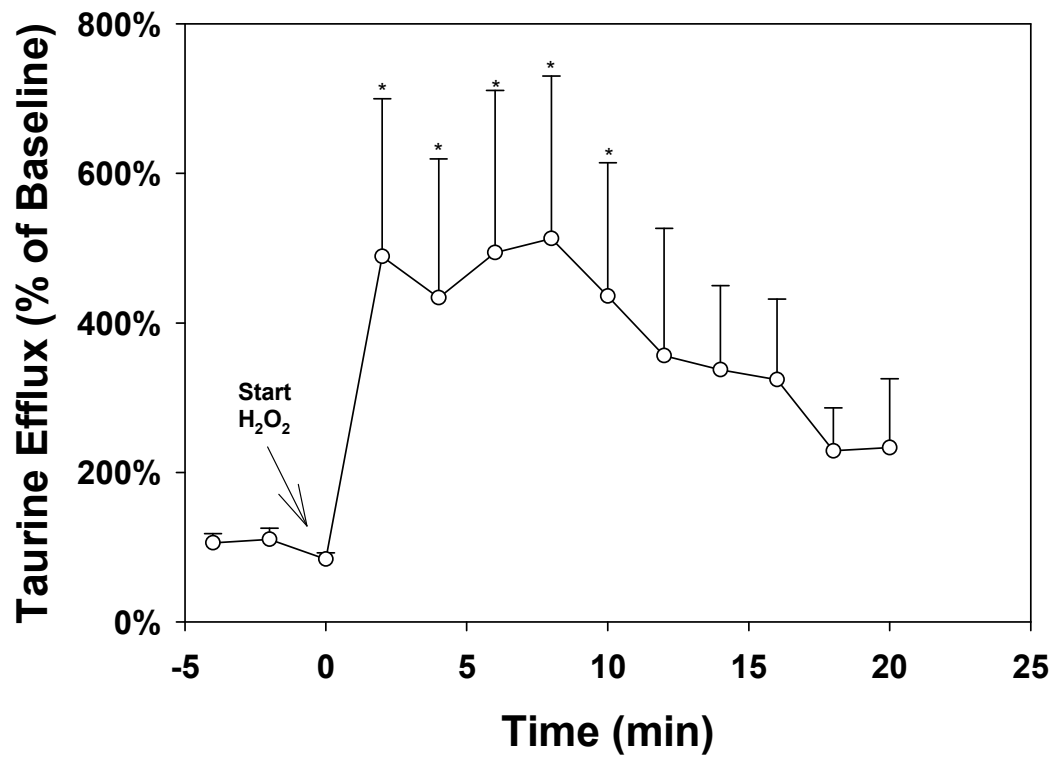


Figure 5

The role of VRAC on volume change in hippocampal slices during oxidative stress. Taurine repleted hippocampal slices were perfused with aCSF containing DCPIB (20 μ M) beginning at time $t = -15$ min and then perfused with aCSF containing 2 mM H_2O_2 from $t = 0$ min to the end of the data sampling period shown. Values are the mean \pm SEM for independent experiments. The intrinsic optical signal (IOS) was calculated for areas of the stratum radiatum in the CA1 region of the hippocampus. Slices treated with DCPIB (solid symbols, $n = 4$) showed a significant increase in IOS during exposure to H_2O_2 over 25 min, while slices incubated with 1 mM taurine (hollow symbols) alone showed little change in IOS ($1.08 \pm 0.61\%$, $n = 12$). Regression lines were calculated from the relative intensity values between 0 min and 25 min. * indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to H_2O_2 treatment.

Figure 5

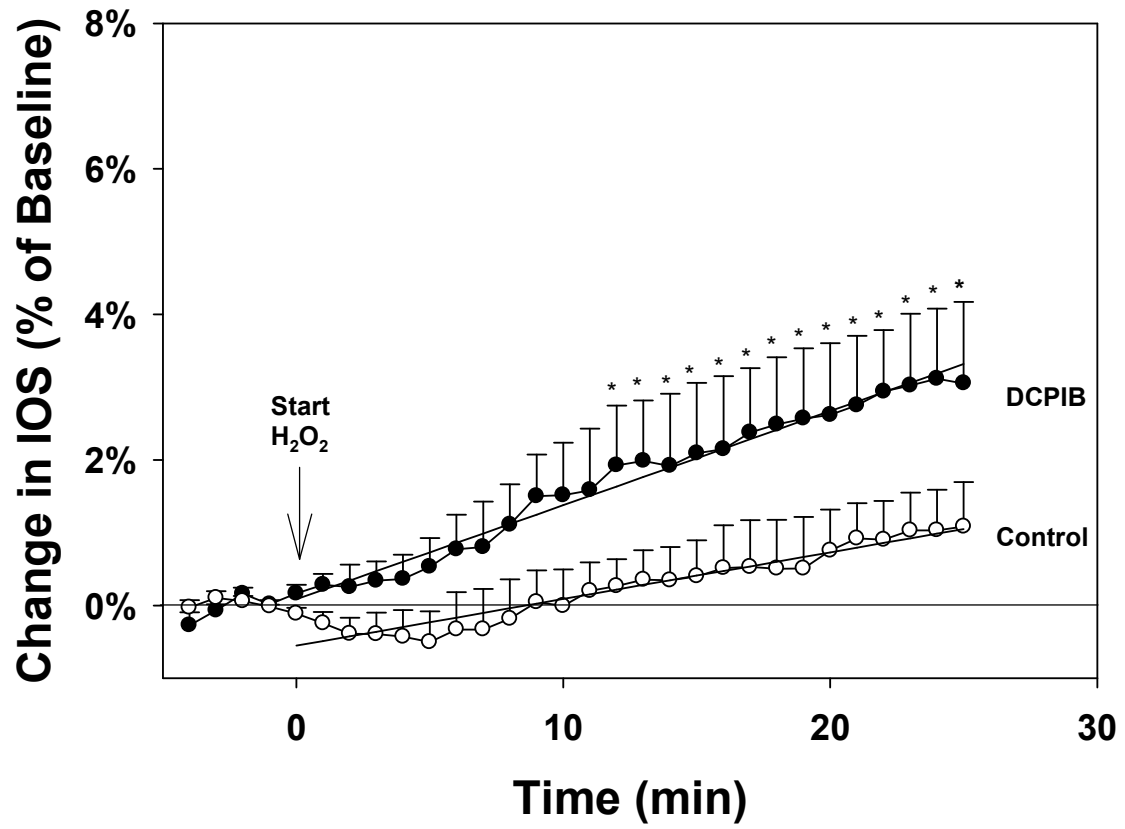
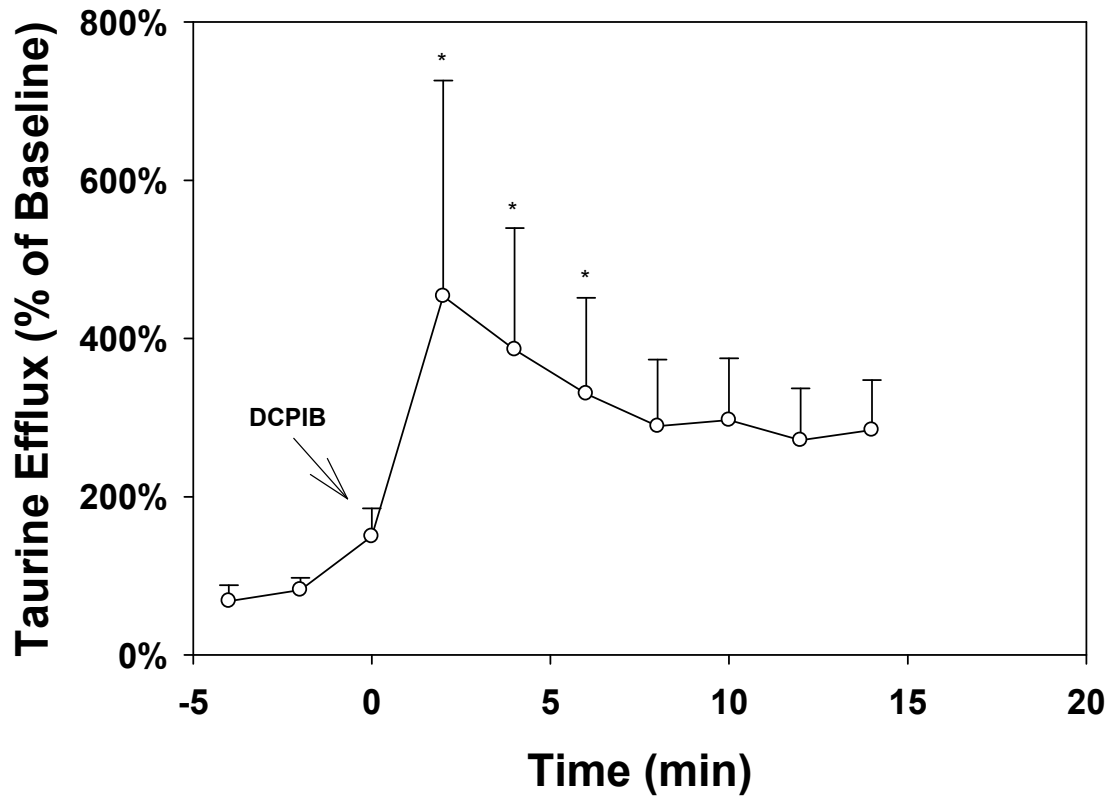


Figure 6

Transient taurine efflux from hippocampal slices during treatment with VRAC antagonist. Hippocampal slices were prepared to maintain normal taurine contents prior to DCPIB (20 μ M) exposure at $t = 0$. Values are the mean \pm SEM. Taurine concentrations were measured in the effluent aCSF prior to and during DCPIB exposure. The taurine concentration in the aCSF perfusate increased significantly ($453.42 \pm 272.65\%$, $n = 4$) within 5 min DCPIB exposure, but gradually returned within 15 min to a stable value not significantly greater than the baseline measured prior to DCPIB exposure.* indicates values which are significantly different from five baseline measurements determined at 1 min intervals prior to DCPIB treatment.

Figure 6



occurred during exposure to oxidative stress. In fact, taurine efflux actually decreased during the H₂O₂ exposure in the presence of DCPIB ($-59.8 \pm 7.0\%$, n = 4) [Figure 7].

We investigated the effect of oxidative stress on taurine efflux from hippocampal tissue slices during exposure to hyperosmotic conditions. The osmolarity of the aCSF was increased to 350 mOsm with the addition of mannitol for 15 min prior to and throughout treatment with 2 mM H₂O₂ in the perfusate. Mannitol treatment significantly and rapidly decreased IOS within the first 10 min of exposure in both controls, and in slices exposed to H₂O₂ after 15 min [Figure 8]. A subsequent slow trend toward baseline volume was also observed in both control slices and slices exposed to H₂O₂ after 15 min. To assess the effect of H₂O₂ against controls in these hyperosmotic conditions, slope analysis was calculated to determine if there was a change in rate of recovery toward baseline as a result of H₂O₂ exposure. In this slope comparison, the graph does show a slight inflection in the rate of recovery toward baseline in the H₂O₂ slices compared to controls. However, statistical comparison of these data sets indicates that after the initial 15 min of H₂O₂ exposure, there is no difference in the slopes between the remaining 15 min and 30 min of H₂O₂ exposure. Taurine efflux remained unchanged throughout the duration of the experiment for slices treated with H₂O₂ and for control slices [Figure 9].

3. Specific Aim III

In specific aim III we examined the role of glutamate signaling in volume regulation by measuring volume and taurine efflux from hippocampal slices exposed to either NMDA or AMPA receptor blockers.

Figure 7

Taurine efflux from hippocampal slices treated with VRAC antagonist during oxidative stress. Hippocampal slices were prepared to maintain normal taurine contents prior to DCPIB (20 μ M) exposure at $t = -15$ min. Slices then were perfused with aCSF containing aCSF plus 2 mM H_2O_2 from $t = 0$ min to the end of the data sampling period shown. Values are the mean \pm SEM for 4 independent experiments. The taurine concentration in the aCSF perfusate decreased significantly after 10 min of H_2O_2 exposure and remained decreased throughout the sampling period. * indicates values which are significantly different from the baseline measurements determined prior to H_2O_2 treatment.

Figure 7

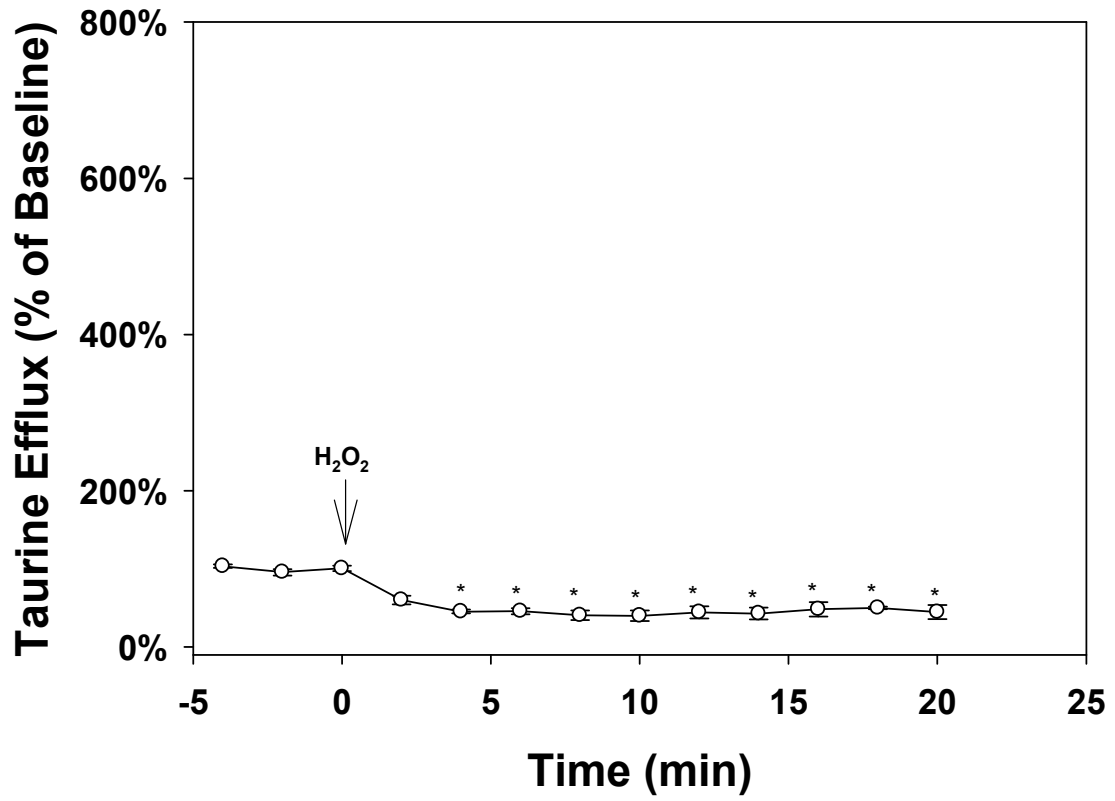


Figure 8

Volume changes in hippocampal slices in hypertonic media during oxidative stress.

Hippocampal slices were prepared to maintain normal taurine contents. Slices were then perfused with 350 mOsm mannitol aCSF from $t = 0$ to the end of the data sampling period shown. Some slices (hollow symbols, $n = 6$) were perfused with aCSF containing 2 mM H_2O_2 from $t = 15$, while other control slices (solid symbols, $n = 5$) were not exposed to H_2O_2 . Values are mean \pm SEM of independent experiments. Reduction in IOS signal expresses the change in IOS at each time point normalized to the maximal decrease in volume during mannitol exposure. Regression lines were calculated from the mean values of the relative intensity values between $t = 15$ min and 30 min but are shown over longer time periods for clarity. The slopes of these regression lines are not significantly different for slices with or without H_2O_2 exposure.

Figure 8

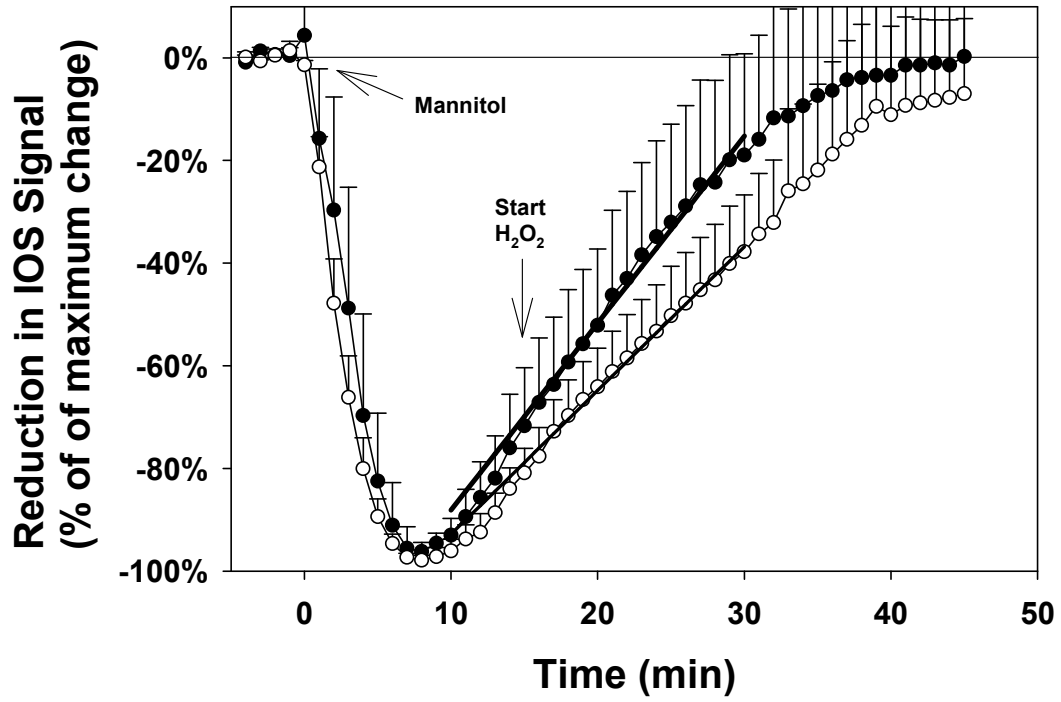
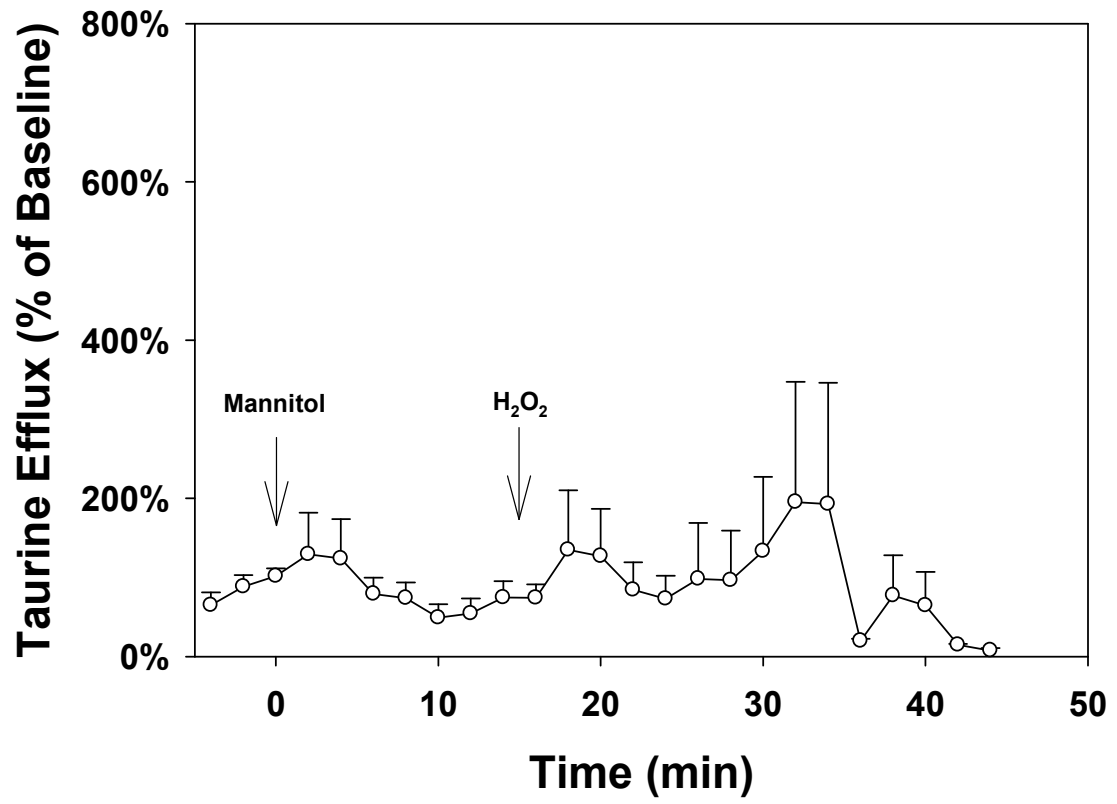


Figure 9

Taurine efflux from hippocampal slices in hypertonic media during oxidative stress

Hippocampal slices were prepared with 1 mM taurine to maintain normal taurine contents. Slices ($n = 5$) were then perfused with aCSF made hypertonic (350 mOsm) by adding mannitol beginning at $t = 0$, and then perfused with this same solution plus 2 mM H_2O_2 from $t = 15$ min until the end of the data sampling period shown. There are no statistically significant changes in taurine efflux observed either after the mannitol treatment, or after exposure to H_2O_2 .

Figure 9



AMPA receptors and NMDA receptors are coupled to hippocampal swelling during oxidative stress in taurine depleted slices

It has been established that activation of NMDA receptors causes brain tissue edema *in vitro* [Brahma et al, 2000]. As a positive control, we exposed our hippocampal tissue slices to NMDA (100 μ M) and observed a significant increase in IOS ($+11.78 \pm 1.95\%$, $n = 6$) within 5 minutes of NMDA exposure and then a gradual trend toward baseline [Figure 10].

Next, hippocampal slices were exposed to an inhibitor of either NMDA-receptors (10 μ M MK-801) or AMPA-receptors (25 μ M CNQX) [Macgregor et al. 2003] during H_2O_2 exposure. In taurine depleted slices, a significant change in IOS was observed after 25 minutes when 2 mM H_2O_2 was added to the perfusate in the presence of CNQX ($+1.19 \pm 0.39\%$, $n = 6$) [Figure 11]. By multiple linear regression we calculated a rate of change in IOS of $0.137 (\pm 0.019)$ percent/min in taurine depleted slices without CNQX treatment and a rate of $0.031 (\pm 0.010)$ percent/min in taurine depleted slices treated with CNQX during H_2O_2 exposure. The difference in these rates (-0.105 ± 0.015 percent/min) was statistically significant.

Taurine depleted slices treated with MK-801 during to H_2O_2 exposure also showed a significant increase in IOS of $1.93 \pm 0.26\%$ after 25 min ($n = 6$) [Figure 11]. Multiple linear regression revealed a rate of change in IOS of $0.085 (\pm 0.0101)$ percent/min during H_2O_2 exposure in the presence of MK-801. This rate of change in IOS was smaller than that determined for control slices by $-0.051 (\pm 0.015)$ percent/min,

Figure 10

Volume change in hippocampal slices exposed to NMDA. Taurine depleted hippocampal slices were perfused in aCSF for 45 min prior to exposure to NMDA (100 μ M) t = 0 min. NMDA induces significant swelling ($11.78 \pm 1.95\%$, n = 6) within the first 5 min of exposure followed by a gradual decrease toward the initial baseline. * indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to NMDA treatment.

Figure 10

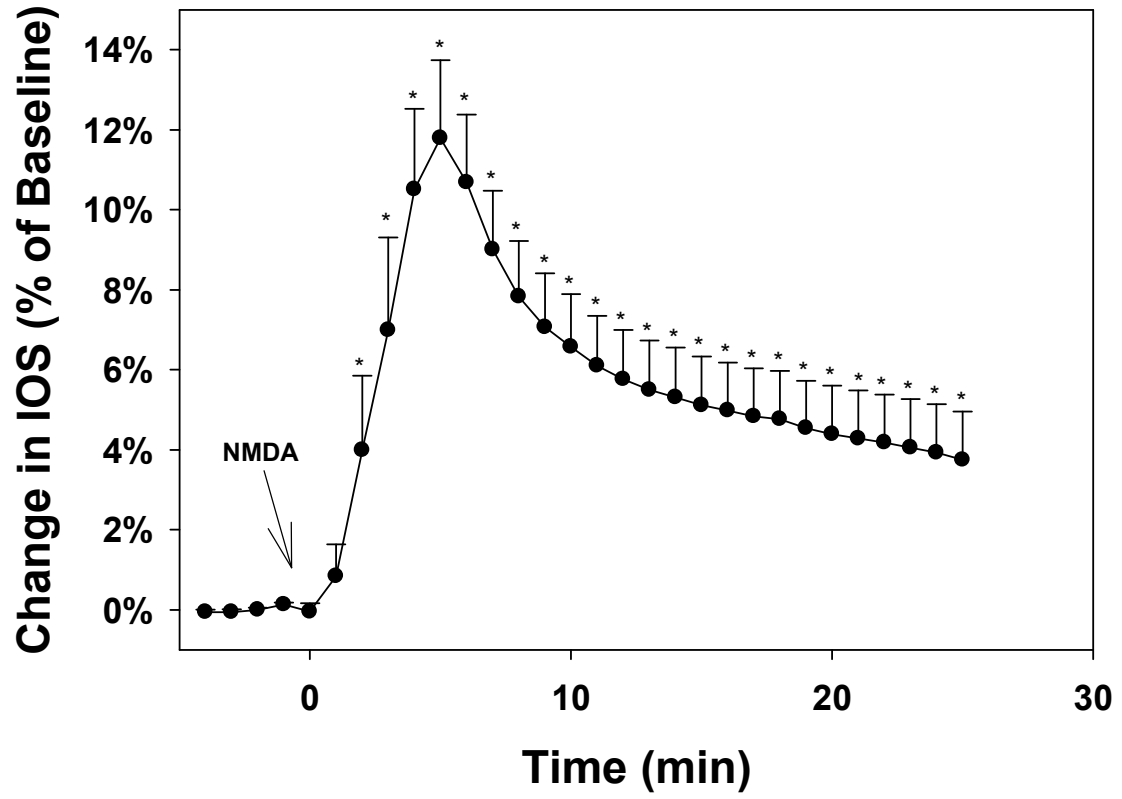


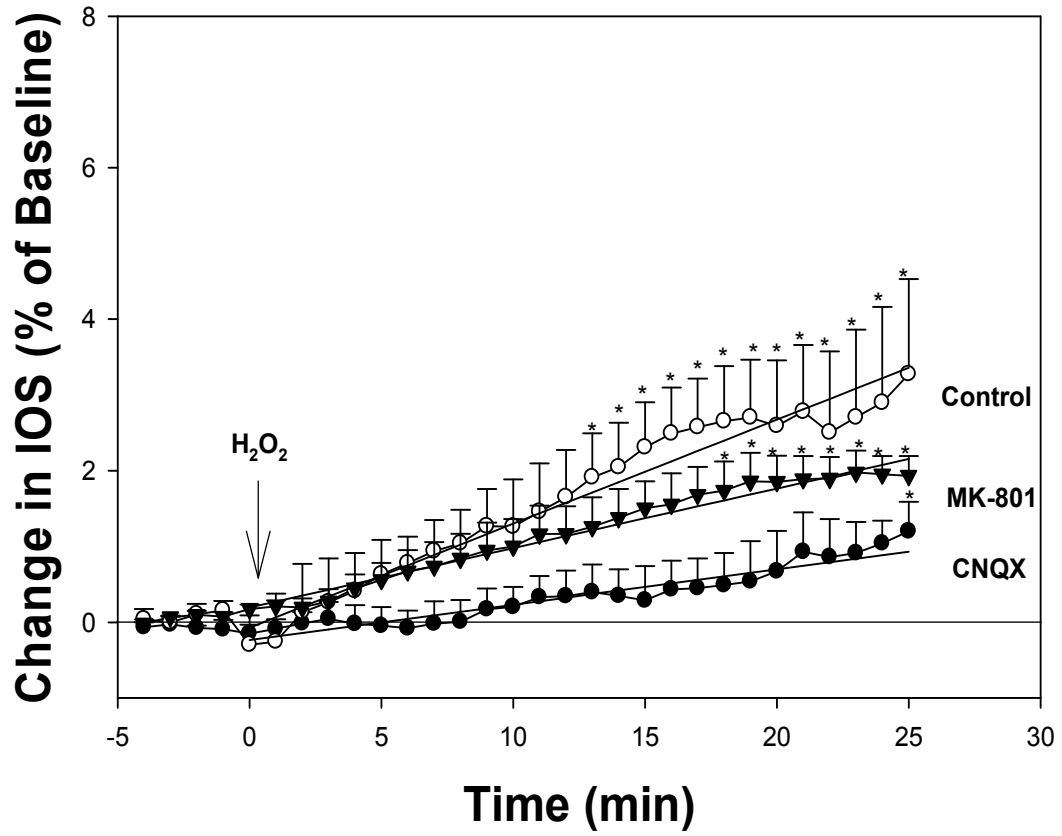
Figure 11

Glutamate receptors and tissue swelling in hippocampal slices during oxidative stress.

Taurine depleted hippocampal slices were treated with CNQX (25 μ M, n = 6) or MK-801 (10 μ M, n = 6) beginning at t = -15 min. Control slices received no drug treatment. Slices then were perfused with aCSF containing the same drug plus 2 mM H₂O₂ from t = 0 min to the end of the data sampling period shown. Values are the mean \pm SEM. The intrinsic optical signal (IOS) was calculated for areas of the stratum radiatum in the CA1 region of the hippocampus. Control slices (n = 4) demonstrated significant changes in IOS throughout the period of H₂O₂ exposure.

Regression lines were calculated from the mean values of the relative intensity values between 0 min and 25 min. * indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to H₂O₂ treatment.

Figure 11



indicating a statistically significant difference compared with control slices. These data are summarized in Figure 12.

Glutamate signaling is coupled to volume regulation during oxidative stress

To examine the role glutamate signaling plays for oxidative stress-induced swelling and volume regulation, taurine repleted hippocampal slices were treated with either an NMDA receptor blocker (10 μ M MK-801) or an AMPA receptor blocker (25 μ M CNQX) prior to and during exposure to 2 mM H₂O₂. In these studies, both volume and taurine efflux were measured throughout the experiment. Similar to what was observed during inhibition of VRAC with DCPIB, during exposure to CNQX or MK-801 hippocampal slices demonstrated an initial increase efflux of taurine [Figure 13 & Figure 14]. However, this response was variable with some slices in each treatment group exhibiting a large amount of taurine efflux after drug exposure while others exhibited almost no change in taurine efflux. After 15 min of continued drug exposure, taurine efflux levels recovered on average to a constant baseline level which was not statistically different than the rate measured prior to drug exposure. Slices were then treated with H₂O₂ in the presence of the glutamate receptor blocker. Changes in IOS were determined for 25 min of H₂O₂ exposure and rates of taurine efflux are reported after 10 min of H₂O₂ exposure as this typically represents time of maximal efflux. In addition, by reporting mean taurine efflux values 10 min after H₂O₂ exposure we present the more immediate effect of H₂O₂ on taurine efflux in the slices. As indicated in results of specific aim I, taurine repleted slices had an increase in IOS of $1.08 \pm 0.61\%$ after 25 min and an increase in taurine efflux of $+335.88 \pm 178.12\%$ after 10 min of H₂O₂ exposure [Figures

Figure 12

Summary of treatment results for glutamate receptor agonists and antagonists: Taurine depleted control hippocampal slices without drug treatments increased IOS by over 3% after 25 min of H₂O₂ exposure. NMDA treatment caused an increase in IOS to a maximum of nearly 12%. In the presence of the NMDA-R antagonists MK-801 or CNQX, the H₂O₂-induced increase in IOS was less than that observed in control slices. Values are the mean ± SEM for individual experimnts. ** indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to H₂O₂ treatment.

Figure 12

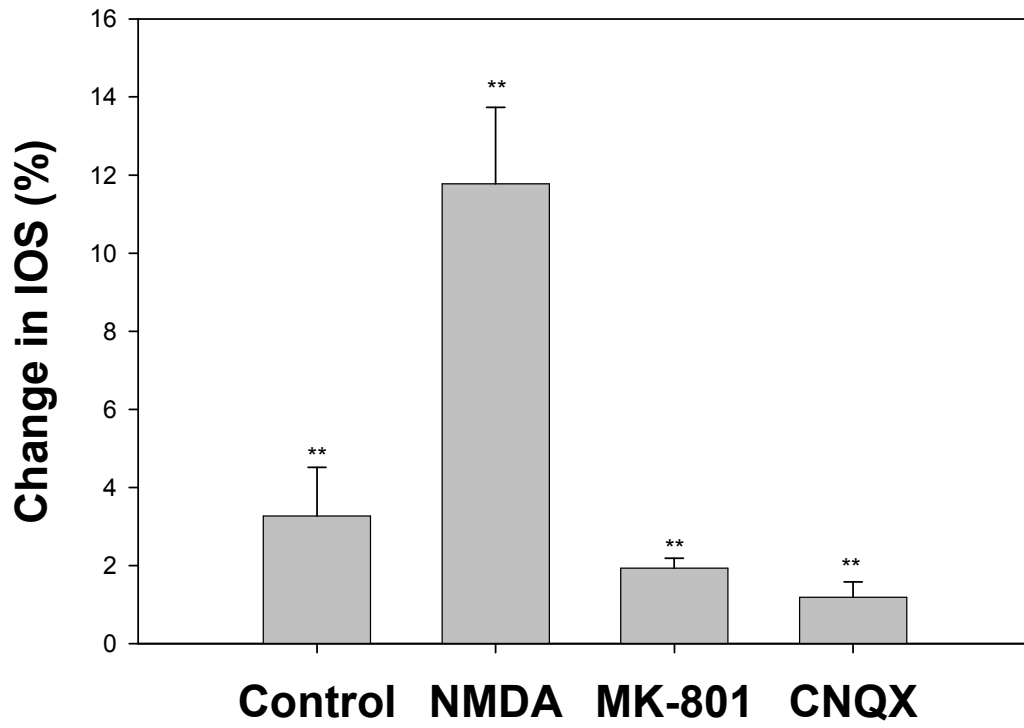


Figure 13

Transient taurine efflux from hippocampal slices during exposure to AMPA antagonist.

Hippocampal slices were prepared to maintain normal taurine contents prior to CNQX (25 μ M, n = 4) exposure at t = 0. Values are the mean \pm SEM for 4 independent experiments. Taurine concentrations were measured in the effluent aCSF perfusate. The taurine concentration in the aCSF perfusate increased significantly to a maximum within 5 min CNQX exposure, but gradually returned to a stable value within 15 min. This value was not significantly greater than the baseline prior to H₂O₂ exposure. * indicates values which are significantly different from the baseline measurements determined prior to CNQX treatment.

Figure 13

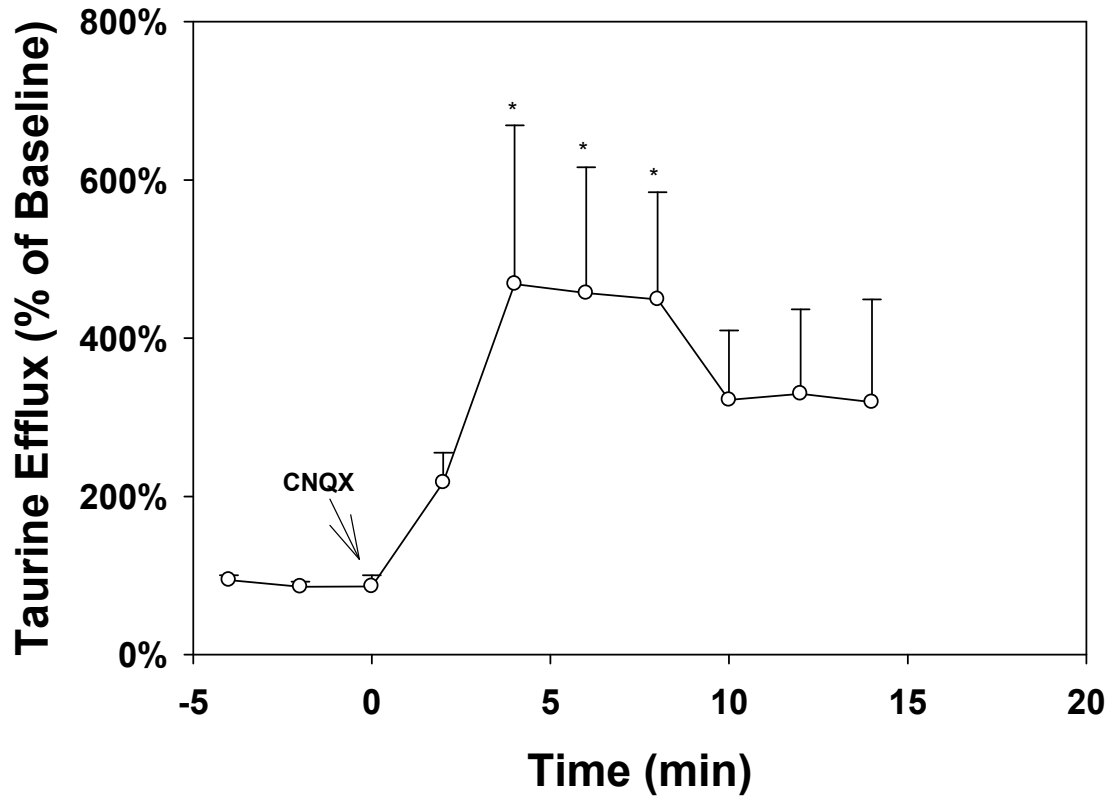
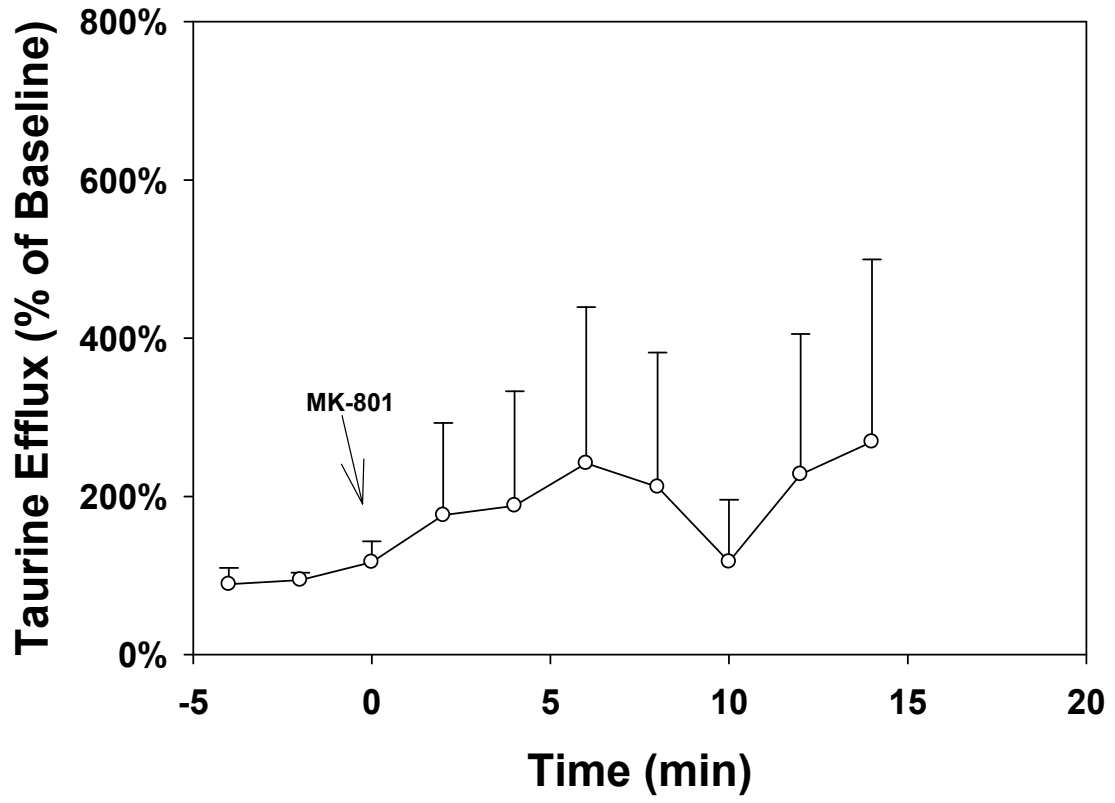


Figure 14

Transient taurine efflux from hippocampal slices during NMDA antagonist. Hippocampal slices were prepared with 1 mM taurine to maintain normal taurine contents prior to MK-801 (10 μ M, n = 4) exposure at t = 0. Values are the mean \pm SEM for 4 independent studies. Taurine concentrations were measured in the effluent of the aCSF perfusate. The taurine concentration in the aCSF perfusate showed upward trend peaking at an increase of 168% \pm 230.5% above baseline, but this increase did not reach statistically significant change during the 15 min of MK-801 exposure.

Figure14



3 & 4, Table 2]. Taurine repleted slices exposed to H₂O₂ in the presence of MK-801 exhibited a significantly greater increase in IOS ($+5.01 \pm 2.36\%$, $n = 5$), indicating a larger degree of swelling compared with control slices [Figure 15]. By multiple linear regression the rate of change in IOS of $0.064 (\pm 0.012)$ percent/min in taurine repleted control slices increased to $0.213 (\pm 0.023)$ percent/min in taurine repleted slices exposed to MK-801. The difference in these rates of change in IOS (0.149 ± 0.027 percent/min) was statistically significant. MK-801-treated slices also exhibited a net decrease in taurine efflux during H₂O₂ exposure ($43.31 \pm 13.43\%$, $n = 4$) [Figure 16, Table 2].

When taurine repleted slices were exposed to H₂O₂ in the presence of CNQX, they also demonstrated a significant increase in IOS after 25 min ($2.54 \pm 0.78\%$, $n = 5$) [Figure 15]. By multiple linear regression, the rate of change in IOS was $0.115 (\pm 0.017)$ percent/min in taurine repleted slices treated with CNQX during H₂O₂ exposure. The difference in rate compared with that of control slices was $0.051 (\pm 0.020)$ percent/min and was statistically significant. Further, slices treated with CNQX exhibited decreased taurine efflux rate compared with controls, ($-58.06 \pm 16.23\%$, $n = 4$) [Figure 16, Table 2]. In contrast to the significant variability in taurine efflux observed in slices as a result of the drug exposures, all slices responded similarly in regards to taurine efflux after exposure to H₂O₂ regardless of the initial drug-induced efflux.

Table 2

Summary of drug effects on tissue swelling and taurine efflux from the hippocampus during oxidative stress. All experiments were performed using taurine repleted hippocampal slices . Changes in IOS measured in the stratum radiatum of the CA1 region and the relative rate of taurine efflux were determined after 25 min and 10 min of H₂O₂ exposure, respectively. These represent time points which resulted in peak responses observed in control slices with no drug treatment. Values are the mean \pm SEM. * Indicates values which are significantly different from baseline measurements determined prior to H₂O₂ treatment. ++ indicates a statistically significant difference between the change in IOS for slices receiving a drug treatment compared with that measured in control slices.

Table 2

	Change in IOS following 25 min of H ₂ O ₂ exposure in hippocampal slices	Change in taurine efflux following 10 min of H ₂ O ₂ exposure in hippocampal slices
Control	1.08 ± 0.61% (n = 12)	335.88 ± 178.12%* (n = 3)
CNQX	2.54 ± 0.78%* ⁺⁺⁺ (n = 5)	-58.06 ± 16.23%* (n = 4)
MK-801	5.01 ± 2.36%* ⁺⁺⁺ (n = 5)	-43.31 ± 13.43% (n = 4)
DCPIB	3.05 ± 1.12%* ⁺⁺⁺ (n = 4)	-59.8 ± 7.0%* (n = 4)

Figure 15

The role of AMPA and NMDA receptors in volume changes during oxidative stress.

Taurine repleted hippocampal slices were treated with CNQX (25 μM) or MK-801 (10 μM) beginning at $t = -15$ min. Control slices received no drug treatment. All slices were perfused with aCSF containing 2 mM H_2O_2 from $t = 0$ min to the end of the data sampling period shown.

Values are the mean \pm SEM independent measurements. IOS was calculated for areas of the stratum radiatum in the CA1 region of the hippocampus. Slices treated with CNQX (triangle dark symbols, $n = 5$) or MK-801 (round dark symbols, $n = 5$) showed a significantly greater increase in IOS during exposure to H_2O_2 , compared with control slices (hollow symbols, $n = 12$). Regression lines were calculated from the relative intensity values between 0 min and 25 min. * indicates values which are significantly different from the average of five baseline measurements determined at 1 min intervals prior to H_2O_2 treatment.

Figure 15

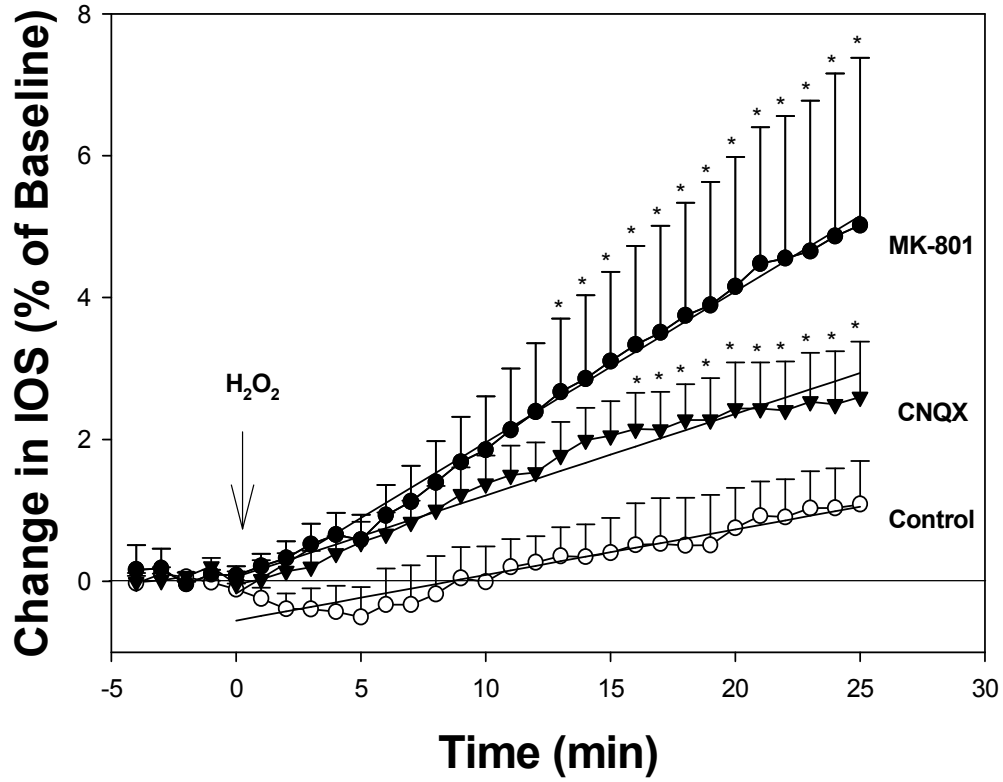
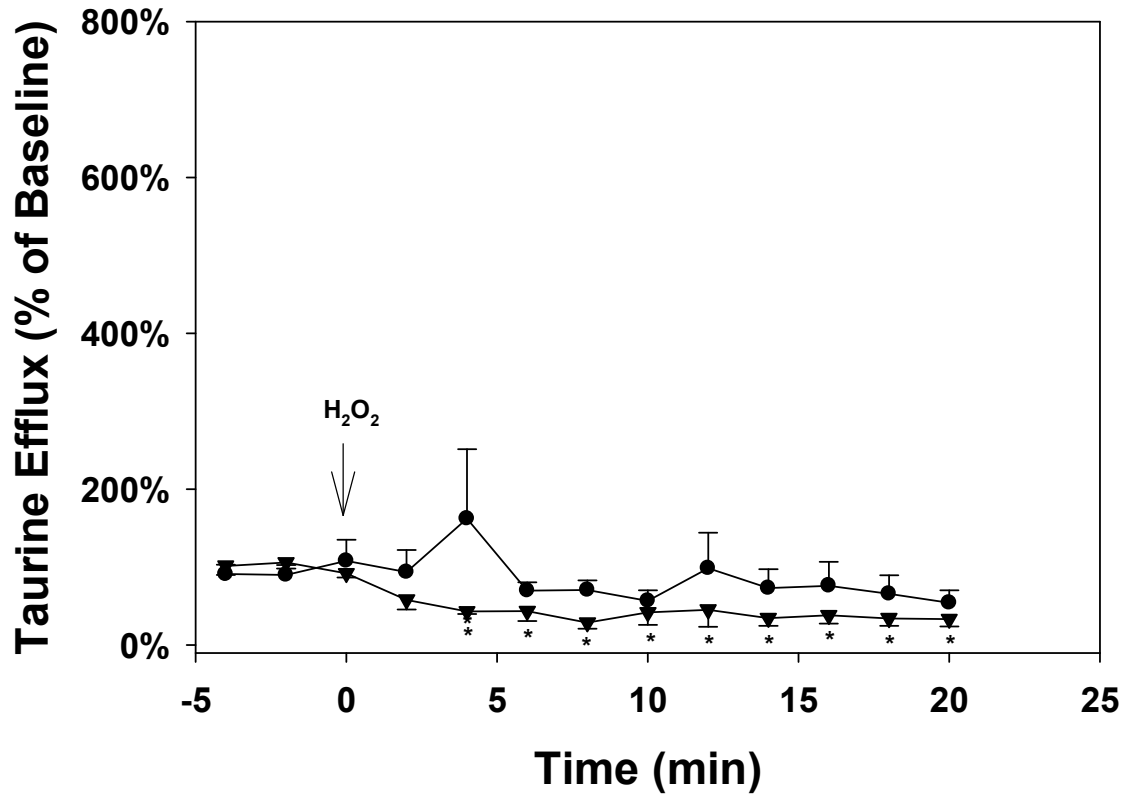


Figure 16

NMDA and AMPA receptors in taurine efflux during oxidative stress. Taurine repleted hippocampal slices were perfused with MK-801 (10 μ M) and CNQX (25 μ M) starting at t = -15 min. Slices then were perfused with aCSF containing 2 mM H₂O₂ from t = 0 min to the end of the data sampling period shown. Values are the mean \pm SEM of four independent experiments. Taurine concentrations were measured in the effluent of the aCSF perfusate and are expressed relative to baseline measurements determined prior to H₂O₂ exposure. The taurine concentration in the aCSF perfusate in CNQX samples (triangle dark symbols, n = 4) decreased significantly at the start of H₂O₂ exposure, and remained significantly decreased throughout the sampling period. The taurine concentration in the aCSF perfusate with MK-801 (solid round symbols, n = 4) showed a gradual downward trend during H₂O₂ exposure, but this change was not statistically significantly from the concentration measured prior to H₂O₂ exposure. * indicates values which are significantly different from baseline measurements determined prior to H₂O₂ treatment.

Figure 16



CHAPTER VI
DISCUSSION

This study illuminates several mechanisms involved in swelling and volume regulation in the hippocampus exposed to oxidative stress. We first demonstrate that taurine is necessary for volume regulation during oxidative stress and that the taurine efflux is regulated via VRAC to control swelling. We demonstrate that increased taurine efflux during oxidative stress is due to tissue swelling, rather than direct activation of VRAC channels by H₂O₂. Finally, the results of our experiments indicate that activation of glutamate receptors is coupled to hippocampal swelling, but also plays a significant role in volume regulation during oxidative stress [Tucker et al. 2010]. This discussion will elucidate the results of this study and provide a better understanding of the relationships between the processes of oxidative stress, cell swelling, glutamate signaling and volume regulation.

1. Taurine is necessary for brain slice volume regulation during oxidative stress

We examined the role of taurine in swelling and volume regulation during oxidative stress by exposing hippocampal slice preparations to H₂O₂. Taurine's role in cell volume regulation is well established in the CNS. Cell studies have indicated that taurine plays a significant role in volume regulation in both astrocytes and neurons [Kimelberg et al. 1990; Vitarella et al. 1994; Pasantes-Morales et al. 1994]. In brain tissue, *in situ* and *in vitro* studies indicate that taurine is the most important osmotically active organic molecule involved in volume regulation during both hypoosmotic hyponatremia, and hyperosmotic dehydration [Verbalis et al. 1991; Bedford et al. 1993]. Additional studies have shown that exposure of brain tissue to hypoosmotic conditions causes a reduction in

brain taurine content [Kimelberg et al. 1995], and that taurine efflux is enhanced in both cerebral cortical slices and hippocampal tissue slices exposed to hypoosmotic conditions [Franco et al. 2000; Law et al. 1994]. Thus, our present understanding of osmotically-induced swelling and volume regulation via loss of taurine is well documented. However, what is less established is whether the mechanisms behind oxidatively induced swelling and volume regulation under isoosmotic conditions are the same as those in hypoosmotically induced swelling and whether taurine plays the same role in enhancing volume regulation during oxidative stress as it does in other causes of tissue swelling.

The results of our studies indicate that when hippocampal tissue taurine content is diminished, there is a reduced or absent volume regulatory response to swelling induced by oxidative stress. Further, our results indicate that restoration of physiological levels of taurine enhances volume regulation in response to oxidatively induced swelling. These results are consistent with studies by Kreisman et al. [2003] who observed that restoration of hippocampal tissue taurine to physiological levels enhanced RVD in response to hypoosmotic swelling. With our present data we confirm that, similar to slices exposed to hypoosmotic conditions, the release of taurine from intracellular stores is critical for isoosmotic volume regulation of the hippocampus exposed to oxidative stress.

2. Taurine efflux during oxidative stress is mediated by VRAC

It is well established that hypoosmotically induced taurine release is mediated via VRAC [Jackson et al. 1996; Okada et al. 2001]. Studies show VRAC-mediated taurine

release in cultured hippocampal astrocytes, hippocampal neurons, hippocampal slices, and in rat cortex [Li et al. 2008; Li et al. 2004; Kriesman et al. 2003; Stutzin et al. 2006]. While numerous cell and tissue studies demonstrate VRAC-mediated taurine efflux in the hypoosmotic swelling model, there are limited studies regarding the role of VRAC in volume control during oxidatively induced swelling [Haskew-Layton et al. 2005]. Some of the evidence that taurine efflux is mediated via VRAC largely stems from cultured neuronal and glial cells, which show that swelling-activated taurine fluxes are inhibited by a variety of VRAC blockers [Stutzin et al. 2006]. In our study we inhibited oxidative stress-induced taurine efflux by the VRAC antagonist DCPIB. DCPIB is a selective VRAC blocker found to block VRAC currents in several cell lines, but not endogenous or heterologously expressed CIC-1, CIC-2, CIC-4, CIC-5, CFTR, or calcium-activated Cl^- channels [Decher et al. 2001]. In our experiments, tissue swelling was observed during oxidative stress in the presence of DCPIB, indicating that VRAC activity is necessary for the volume regulatory response during oxidative stress. These results also indicate VRAC-mediated taurine efflux is as significant for attenuating tissue swelling induced by oxidative stress as it is during hypoosmotic exposure.

In this work we also address whether the increase in taurine release observed during oxidative stress is due to cell swelling or by direct activation of VRAC by H_2O_2 . Studies have shown that ROS may play a significant role as an intracellular messenger regulating RVD [Lambert et al. 2003]. Others also have established that H_2O_2 potentiated the swelling-induced taurine efflux in hypoosmotic medium [Stutzin et al. 2006]. Further, a recent study found that VRAC may be activated or positively modulated by ROS, particularly H_2O_2 , in microglial cells [Harrigan et al. 2008; Varela et al. 2004]. In order to

establish whether ROS activates VRAC independently of swelling, the tissue slices in these experiments were exposed to a hypertonic treatment of mannitol prior to, and throughout, H₂O₂ exposure. With this experimental model, we were able to maintain hippocampal tissue in a non-swollen status as we monitored taurine efflux after H₂O₂ exposure. Our results show that taurine efflux was not increased during oxidative stress after pre-treatment with hyperosmolar aCSF. This suggests, in this non-swollen state, H₂O₂ is not acting directly on VRAC to induce taurine efflux. These results appear to contradict the studies of Lambert et al. [2002] and Stutzin et al. [2006] which both suggest a direct activation of taurine release via H₂O₂. While these studies observed significant taurine release after H₂O₂ exposure, neither study accounted for H₂O₂-induced swelling, which also would be expected to lead to taurine release. In this respect these studies are limited because cell volume was not measured, and thus, it is not clear whether taurine efflux was induced directly by H₂O₂ exposure, or indirectly by cell swelling. By monitoring and maintaining the tissue in an osmotically ‘non-swollen’ state during H₂O₂ exposure, our experiment removes the swelling component in the system. Our model provides a more accurate measure of the effect of H₂O₂ in these tissue slices in the absence of swelling.

During hyperosmotic treatment, H₂O₂ did not cause additional swelling. Figure 8 shows a slight deflection in the recovery line shortly after the application of H₂O₂ leading to a slower recovery to baseline compared to controls. This may indicate that oxidative stress has an effect on the RVI process. Currently, there is limited literature indicating that cell shrinkage inhibits taurine release, or that cell shrinkage alters the activation of VRAC. Tissues which have been osmotically shrunk prior to H₂O₂ exposure, as we did in

our experiments, may have activated regulatory volume increase (RVI) mechanisms which could potentially affect taurine release regardless of H₂O₂ exposure. This represents a limitation in our use of mannitol in studying the effects of H₂O₂ in the ‘non-swollen’ environment. It is not unreasonable to propose that taurine release is inactivated as a counter-regulatory mechanism when cells are osmotically shrunken and undergoing RVI. There is an absence of studies that have examined the effects of H₂O₂ exposure in volume regulation in a model that simultaneously limits swelling. Future experimentation is necessary in both cell culture and tissues to isolate the direct effects of H₂O₂ in the absence of swelling.

3. AMPA and NMDA receptors are coupled to hippocampal swelling during oxidative stress

Glutamate receptor inhibitors have been shown to significantly diminish swelling in hippocampal tissue slices exposed to oxidative stress [MacGregor et al. 2003]; however, the individual contributions of NMDA and AMPA during H₂O₂ have not been elucidated. Further, glutamate-receptor activation has been shown to cause edema in brain slices *in vitro* [Brahma et al. 2000] and to contribute to the initial cell swelling observed during anoxic depolarization in slices [Werth et al. 1998; MacGregor et al. 2003]. We also have witnessed hippocampal tissue swelling after treatment with NMDA (100 μM), presumably mediated by glutamate receptors. The mechanisms by which glutamate may contribute to brain edema are many, from the initial Na⁺ entry and consequent swelling that accompanies ionotropic glutamate-receptor activation, [Werth et

al. 1998] to the activation of a number of secondary processes, including mitochondrial dysfunction, Ca²⁺ loading, and ROS production [Macgregor et al. 2003; Prehn et al. 1998; Nichols et al. 2000].

Our data suggest that both AMPA, and to a lesser extent NMDA sub-types of ionotropic glutamate receptors contribute to hippocampal swelling induced by oxidative stress in taurine-depleted slices. Consistent with data from MacGregor et al. 2003, work on the role of glutamate receptors in *in vitro* ischemia models, our results indicate a significant role of glutamate-receptor activation in the formation of brain tissue edema during oxidative stress. The greater swelling effect of the AMPA receptor may be explained by the effect of AMPA on membrane potential and thus, its interaction with the NMDA pathway. Because NMDA activation requires the simultaneous presence of both agonist and membrane depolarization, any inhibition of AMPA-stimulated depolarization will have secondary effects on NMDA receptor activation. In this regard, blocking an AMPA receptor has a two-fold effect on tissue swelling, blocking both AMPA itself and secondarily inhibiting NMDA via inhibition of membrane depolarization. MacGregor et al. [2003] showed that a cocktail of NMDA antagonist, AMPA antagonist, mitochondrial permeability antagonist, and antioxidant was more effective in attenuating edema than any single treatment alone. Their study indicated a significant, but not exclusive role for glutamate in ischemia-induced brain tissue swelling [Macgregor et al. 2003]. Our data further indicates a more prominent role of AMPA in swelling during oxidative stress.

4. NMDA-receptor signaling is coupled to volume regulation during oxidative stress

Ionotropic glutamate receptor agonists can effectively enhance taurine release. The effects of these drugs have been shown to be glutamate receptor-mediated in adult mouse hippocampal slices under isoosmotic conditions [Saransaari et al. 1997]. Further, works by Morales et al. [2007] established that glutamate receptors are involved in stimulating taurine efflux from the substantia nigra exposed to hypoosmotic conditions. To date, however, there are limited studies that address the specific role of glutamate receptors on taurine efflux in tissue swollen by oxidative stress. To effectively study volume regulation of normal hippocampal tissue, taurine concentrations were restored to physiological levels prior to H₂O₂ exposure [Kreisman et al. 2003].

Our results indicate that when taurine-repleted slices were exposed to H₂O₂ in the presence of the NMDA antagonist MK-801, significant swelling occurred. In these same slices the rate of taurine efflux was not altered by oxidative stress. Since taurine efflux was not increased during the period of oxidative stress, we conclude that activation of NMDA receptors is necessary for effective volume regulation via organic osmolyte efflux in the presence of H₂O₂. Recent studies indicate that synaptic NMDA receptor activity boosts intrinsic anti-oxidant defenses in CNS [Papadia et al. 2008]. In the presence of NMDA receptor antagonist during oxidative stress, CNS tissue has a diminished capacity to defend itself against the deleterious effects precipitated by reactive oxygen species. Thus, the role of NMDA receptor activation in swelling and volume regulation during oxidative stress may be multi-faceted. First, our data suggest

that NMDA receptor activation is necessary for proper volume regulation and taurine efflux during H₂O₂-induced swelling. Second, activation of NMDA receptors enhances anti-oxidant defenses, which could provide neuro-protection inhibiting swelling caused by of H₂O₂-induced injury.

5. AMPA-receptor signaling is coupled to volume regulation during oxidative stress

Because swelling induced by oxidative stress was markedly diminished by CNQX in taurine-depleted slices, the role that AMPA receptors may play for volume regulation resulting from increased taurine efflux cannot be evaluated definitively. However, when taurine-treated slices were exposed to H₂O₂ in the presence of CNQX, significant swelling was again observed compared to control slices, and the rate of taurine efflux was decreased. The decrease of taurine efflux upon H₂O₂ exposure in the presence of CNQX suggests that AMPA receptors are also necessary for effective volume regulation mediated by loss of intracellular taurine.

The decrease in H₂O₂-induced taurine release in the presence of CNQX is consistent with previous studies that reported an attenuated taurine response to hypoosmotic swelling in tissues treated with an AMPA-kainate glutamate receptor antagonist [Saransaari et al. 1997]. This implies that glutamatergic mechanisms involved in volume regulation are similar during osmotically and oxidatively-induced swelling. We propose that oxidatively-induced swelling facilitates localized glutamate release, which, acting on ionotropic glutamate receptors, increases the taurine osmo-regulatory

response. This mechanism is similar to a proposal by Morales et al. [2007], for hypoosmotically induced taurine release in the substantia nigra.

6. Glutamate and purinergic receptor-mediated volume regulation – a possible mechanism for glutamate effects on cell volume during oxidative stress

Our results indicate that glutamate receptor activation is involved in both tissue swelling and volume regulation in the hippocampus during oxidative stress. A mechanism linking glutamate signaling to cell volume regulation has been proposed by Uckermann et al. 2006. In a study of osmotic glial cell swelling in rat retinal cells, Uckermann et al. 2006 observed that glutamate had an inhibitory effect on osmotic glial cell swelling, and that the mechanism involved a purinergic signaling pathway. They proposed a neuron-to-glia glutamate signaling cascade that inhibited osmotic glial cell swelling in post-ischemic retinal cells exposed to hypoosmotic conditions. The study showed that neuronal glutamate activated mGluRs on glial cells, causing a release of ATP and a signaling cascade resulting in the efflux of K^+ and Cl^- . The observed release of ions was accompanied by water efflux, resulting in regulatory volume decrease. Although this study does not indicate which specific cells were swelling and which were releasing taurine, our results suggest that glutamate-mediated volume regulation may occur in the hippocampus. This may involve a neuron-to-glia signaling pathway in tissue swelling induced by oxidative stress in a mechanism similar to that proposed in Uckermann et al. 2006.

ATP has been proposed as an autocrine signaling molecule in volume regulatory mechanisms in many cell types [Wang et al. 1996; Darby et al. 2003]. ATP is released from a variety of cells upon hypoosmotically induced swelling [Mitchell et al. 2001; Sabirov et al. 2001; Phillis et al. 2002] with an increase in concentration large enough to activate purinergic receptors [North et al. 2000]. Purinergic receptors have been coupled to activation of anion channels in many cell types, including hippocampal neurons and in astrocytes [Li et al. 2004; Mongin et al. 2002]. Extracellular ATP has also been found to activate chloride and taurine conductances in cultured astrocytes [Li et al. 2004]. Finally, purinergic receptor activation has been observed to activate anion channels (VRAC) established in this and previous studies to allow release of taurine during swelling induced by oxidative stress. With this increasing insight into the role of ATP in cell volume regulation, future works are needed to broaden our understanding in this area.

CHAPTER VII
LIMITATIONS AND
FUTURE WORKS

There were several limitations within this research project. The first limitation was the use of H_2O_2 as our source for ROS in the induction of oxidative stress. H_2O_2 exposure was chosen because it commonly increases during oxidative stress *in vivo*, and is commonly used for induction of experimental oxidative stress *in vitro* [Pellmar et al. 1991]. H_2O_2 is both the product of the catalyzation of reactive superoxide radicals by SOD and is, itself, easily converted to the highly reactive and destructive hydroxyl radical. However, H_2O_2 represents only one of many classes of ROS that can elicit oxidative stress and has also been shown to act as a signaling molecule. Another limitation of the study was the use of mannitol in some experiments to ensure the tissue remains in a 'non-swollen' state during oxidative stress. The significant decrease in tissue volume induced by this treatment may induce RVI mechanisms. Finally, the unexpected observation of increased taurine efflux elicited by the application of drugs is another limitation. This observed increase in taurine efflux was over a range of drug classes that includes glutamate receptor antagonists and chloride channel antagonists and different vehicles used to prepare drug stock solutions. In all cases, the rate of taurine efflux returned to a baseline not significantly larger than that prior to drug exposure. However, because of the neuro-inhibitory properties of taurine, this increased efflux may alter the subsequent response to H_2O_2 observed in our experiments.

Future works may define more specifically the mechanisms mediating glutamate receptor activated tissue swelling and volume regulation. Our experiments focused exclusively on the ionotropic glutamate receptors AMPA and NMDA and did not examine the possible role of metabotropic glutamate receptors. Uckermann et al. [2006] reported that glutaminergic metabotropic receptor activation may mediate cell volume

regulation. In addition, while we did not differentiate neuron and glial responses to oxidative stress the volume regulatory mechanism may differ and warrant examination of cell-specific responses. These studies also are limited by the use of IOS as an index of the volume changes rather than directly measuring tissue volume. Although IOS is an established method of measuring tissue volume, future works could also include optical assessments of cell volume or wet weight/dry weight analysis of water content [Brahma et al. 2000].

CHAPTER VIII
CONCLUSION

Our results indicate that the release of taurine from intracellular stores is critical for isoosmotic volume regulation of the hippocampus exposed to oxidative stress. Further, taurine is lost from hippocampal cells during oxidative stress via the volume activated anion channel (VRAC). Glutamate AMPA receptors, and to a lesser extent NMDA receptors are coupled to hippocampal swelling during oxidative stress. Further, volume regulation of the hippocampus also is regulated, in part, by glutamate signalling via NMDA and AMPA receptors. Establishing the importance of taurine mobilization during swelling induced by oxidative stress and defining a significant role for glutamate receptors in swelling and volume regulation is an important step in understanding the cellular response to cytotoxic brain edema. Future work is needed to further our understanding of the response and adaptation of brain cells to edema leading to improved treatments and recovery from these devastating pathological conditions.

CHAPTER VIII
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