2010

SOMATIC INJURY PRECEDES DISTAL ATROPHY FOLLOWING EXCITOTOXIC HIPPOCAMPAL INSULT

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Abstract of Thesis

Excitotoxicity can lead to increases in intracellular Na^{+} and Ca^{2+} concentrations via the glutamatergic NMDA receptors, which can lead to cell death. Detailing the time-dependent degradation of neuronal components in response to excitotoxic challenge may help elucidate the sequence in which these signaling pathways are initiated and further, associate these pathways with topographic cellular demise. Using organotypic hippocampal slice culture technique, tissue from neonatal rat pups was exposed to NMDA, APV, or co-exposed for 24, 72 or 120 hours. Fluorescent microscopy of propidium iodide (PI) was used to evaluate neuronal membrane damage, changes in the density of mature soma (NeuN) and NMDA NR2B subunits were measured using immunohistochemical procedures. After 24 hours of exposure, the CA1 showed an increased PI signal and a decrease in NeuN marker, indicating somatic injury occurs shortly after excitotoxic challenge; these effects were blocked by co-administration of APV. Also in the CA1, loss of NR2B subunits, heavily expressed in dendritic processes, declined following 72 hours of exposure. Because somatic injury precedes loss of distal NR2B subunits, it is possible that these events involve different mechanisms, findings that may be relevant in the development of therapies to target neurodegeneration resulting from excitotoxicity.

Keywords: NMDA, Excitotoxicity, Organotypics, Hippocampus, EtOH

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The Graduate School

University of Kentucky

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SOMATIC INJURY PRECEDES DISTAL ATROPHY FOLLOWING EXCITOTOXIC HIPPOCAMPAL INSULT

THESIS

A thesis submitted in partial fulfillment of the
Requirements for the degree of Master of Science in the
Experimental Psychology Department
at the University of Kentucky

By

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2010

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Chapter One: Introduction

Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter found in the mammalian central nervous system (CNS). The activation of glutamatergic receptors is a crucial component in the proliferation, maintenance, and survival of neurons (Cooper et. al., 2003; Hardingham & Bading, 2003). However, when neurons are chronically exposed to glutamate or exposed to excessive concentrations of the neurotransmitter, they can become over stimulated, a condition defined by Olney as excitotoxicity (Choi, 1992; Olney, 1986). Conditions suspected to involve glutamate induced excitotoxicity include alcohol withdrawal (Prendergast et al., 2004), hypoxia-ischemia (Hertz 2008), epilepsy (Olney et al, 1998), traumatic brain injury (TBI) (Hardingham & Bading, 2003) and neurodegenerative diseases including Alzheimer's (Albensi et al, 2004; Dong et al, 2009). Excitotoxicity can lead to the swelling of the cell, loss of cellular components via activation of multiple signaling pathways including proteases, lipases, and the disruption of mitochondrial membrane potential, all of which can culminate in cell death (Albensi, 2007; Choi et. al., 1987; Forder, 2009; Hardingham & Bading, 2003). The following research sought to detail the time-dependent degradation of neuronal components in response to excitotoxic challenge in order to elucidate the sequence in which signaling pathways are initiated and further, associate these pathways with topographic cellular demise. This temporal and topographical mapping may identify crucial mechanisms in the death cascade, which could then be targeted to halt the neurodegeneration that results from excitotoxicity.

Glutamate Mediated Calcium Activity

Cell Membrane Permeability to Calcium Ions

Glutamate induced excitotoxicity is a calcium (Ca^{2+}) mediated event in which intracellular Ca^{2+} concentrations increase to toxic levels from which the cell cannot recover. There is a 10,000 fold Ca^{2+} concentration gradient across the plasma membrane, with concentrations around 1-2 mM outside the cell and between 10-100 nM in the cytoplasm (Marcoux & Choi 2002; Dingledine, et al., 1999). A key component in Ca^{2+} regulation is the plasma membrane calcium-ATPase (PMCA) (Choi, 1992; Dingledine, et al., 1999). The PMCA transports Ca^{2+} from the cytosol into the extracellular space and is regulated by several intracellular signaling pathways. However, because the PMCA is a high affinity but low capacity transporter, its main role is to regulate Ca^{2+} concentration when the cell is at rest. Following a depolarizing event, it is the fast acting Na^{+}/Ca^{2+} exchanger that is capable of moving large amounts of Ca^{2+} into the extracellular space (Feldman et al, 1996). The Na^{+}/K^{+} -ATPase also works to reset Ca^{2+} levels, though more indirectly. By resetting the cells membrane to its resting potential, thereby inhibiting depolarization, the Na^{+}/K^{+} -ATase makes the membrane less permeable to Ca^{2+} influx via voltage-dependent calcium channels (VDCC) and *N*-methyl-D-aspartate (NMDA) receptors (Cooper et. al., 2003). Gamma-aminobutyric acid (GABA) receptors also contribute to stabilizing the plasma membrane and reducing Ca^{2+} permeability by inhibiting depolarizing events (Cooper et. al., 2003). The effects of Na^{+} coupled glutamate transporters located on astrocytes also help protect the cell from excitotoxicity

by removing glutamate from the synaptic cleft, thereby limiting its ability to affect postsynaptic membrane calcium permeability (Marcoux & Choi 2002).

Intracellular Reaction to Increases in Calcium Ions

Once cytosolic Ca^{2+} levels have increased beyond physiological levels, the endoplasmic reticulum (ER) and mitochondria, two major organelles critical to cellular function, can become compromised. The ER is continually transporting cytosolic Ca^{2+} across its membrane by means of the smooth ER Ca^{2+}ATP-ase (SERCA) to maintain a 100 fold higher Ca^{2+} concentration inside the ER than in the cytosol (Marcoux & Choi 2002). The ER membrane also has two ligand gated Ca^{2+} channels, inositol trisphosphate (IP_3) and ryanodine receptors, which mediate the release of Ca^{2+} stores from the ER. The IP_3 receptor is activated when bound by IP_3, which is released in response to G-protein coupled glutamate receptor activation. The ryanodine receptor is activated in response to elevated intracellular Ca^{2+} concentrations (Marcoux & Choi 2002). Activation of these receptors not only increases cytosolic Ca^{2+} concentrations, but can also lead to ER stress. This stress can release ER membrane bound caspase-12, which in turn can activate executioner caspases, which induce apoptosis (Dingledine, et al., 1999).

The mitochondria are also sensitive to disruptions in Ca2+ homeostasis. During times of normal activity, mitochondrial Ca^{2+} concentrations are ~100nM (Dingledine, et al., 1999). However, upon influx of Ca^{2+} (ie, activation of glutamatergic receptors) mitochondria function as a buffer, rapidly moving ions down an electrochemical gradient via a uniporter, into the mitochondria (Bernardi & Rasola 2007). In addition, mitochondria possess a Na^{+} /Ca^{2+} exchanger that functions bi-directionally in reaction to

physiological or pathological conditions. Despite these dynamic mechanisms, mitochondria can become compromised under high intracellular Ca^{2+} concentrations and mitochondrial permeability transition pores (mPTP) can develop, a topic reviewed by Crompton (1999). The formation of these pores can lead to cell death by either necrosis or apoptosis. mPTP can permanently disrupt mitochondrial homeostasis and therefore, the synthesis of ATP. Once a cell can no longer generate ATP, overall cell homeostasis is compromised and necrosis can ensue (Crompton 1999; Dingledine, et al., 1999). mPTP formation can also allow the release of several apoptotic molecules, initiating either caspase-dependent or -independent pathways. The former pathway is initiated with cytochrome c (cyt c) release through mPTP; the latter is initiated by the release of apoptosis-inducing factor (AIF) through mPTP (Crompton 1999; Dingledine, et al., 1999, Renolleau et al, 2008).

Glutamate Receptors and Excitotoxicity

Glutamate receptors are referred to as excitatory amino acid transmitters (EAA) and can be classified into two categories, metabotropic and ionotropic; both are involved in glutamate mediated excitotoxicity. The metabotropic glutamate receptor (mGluR) family is distinct from ionotropic EAA receptors in that they are linked to G proteins and bring about intracellular changes via second messenger systems (Cooper et al, 2003; Feldman et al, 1997). The mGluR family is divided into 3 groups and contains a total of 7 receptors subclasses. Only the $mGluR_1$ and $mGluR_5$ receptor subclasses, which belong to Group I, have excitatory actions and are therefore the only mGluR receptors implicated

in the excitotoxic pathway. These receptors are found on postsynaptic membranes and are G_q protein coupled.

The $mGluR_{(1,5)}$ pathway is initiated when glutamate binding causes a conformational change that results in the release of the G_q alpha subunit. As illustrated by Albert and others (2002), this alpha subunit phosphorylates nearby phospholipase C, which then cleaves membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). As previously mentioned, the ER has IP_3 gated Ca^{2+} channels on its membrane. When bound, the channel opens to release Ca^{2+} filled vesicles into the cytoplasm. DAG combines with Ca^{2+} to form a membrane bound DAG/ Ca^{2+} complex that is able to activate protein kinase C (PKC) (Albert et al, 2002). PKC acts to phosphorylate proteins thereby changing membrane permeability to extracellular ions, including Ca^{2+}. In addition to increasing the intracellular Ca^{2+} load, DAG is also cleaved to form arachidonic acid (AA). AA can change membrane permeability, effect ion pumps, and activate protein kinases. The cleavage of AA initiates the inflammation pathway, producing eiconsinoids such as prostaglandin and leukotrienes, which can also lead to cell death (Marcoux & Choi, 2002).

There are three ionotropic EAA receptors, *N*-methyl-D-aspartate (NMDA), *α*–amino-3hydroxy-5-methyl-4isoxazoleproponic acid (AMPA), and kainic acid (KA) receptors (Mody, 1995); all three participate in producing glutamate mediated excitotoxicity (Marcoux & Choi, 2002). The receptors have three transmembrane domains with the N terminus located extracellularly and an intracellular C terminus. All receptors have been named for and can be pharmacologically distinguished by their

preferred agonist. In fact, the receptors belonging to the AMPA and KA families are difficult to distinguish from one another outside of pharmacological discrimination; this is largely due to a high amount of heterogeneity within the two. Such is the case that they are often referred to jointly as non-NMDA receptors. Both of these non-NMDA receptors are voltage-independent, ligand-gated ion channels that mediate fast excitatory synaptic transmission (Cooper et al, 2003; Feldman et al, 1997). When activated they allow for the influx of extracellular Na^+. In addition, activation of these receptors initiates a localized depolarization of the cellular membrane, a fact that is of functional importance to NMDA receptor activity. The NMDA receptor is channel bound by magnesium (Mg^+) ions. This block is released upon membrane depolarization, which physiologically follows AMPA receptor activation (Koch et al, 2004). Once the receptor channel is unblocked and in the presence of glycine and glutamate, the ion pore allows Ca^{2+} and Na^+ to enter the cell, the latter in lesser amounts, as well as a minor efflux of potassium (K^+) (Cooper et al, 2003; Feldman et al, 1997).

The ionotropic EAA receptor mediated excitotoxic pathway has been characterized as being composed of two components, swelling followed by cell degeneration (Crews & Chandler, 1993; Olney, 1986). In this model the stages are separated dependent upon time and ion involvement. Minutes after an initial glutamate insult, excessive glutamatergic activation of AMPAR and KAR is believed to allow for the influx of excessive amounts of Na^+ followed by a passive influx of Cl^- and water, all combining to account for an increase in cellular volume (Maulucci-Gedde & Choi 1987). However, this acute swelling is not always sufficient to produce cell death (Albensi 2007; Choi 1992). In fact, death is often not observed until a period of hours following

excitotoxic triggers, if at all. The movement of Ca^{2+} via NMDA receptor activation has been implicated as the crucial component in the ultimate demise seen in association with glutamate induced excitotoxic cell death.

Formation and Composition of the NMDA Receptor

As previously mentioned, the NMDA receptor is both ligand gated and voltage-dependent. This dual activation is dependent upon both pre- and post-synaptic activity. The voltage sensitive Mg^{+} block is removed by local depolarizations that occur in the post-synaptic membrane, the ligand is provided by pre-synaptic release of glutamate. Due to these unique properties, the NMDA receptor is referred to as a "coincidence detector" (Köhr, 2006). It is this ability of the receptor that allows it to produce long term potentiation (LTP) (Dong, 2009). The receptor is also unique among the glutamate receptors in that, in humans, it requires two of its known subunits, the NR1 and NR2, in its channel structure to be functional (McIlhinney et. al., 2003). A third and less frequently expressed subunit type, the NR3, is believed to form a tretrameric NR1/NR2/NR3 complex (Paoletti & Neyton, 2007). All NMDA receptors require the co-agonists glycine and glutamate to be bound at the NR1 and NR2 receptor subunits, respectively, for maximal activation. If dual binding occurs during a localized depolarizing event, ion permeability changes and the channel pore will allow the entry of $Ca^{2+}$and Na^{+}, and small amounts of K^{+} are allowed to exit the cell.

The NR1 subunit is encoded by a single gene that can be spliced to form 7 different variants. The exact number of subunits required to produce a functional channel pore remains unclear, however, evidence supports either di-heteromeric (NR1/NR2A or

NR1/NR2B) or tri-heteromeric (NR1/NR2A/NR2B) structures (Al-Hallaq et al, 2007). Recent studies indicate that, in young rat hippocampi approximately two-thirds of NR2 containing NMDA receptors are of the NR1/NR2A or NR1/NR2B structure while one third are NR1/NR2A/NR2B tri-heteromeric complexes (Al-Hallaq et al, 2007). The NMDA receptor is complex, possessing many different recognition sites through which its function can be modified. All NMDA receptors subunits contain large extracellular region that consist of two domains, the N-terminus domain and the agonist-binding domain (ABD), as well as an ion channel domain, which is contained within the cellular membranes (for review see Arundine & Tymianski, 2004; and also Paoletti & Neyton, 2007). Non-competitive antagonists including MK-801, mematine, and ketamine, as well as the endogenous Mg^{+} interact at the ion channel domain, the latter in a voltage-dependent manner. As previously mentioned, activation of NMDA receptors require the competitive co-gonists glycine (or D-serine) and glutamate. Glycine binds at the ABD of NR1and NR3 subunits, while glutamate binds at NR2 subunit ABDs. The N-terminus of the NR1 and NR2 subunits contain allosteric modulatory site for polyamines (Li et al, 2007) this site is also used by the competitive antagonist ifenprodil (NR2B selective). The N-terminus of NR2 subunit also has an inhibitory site for endogenous Zn^{+} (NR2A and NR2B).

Function and Regulation of the NR2B Receptor Subunit

The NMDA receptor is believed to be comprised of two obligatory NR1 subunits and two regulatory NR2 subunits. The NR2 subunit has four variants (NR2A, -B, -C, and -D), which are encoded by four different genes (Dingledine et al., 1999). The NR2A and

NR2B di-heteromeric complexes are the most common expressed in the CNS (Mony et. al., 2009; Yashiro & Philpot, 2008). The prenatal and early postnatal rat brain contains a higher ratio of NR2B as compared to NR2A subunits. However, with development, this ratio changes in both an activity-dependent and independent manner (for review see Yashiro & Philpot, 2008; Hardingham, 2009). Activation of the NMDA receptor is associated with increased expression of NR2A mRNA. Therefore, it is thought that when the NMDA receptor is active, NR2A subunits are expressed and become inserted at the synapse, changing the ratio of NR2B to NR2A subunits. Additionally, NR2B subunit expression has also been shown to down regulate with development in an activity-independent manner (Hardingham, 2009).

The subunit composition of the NMDA receptor has direct implications toward its channel kinetics (Erreger et al., 2005; Vicini et al., 1998), synaptic localization (Köhr, 2006), and protein binding partners (Hardingham et. al., 2002; Vanhoutte & Bading, 2003; Ivonov et al., 2006). Using recombinant NMDA receptors, Erreger and others have shown that NR1/NR2A heteromers had faster gating (open/close) time than did NR1/NR2B. However, NR1/NR2B heteromers had longer activation duration, therefore greater total conductance of Ca^{2+}. Although subunit composition is important to the function of the NMDA receptor, another factor is the receptor location. NR2B containing NMDA receptors are found in higher concentration in the extrasynaptic space than are NR2A heteromers (for review see Köhr, 2006). It appears that both location and subunit composition together indicate the intracellular signaling pathways that are activated by the NMDA receptor. Several studies have suggested an opposing role for synaptic versus extrasynaptic NMDA receptor activation. Synaptically located NR1A containing NMDA

receptors have been shown to activate cAMP response element binding protein (CREB; Hardingham et al., 2002) and, via CREB activation, brain-derived neurotrophic factor (BDNF) gene expression (Vanhoutte & Bading 2003), as well as extracellular signal-regulated kinases (ERK; Ivanov et al., 2009). In contrast, activation of the extrasynaptic NR2B subunit appears to shut off CREB activation and thereby decrease the expression of BDNF. Similarly, activation of extrasynaptic NR2B containing NMDA receptors triggers the inactivation of ERK activity. Lastly, using the Xenopus oocyte as an expression system, Williams (1994) found that the NR2B containing NMDA receptor is stimulatory when both active and bound by the polyamine spermine, a characteristic not found in the NR2A heteromer.

Chapter Two: The Role of Excitotoxicity in Disease

Excitotoxicity and EtOH

The affects of EtOH on the glutamatergic system are complex and still being defined. It is believed that EtOH acts as an allosteric modulator at NMDA receptors. Acutely, EtOH acts to inhibit NMDA receptor activity; however, chronic EtOH exposure enhances activity at the receptor (for review see Kumari and Ticku, 2000). In response to acute inhibition to EtOH, the cell is believed to upregulate NMDA receptors in order to maintain homeostatic intracellular Ca^{+} levels. When EtOH, and therefore inhibition, is removed during withdrawal, the increased abundance of NMDA receptors can be activated to increase intracellular Ca^{+} to cytotoxic levels. As previously discussed, NR2B containing NMDA receptors have a greater capacity for Ca^{2+} conductance than other NMDA receptor subunit compositions. In addition to this, the polyamine binding site on the N-terminus of the NR2B that can potentiate NMDA receptor activity; EtOH exposure increases the amount of polyamines present in the brain. Therefore, increased polyamines occurring in the presence of NR2B subunits can additively contribute to rises in intracellular Ca^{2+}. Finally, data indicate extrasynaptically located NR2B subunits can oppose some neurotrophic effects generated by cells in response to injury.

Excitotoxicity and Epileptic Seizures

Epilepsy is a diverse brain disorder that has been classified into over 40 distinct types based on several different factors including age of onset, characteristic symptoms and signs, seizure types, cause, and electroencephalographic pattern (for review see

McNamara, 1999). Genetics play an important role in epilepsy, with more than a dozen gene mutations identified as being linked to epileptic phenotypes.

In 1983, Olney and colleagues replicated both status epilepticus and epileptic seizure activity by electrically stimulating the perforant pathway of the hippocampus, the structures major route for receiving information, for either 2 hours continuously or intermittently over a 24 hour period. The group found neuronal degeneration, both somatic and dendritic, in the pyramidal cells of the CA3 and CA1 as well as evidence of neuron induced glial damage in the same areas. These changes were attributed to over activation of the glutamatergic neurons of the hippocampus, which produced excitotoxicity. Over activation of NMDA receptors during seizure activity can lead to the activation of proteases, damage to DNA, the creation of mPTP and subsequent activation of apoptotic cascades, all which can lead to cell death (reviewed by Fujikawa, 2005).

Excitotoxicity and Traumatic Brian Injury

Traumatic brain injury (TBI) can be classified into two categories (1) primary, which occurs at the time of injury and includes lacerations, fractures of the skull, hemorrhage, and surface contusions of the brain and (2) secondary, which can be initiated at the moment of injury but which develops over a subsequent period of time (Cater et al, 2007; Graham et al, 2000). Secondary injuries include edema, infection, ischemic insults, oxidative stress, and changes in endogenous neurotransmitters, including glutamate (Yi, et al., 2006). As previously stated, glutamate is the most abundant neurotransmitter in the human CNS. As would stand to reason, there are also an abundance of glutamatergic neurons in the CNS, as well as astrocytes, which transport

and breakdown glutamate. When these neurons and astrocytes become damaged during either primary or secondary insult presented by TBI, their contents, including glutamate, can spill into the extracellular milieu. The rise in glutamate activity can further exacerbate the TBI by producing and excitotoxic environment. Therapeutics are directed at mediating these secondary effects includes targeting those mechanisms common to NMDA mediated excitotoxicity.

Excitotoxicity and Hypoxia-Ischemia

Hypoxic-ischemic (HI) conditions, when the brain is deprived of oxygen and glucose, can result in extracellular glutamate concentrations that deviate from homeostatic levels due in part to the rupture of glutamatergic neurons. When these neurons rupture, the neurtotransmitters they contain, including glutamate, are spilled into the extracellular milieu, where they are free to affect neighboring neurons (Hardingham & Bading, 2003). Also a factor in HI damage is the injury sustained by astrocytes, which normally function to remove glutamate from the synaptic space, thus allowing prolonged post synaptic stimulation (Hertz, 2008). The NR2B NMDA receptor subunit, in particular, has been implicated in contributing to elevated intracellular Ca^{2+}. Reasons for this may due to the activation by the NR2B containing NMDA receptor complex by polyamines, which are expressed following brain injury (Li et al, 2007). However, the rise in intracellular Ca^{2+} cannot solely be contributed to increased *extracellular* glutamate levels. This was elucidated by Mitani (1993) in experiments using hippocampal slices from gerbils exposed to ischemic-like conditions (exposure to medium absent of glucose and oxygen). The hippocampal tissue showed a significant rise in intracellular Ca^{2+} levels both in the presence and absence of media containing glutamate. This intracellular

release of Ca^{2+} is thought to be attributed to activation of ER receptors, which release Ca^{2+} stores from the ER and to the dysregulation of mitochondria. Mitochondrial dysregulation can lead to increased use but decreased production of ATP, which can break down the mitochondrial membrane, thereby releasing Ca^{2+} stores. Rises in cytosolic Ca^{2+} via ER and mitochondria release can further cause the formation of mPTP and subsequent release of cyt c, as well as activation of AIF, all culminating in excitotoxic cell death (for review see Arundine &Tianski, 2004).

Excitotoxicity and Alzheimer's Disease

The first indications that implicated the glutamatergic system in Alzheimer's disease (AD) were proposed by Greenamyre et al. in 1988. Upon autopsy of individuals who had died with AD, the group found a decrease in available glutamate binding sites that resulted in reduced glutamate binding in the cortical regions of the brain as compared to individuals who had no neurological disease. Based upon these and other similar findings, the glutamatergic hypothesis of dementia was proposed (Albensi et al, 2004). Molecular hallmarks of AD include extracellular β-amyloid peptide (Aβ) plaque deposits and intracellular neurofibrillary tangles (NFT) in the hippocampus and other brain regions necessary to cognitive and memory function (as reviewed by Dong et al, 2009). It is suspected that Aβ plaques either sensitize or activate NMDA receptors (Koch et al, 2004) and lead to widespread apoptosis in the pyramidal cells of the CA1 (Miguel-Hidalgo et al, 2002). The alteration of NMDA receptors allow their activation at μM concentrations of glutamate as opposed to the mM concentrations required under physiological conditions, producing excitotoxicity (Dong et al, 2009). In the

hippocampus, neuropathic activity of NMDA receptors leads to memory and cognitive deficits because constant activation prevents the receptors from being "co-incidence detectors", thus preventing LTP (Dong et al, 2009). Further evidence implicating the NMDA receptor in AD is the action of the therapeutic drug memantine. Memantine is low affinity blocker of the NMDA channel and works to "re-regulate" NMDA receptors that have been sensitized by Aβ plaques (Koch et al, 2004). The goals of therapeutic drugs that target NMDA receptors being developed to treat AD such as memantine, are to prevent excitotoxicity and the downstream intracellular cascades that lead to mitochondrial dysfunction, caspase activation, and cell death.

Chapter 3: Summary and Hypothesis

Summary

Because Ca^{2+} has the ability to activate many different cell signaling pathways, regulation of this cation is crucial to cell survival and proper neuronal function. Excessive glutamatergic activity can bring about the disruption of Ca^{2+} homeostasis; this disruption can lead to excitotoxicity, a condition that can be fatal to cells. Each EAA receptor represents a different pathway through which cytosolic Ca^{2+} levels can become elevated, thereby increasing the possibility of cell death. However, the NMDA receptor is unique in its ability to produce excitotoxicity. Because it contains a dense population of NMDA receptors and is therefore exquisitely susceptible excitotoxicity, the hippocampus is an ideal structure in which to study the phenomenon. This brain structure is composed of three subregions, the granule cells of the DG and the pyramidal cells of the CA1 and CA3, of these, the CA1 has been shown most susceptible to excitotoxicity (Butler et al., 2009). It has been suggested that this regional difference can be attributed to the larger population of mature neurons and dendritic processes found in the CA1. In addition, the pyramidal cells of this subregion contain more NR1/NR2B heteromers than are found in other subregions, a configuration that confers greater conductance of Ca^{2+} across the membrane and a characteristic that is potentiated in the presence of polyamines.

The proposed studies will examine the time-dependence of neuronal nuclear degradation (ie. PI fluorescence, NeuN staining) and loss of NR2B subunit density, following an excitotoxic insult to provide a better understanding of the topographical

nature of neuronal death following NMDA receptor activation. The topographical "mapping" of neuronal injury or death following NMDA receptor activation is important as it may elucidate the specific cellular mechanisms that precipitate cellular demise. Identification of these mechanisms is crucial to the development of novel therapeutic strategies to be used in the treatment of disease states that are associated with excitotoxic insult.

Hypotheses

Experiment (1) NMDA, a NMDA receptor agonist, will be administered to hippocampal slices in levels sufficient to produce excitotoxicity; a NMDA receptor antagonist will be co-administered to an experimental group to as a proof of NMDA receptor mediated cell death. After 24, 72, or 120 hours, granule cells of the DG and the pyramidal cells of the CA1 and CA3 regions of the hippocampal formation will be measured to assess cell death (i.e. propidium iodide (PI) fluorescence). Experiment (2) following NMDA administration, immunohistochemical techniques will be used to assess loss of mature neurons (i.e. NeuN staining) over 24, 72, or 120 hour periods. Experiment (3) procedures used in experiment 2 will be replicated using NR2B staining antibodies to assess loss of this subunit over the same time course. It is hypothesized that, in reaction to NMDA: (1) cell death will be region dependent, with highest total loss occurring in the pyramidal cells of the CA1 hippocampal subregion (2) this cell death will be time dependent with the greatest cellular loss occurring after 24 hours (3) the total loss of mature neurons (NeuN staining) will be most substantial in the pyramidal cells of the CA1 hippocampal region (4) this loss of mature neurons will be significant after 24 hours and greatest after 120 hours (5) the total loss of NR2B subunits will be highest in the

pyramidal cells of the CA1 hippocampal region (6) this loss of NR2B subunits will be significant after 24 hours and greatest after 120 hours.

Chapter 4: Methods

Organotypic Hippocampal Slice Culture Preparation

Organotypic hippocampal slices will be obtained from 8-10 day old male and female Sprague Dawley rat pups (Harlan Laboratories, Indianapolis, IN, USA). Pups will be decapitated; the whole brain extracted and placed in chilled dissecting medium comprised of Minimum Essential Medium containing Hanks' salts and L-glutamine (MEM; Gibco, Gaithersburg, MD, USA), 25 mM HEPES (Sigma-Aldrich Co., St. Louis, MO, USA), and 50 µM Penicillin/Streptomycin (Gibco). The bilateral hippocampi will be removed, placed in culture medium composed of dissecting medium with the addition of: 36 mM glucose (Fisher Scientific, USA), 25% (v/v) Hanks' balanced salt solution (Gibco), 25% heat-inactivated horse serum (Sigma-Aldrich Co.), and 0.05% Penicillin/ Streptomycin. Under a dissecting microscope, loose and extra tissue will be removed. Each hippocampus will be coronally sectioned into 200µm slices using the McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK), yielding approximately 12 slices (approximately 24 per animal), and placed into fresh culture medium. Slices will be sorted under a dissecting microscope to remove damaged or partially sectioned specimens. Using transfer pipettes, intact slices will be placed three per well onto porous Teflon membrane inserts (Millicell-CM 0.4 µm; Millipore, Marlborough, MA) that had been incubated in 1 ml of culture media for a period of at least one hour. Excess culture media will be aspirated from the top of the membrane. Plates will be comprised of 6 wells and will yield 18 slices per plate. After plating, slices will remain in an incubator exposed to an atmosphere of 5% carbon dioxide 95% air and

at constant temperature of 37°C for 5 days to allow the tissue to adhere to the membrane and to stabilize the slices. This method was adapted from Stoppini et al., 1991. Care of all animals will be performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), as well as with the University of Kentucky's Institutional Animal Care and Use Committee. All experiments discussed will be replicated using different rat litters.

NMDA Stimulated Excitotoxicity

Excitotoxicity will be induced in cell cultures using NMDA, a selective agonist of the NMDA receptor. NMDA will be chosen over glutamate, the endogenous NMDA receptor ligand, to isolate NMDA receptor activation from other EAA receptors. DL-2-Amino-5-phosphonopentanoic acid (APV), a selective NMDA receptor antagonist, will be used to block the activation of the NMDA receptor. After 5 days in vitro (DIV), inserts will be transferred into plates filled with 1 ml of fresh, pre-incubated media that will contain 3.74 μM propidium iodide (PI) (Sigma-Aldrich) and, in addition, will contain either 50 μM APV (Sigma-Aldrich), 20μM NMDA (Sigma-Aldrich), or 20 μM NMDA and 50 μM APV (NMDA/APV). Cultures will be returned to the incubator and allowed to rest for a period of 24, 72, or 120 hours. After each of these time points, plates will be removed from the incubator and assessed for cell cytotoxicity as described below.

Propidium Iodide Uptake in Treated Cultures

Fluorescent microscopy of propidium iodide (PI) will be used to assess levels of cell cytotoxicity incurred by cell cultures after time dependent exposure to NMDA and/or

APV. Membrane impermeable PI binds to DNA and RNA once the cellular membrane becomes compromised. Once bound to these nucleic acids, the fluorescence of PI is enhanced 20-30 fold (Arndt-Jovin & Jovin, 1989). This altered wavelength allows for non-bound concentrations of PI to be excluded from analysis. The use of this staining technique has been significantly and reliably correlated with other measures of cell death and cytotoxicity (for review, see Zimmer et al., 2000). Uptake of PI will be imaged with SPOT Advanced version 4.0.2 software for Windows (W. Nuhsbaum Inc., McHenry,IL) using a 5x objective on a Leica *DM*IRB microscope (W. Nuhsbaum IL) fitted for fluorescence detection (mercury-arc lamp) and connected to a personal computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). When bound to nucleic acids, the peak excitation wavelength of PI is 535 nm, with emission maximum of 617nm. The wavelength will be excited to between 510-560 nm using a rhodamine band-pass filter. The intensity of PI within each culture will be determined by densitometry using Image J 1.29x (National Institutes of Health, Bethesda, MD). Three cell layer regions of the hippocampal formation were analyzed for cytotoxicity, the granule cells of the dentate gyrus (DG) and the pyramidal cells of the cornu Ammonis-3 (CA3) and cornu Ammonis-1 (CA1). The visual field containing each sub-region of interest will be measured; a background measure will be also taken for each tissue slice analyzed. To reduce environmental variations between slices, the background value will be subtracted from each sub-region value. This will result in an intensity measure for each sub-region of each slice, 9 measures per well, 54 per plate. These values will be recalculated, dividing by intensity values for a control plate, to express the value as a percent of control.

Immunoreactivity of Neuron Specific Nuclear Protein and NMDA NR2B Subunits

Immunohistochemical labeling using monoclonal antibodies will be used to measure the density of both mature neurons and NMDA NR2B subunits. Neuron specific nuclear protein (NeuN) primary antibody will be used to identify mature neurons in the granule cells of the DG and the pyramidal cells of the CA1 and CA3 of the hippocampus. NeuN antibody recognizes the neuron-specific NeuN protein, which is found in the CNS of all vertebrates (Mullen, 1992). NeuN is first evident in neurons shortly after they become post-mitotic and/or shortly after differentiation (Mullen, 1992). Therefore, strong staining of NeuN indicates the presence of mature cells. The monoclonal anti-NR2B primary antibody will be used to measure the density of NMDA NR2B subunits in the same hippocampal regions. Mouse or rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody will be used with NeuN and NR2B antibodies, respectively.

Following PI analysis discussed previously, culture slices will be removed from culture medium for fixation with 10% formalin, during this procedure slices will be preserved on previously plated inserts. Inserts will be immersed in wells containing 1 ml of formalin, an additional 1 ml of formalin will be applied to the top of the insert; inserts will be allowed to rest in formalin for 30 minutes. Inserts will be removed from formalin plate and will be placed in plates containing 1ml of 1x PBS, with 1 ml 1x PBS slowly added to the top of the insert, then drained, a procedure termed washing. This wash will be performed twice before inserts will be transferred to plates containing 1 ml of 1x PBS and stored at 4°C until immunohistochemistry will be conducted. Prior to

immunohistochemistry, slices will be placed in a plate containing 1ml buffer consisting of 1x PBS, 0.005% bovine serum albumin, and 0.1% Triton-X (Sigma-Aldrich), with 1 ml of buffer slowly added to the top of insert. Cultures will be allowed to rest for 45 minutes to permeabilize cell membranes. Following this incubation period, cultures will be transferred into fresh plates containing 1 ml of1 x PBS, 1 ml of buffer and mouse anti-NeuN monoclonal primary antibody (1:200; Chemicon, Temecula, CA) or rabbit anti-NR2B monoclonal primary antibody (1:500; Chemicon, Temecula, CA) will be added slowly to top of inserts; cultures will be stored for 24 hrs at 4°C. Following this incubation period, cultures will be washed twice in 1x PBS and placed into fresh culture plates containing 1 ml of 1 x PBS, 1 ml of buffer and anti-mouse (NeuN; 1:200) or anti-rabbit (NR2B; 1:200) fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma-Aldrich) will be slowly added to top of inserts previously treated with NeuN, those previously treated with NR2B will receive the same treatment but with anti-rabbit FITC (1:200; Sigma-Aldrich). Plates will be stored at 4°C for 24 hours before fluorescent images will be taken. FITC fluorescence will be elicited using a band-pass filter that excites wavelengths of approximately 495 nm (emission wavelength = 520 nm). Images will be taken using a 5× objective lens, as described previously.

Chapter 5: Results

Statistical Analysis

Two-way ANOVA (treatment x region) was conducted to investigate the validity of the *a priori* hypothesis regarding regional differences in reaction to exposure to NMDA. Three-way ANOVA (time x treatment x sex) was conducted to investigate differences within the subregions of the hippocampus. A reduced design, two-way ANOVA (treatment x time), was used if no effect of sex was detected by the omnibus analysis. Following ANOVA analyses, the Fisher's LSD post-hoc test was interpreted when appropriate; significance level was set at $P < 0.05$. All analyses are of a between subjects design and all data is expressed as percent control for ease of interpretation. Experiments were replicated a minimum of two times, all data represent tissue sampled from a minimum of two litters. Tissue from both male and female animals was used.

Results

Propidium Iodide Uptake in Treated Cultures

Densitometric analysis of cell toxicity as measured by PI fluorescence was evaluated in the pyramidal cell layers of the CA1 and CA3 and the granule cell layer of the DG. To test the *a priori* hypothesis that exposure to 20 μM NMDA would produce greater toxicity in the CA1 as compared to the CA3 and DG, a two-way ANOVA (treatment x region) was performed. A significant interaction was found ($F(6, 1262)=25.508$; $P<0.001$). As predicted, increased PI fluorescence was largest in the CA1 (277.545, ±14.489) as compared to the CA3 (211.001, ±17.37) and the DG (132.505, ± 5.759; Fig. 1). Further analysis was conducted to explore differences within the

subregions of the hippocampus. A three-way ANOVA (time x treatment x sex) was conducted in each region (CA1, CA3, DG), which revealed no significant sex effect in any region. A reduced, two-way ANOVA (treatment x time), revealed a significant interaction within the CA1 ($F(6,420)= 16.728$, $P < 0.001$). NMDA treatment resulted in significant toxicity on days one (319.41, ±32.38), three (338.34, ±14.63), and five (173.62, ±12.63; Figure 2A) as compared to controls. These data are consistent with the previously stated hypothesis, which predicted the greatest toxicity would occur on day one. As compared to control values, NMDA/APV was not associated with significant increases in PI fluorescence on days three and five but did reach significance on day one (138.78, ±8.28; Fig. 2A). However, co-administration of NMDA/APV did reduce NMDA induced damage at all time points. APV exposure resulted in no significant change in PI fluorescence as compared to controls at any time point (Table 1). In the CA3, two-way ANOVA (treatment x time) revealed a significant interaction ($F(6,420) = 19.345$, $P < 0.001$). APV and NMDA/APV treatment groups did not differ significantly from control values at any time point (Table 1, Fig. 1B). However, NMDA treatment produced significant toxicity as compared to controls, reaching maximal effect on day one (345.77, ±42.29), which diminished on day three (135.38, ±6.90), from which there was no significant change on day five (148.01, ±6.84, $P<0.508$); Figure 1B). Within the DG, two-way ANOVA (treatment x time) revealed no significant interaction (Figure 1C). However, a main effect for treatment ($F(3, 420)=15.956$, $P<0.001$) was found, with NMDA (132.33, ±3.423) and NMDA/APV (113.512, ±3.439) treatments associated with significantly higher PI fluorescence as compared to control values. However, APV co-administration was effective at reducing NMDA induced damage.

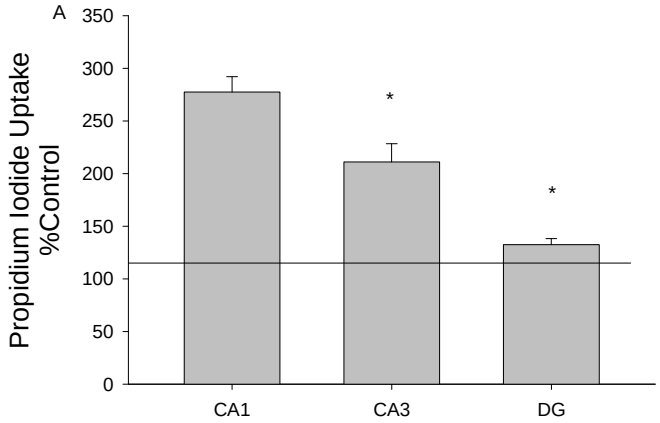

Figure 1: Effects of exposure to 20µM NMDA on PI fluorescence in the pyramidal cells of the CA1 as compared to the pyramidal cells of the CA3 and the granule cells of the DG (* p< .05 vs. CA1).

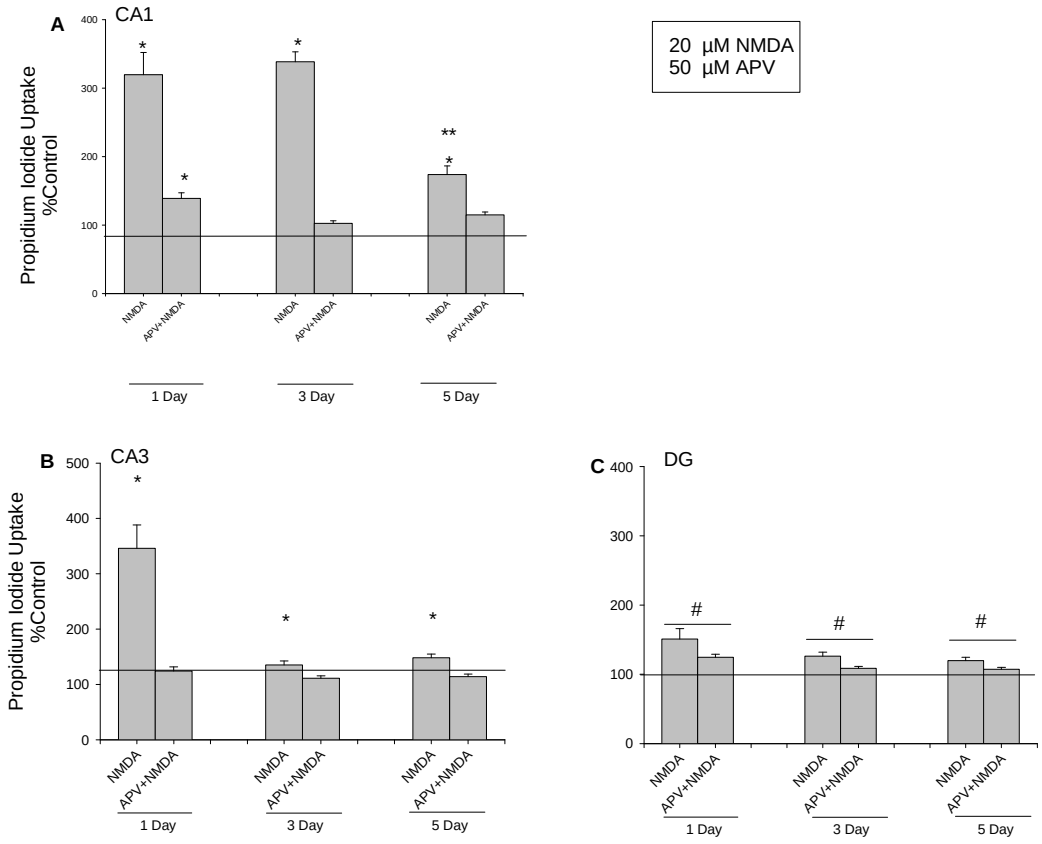

Figure 2A-C: Time dependent effects of exposure to 20µM NMDA or 20µM NMDA + 50µM APV on PI fluorescence in the pyramidal cells of the CA1 (A), CA3(B), and the granule cells of the DG (C). (* p< .05 vs. control; ** p< .05 vs. Day 1; # main effect of treatment p<.05).

Immunoreactivity of Neuron Specific Nuclear Protein

Mature neuron density, as indicated by NeuN immunoreactivity, was analyzed using densitometry for the pyramidal cell layers of the CA3 and CA1 and the granule cell layer of the DG. To test the *a priori* hypothesis that exposure to 20 μM NMDA would produce greater reductions in the density of mature neurons in the CA1 as compared to the CA3 and DG, a two-way ANOVA (treatment x region) was performed, a significant interaction was found ($F(6, 2075)=14.807$; $P<0.001$). As predicted, reduction in NeuN reactivity was largest in the CA1 (52.57, ±1.42) as compared to the CA3 (76.36, ±1.80) and DG (76.37, ±1.37). No significant difference was found between the latter two regions (Fig. 3). Further analysis was conducted to explore differences within the subregions of the hippocampus. A three-way ANOVA (time x treatment x sex) was conducted in each region (CA1, CA3, DG), which revealed no significant sex effect. A reduced design, two-way ANOVA (treatment x time), revealed a significant interaction within the CA1 ($F(6, 691)= 6.13$, $P<0.001$). In this region, NMDA treatment was shown to significantly reduce the density of mature neurons relative to controls at all time points. Supporting the hypothesis regarding time-dependent loss in this region, significant declines in the density of mature neurons was observed at day one (62.04, ±2.67). Contrary to the hypothesis, which stated that the largest decline would be evident at day five, there was a decline at day three (47.82, ±2.84), from which there was no significant change on day five (47.95, ±2.58; Fig. 3A). NMDA/APV treatment group values were significantly different from control values, with an increase in NeuN immunofluorescence at day five (116.65, ±4.68; Fig. 3A), illustrating that APV blocked NMDA mediated reduction in mature neurons. No significant effect was associated with

APV treatment in the CA1 at any time point (Table 2). Two-way ANOVA (treatment x time) in the CA3 revealed no significant interaction, however, there was a main effect of treatment ($F(3, 691)=46.451$, $P<0.001$). Significant treatment effects were seen upon exposure to NMDA (76.252, ±1.884) and APV (108.83, ±2.93; Fig. 3B; Table 2). Two-way ANOVA (treatment x time) in the DG also revealed no significant interaction. However, there was a main effect of time ($F(2,691)=7.44$, $P<0.001$) and treatment ($F(3,691)=56.625$, $P<0.001$). NMDA treatment (73.346 ±1.423) produced significantly lower NeuN immunofluorescence as compared to control group in this region (Fig. 3C).

Imunoreactivity of NMDAR2B Subunits

Density of NMDAR2B subunits, as indicated by NR2B immunoreactivity, was analyzed using densitometry in the pyramidal cell layers of the CA1 and CA3 and the granule cell layer of the DG. The *a priori* hypothesis, which stated that exposure to 20 μM NMDA would result in a

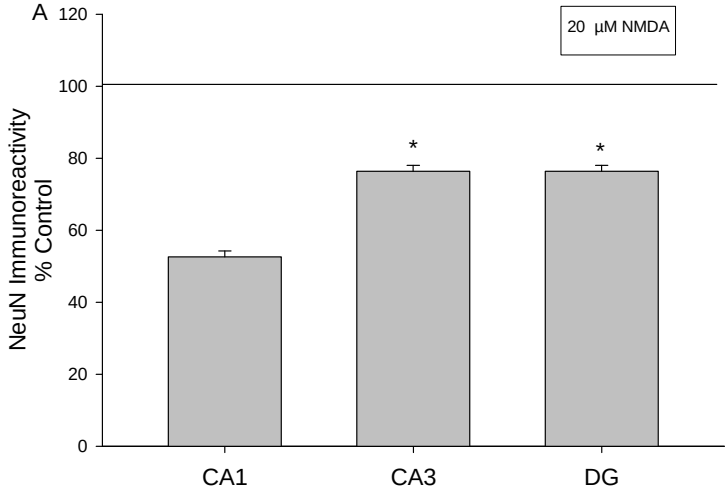

Figure 3: Effects of exposure to 20μM NMDA on NeuN immunofluorescence in the pyramidal cells of the CA1 as compared to the pyramidal cells of the CA3 and the granule cells of the DG (* $p< .05$ vs. CA1).

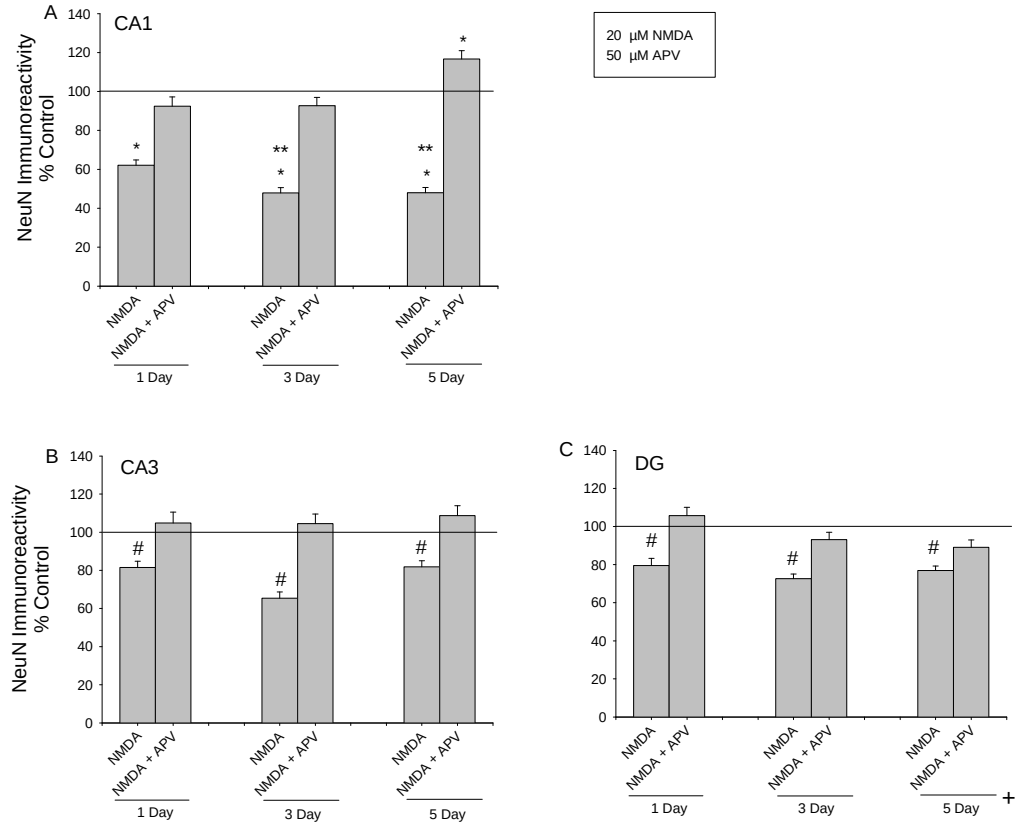

Figure 4A-C: Time dependent effects of exposure to 20µM NMDA or 20µM NMDA + 50µM APV on NeuN protein immunofluorescence in the pyramidal cells of the CA1 (A), CA3(B), and the granule cells of the DG (C). (* p< .05 vs. control; ** p< .05 vs. Day 1, # main effect of treatment p<.05, + main effect of time p<.05).

greater reduction of NR2B subunit protein in the CA1 as compared to the CA3 and DG, was tested by conducting a two-way ANOVA (treatment x region). A significant interaction was found (F(6, 2183)=8.881, P,0.001), with a greater loss of NR2B protein occurring in response to NMDA exposure in the CA1 (83.699, ± 1.106) as compared to CA3 (93.665, ± 1.106) or DG (92.816, ± 1.106; Fig. 5), consistent with the hypothesis. Analysis exploring differences within the subregions of the hippocampus was initially conducted using a three-way ANOVA (time x treatment x sex), for each region. No significant sex effect was found in any region, therefore, a reduced design, two-way

ANOVA (time x treatment), was used for subsequent analysis. Within the CA1, two-way ANOVA (treatment x time) revealed a significant interaction ($F(6, 727)= 11.24$, $P<0.001$). NMDA treatment significantly reduced NR2B density on days three (73.885, ± 1.840) and five (77.184, ±1.861; Fig. 6A). NMDA/APV treated group did not differ significantly from control group any time point (Fig. 6A), verifying that APV was effective in this area of mediating NMDA induced loss of NR2B subunits. APV treatment significantly altered NR2B subunit density on day one (112.38, ±2.86) and day five (90.96, ±2.93; Table 3). Two-way ANOVA (treatment x time) revealed a significant interaction within the CA3 hippocampal region ($F(6,727)=7.18$, $P<0.001$). NMDA treatment significantly changed NR2B subunit density on days one (105.48, ± 1.792), three (88.90, ±1.812), and five (86.17, ±1.834; Fig. 6B). Treatment with NMDA/APV was shown to significantly reduce NR2B subunit density on day three (90.96, ±2.817; Fig. 6B). APV resulted in altered subunit levels on days three (89.01, ±2.27) and five (89.01, ±2.76; Table 3). Two-way ANOVA (treatment x time) conducted in the DG revealed a significant interaction ($F(6, 727)=5,820$, $P<0.001$). NMDA exposure was shown to reduce NR2B subunit protein on day three (84.995, ±1.725) and day five (89.866, ±1.745). Additionally, exposure to NMDA/APV significantly reduced NR2B subunit protein on day three (88.773, ± 2.681). Treatment with APV was associated with decreased NR2B immunoreactivity on day three (89.01, ± 2.72) and day five (89.01, ± 2.76).

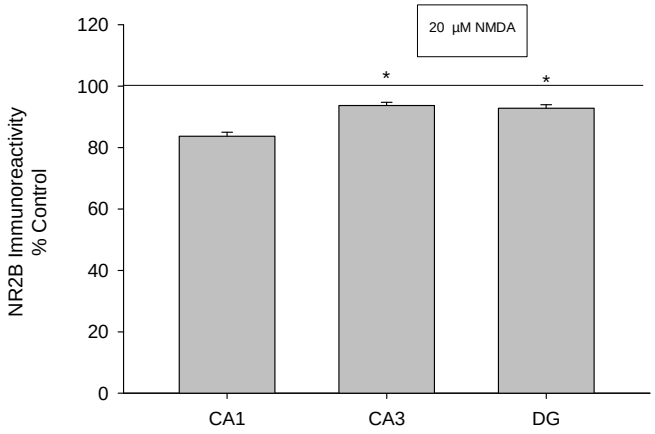

Figure 5: Effects of exposure to 20µM NMDA or 20µM NMDA + 50µM APV on NR2B protein immunofluorescence in the pyramidal cells of the CA1 (A), CA3(B), and the granule cells of the DG (C) (* p< .05 vs. CA1).

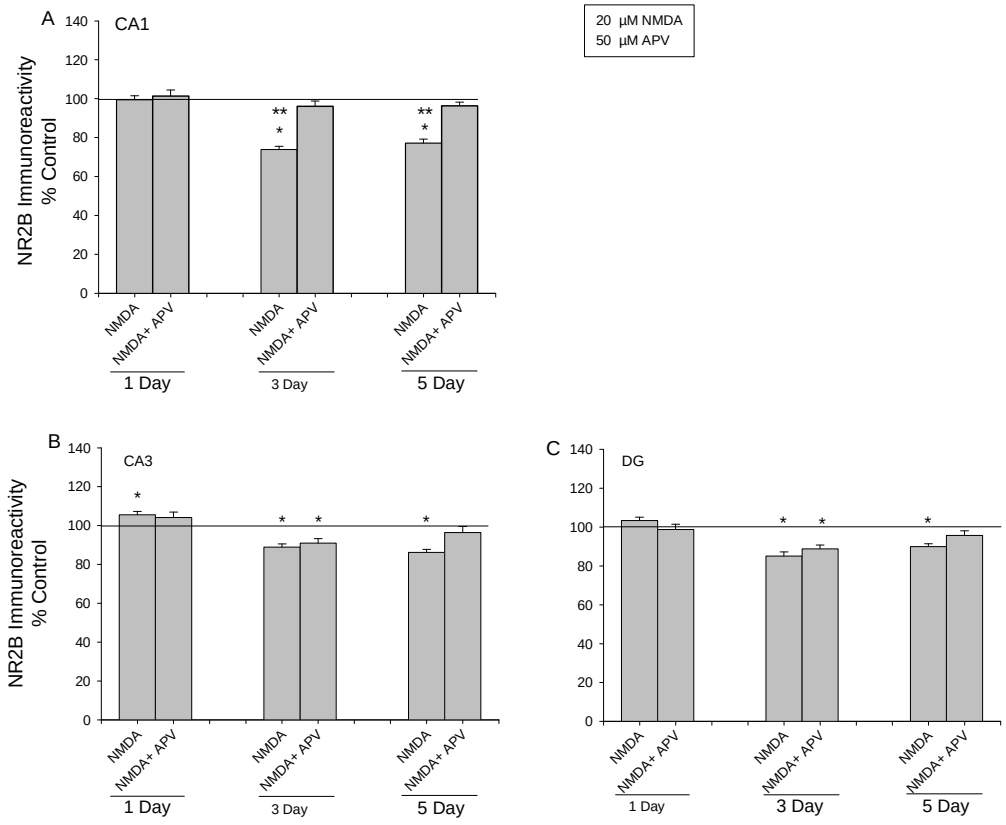

Figure 6 A-C: Effects of exposure to 20µM NMDA on NR2B immunofluorescence in the pyramidal cells of the CA1 as compared to the pyramidal cells of the CA3 and the granule cells of the DG on day1(A), day 2 (B), and day 3 (C) (* p< .05 vs. CA1;**p<.05 vs. Day 1).

Table 1: PI uptake in response to 50 μM APV treatment. Values expressed as percent control. ($p < .05$ vs. control, # indicates main effect of treatment)

	Treatment Period		
	1 Day	3 Days	5 Days
Subregion			
CA1	100.06 ± 5.06	98.78 ± 2.90	122.3 ± 4.92
CA3	98.03 ± 4.11$^{\#}$	101.47 ± 4.77$^{\#}$	109.28 ± 4.05$^{\#}$
DG	106.5 ± 3.65	109.06 ± 6.10	108.7 ± 4.11

Table 2: NeuN immunoreactivity in response to 50 μM APV treatment. Values expressed as percent control. (* $p < .05$ vs. control)

	Treatment Period		
	1 Day	3 Days	5 Days
Subregion			
CA1	102.45 ± 4.85	100.726 ±5.46	92.637 ± 5.31
CA3	108.81 ± 6.05	102.749 ± 6.46	114.93 ± 6.34
DG	108.86 ± 5.91	94.716 ± 4.45	99.69 ± 2.86

Table 3: NMDA NR2B subunit immunoreactivity in response to 50 μM APV treatment. Values expressed as percent control. (* $p < .05$ vs. control)

	Treatment Period		
	1 Day	3 Days	5 Days
Subregion			
CA1	112.38 ± 2.41*	97.76 ± 2.55	90.96 ± 2.44*
CA3	102.91 ± 2.22	94.57 ± 2.21	83.55 ± 4.32*
DG	101.81 ±1.99	89.01 ± 2.47 *	89.01 ± 2.20*

Chapter 6: Discussion

Glutamate mediated activity is a crucial component in the proliferation, maintenance, and survival of neurons (Cooper et. al., 2003; Hardingham & Bading, 2003). This is due in part to its ability to provide much needed calcium Ca^{2+} to the intracellular environment. At physiological levels, intracellular Ca^{2+} is vital to the phosphorylation and synthesis of proteins, cellular communication, and gene expression. However, overexposure to glutamate or exposure to excessive concentrations of the neurotransmitter, can allow intracellular Ca^{2+} to exceed physiological levels, a condition defined by Olney as excitotoxicity (Choi, 1992; Olney, 1986). Pathological intracellular Ca^{2+} concentrations can lead to the loss of cellular components via activation of multiple signaling pathways, proteases, lipases, as well as to the disruption of mitochondrial membrane potential, all of which can culminate in cell death (Albensi, 2007; Choi et. al., 1987; Forder, 2009; Hardingham & Bading, 2003). Due to its crucial role in cell viability there are several mechanisms in place dedicated to maintaining Ca^{2+} homeostasis. These include the PMCA transporter and Na^{+}/Ca^{2+} exchanger, which both work to remove Ca^{2+} from the cytosol (Choi, 1992; Dingledine et al., 1999). Additionally, the Na^{+}/Ca^{2+} exchanger works to reset resting membrane potential, thereby limiting activation of voltage dependent calcium channels and NMDA receptors (Choi, 1992; Dingledine et al., 1999). The family of glutamatergic receptors is also important to Ca^{2+} homeostasis (Cooper et al. 2003). These receptors can change intracellular Ca^{2+} concentrations either directly, as with the previously mentioned NMDA receptor, or indirectly, as with the mGluR and AMPA/KA receptors. mGluRs are capable of disrupting Ca^{2+} homeostasis by activating IP_3, an agonist capable of releasing intracellular stores of Ca^{2+}. Additionally,

because antagonizing these receptors dampens NMDA activity, the mGluRs are suspected to interact with the intracellular NMDA receptor domain to modulate activity (Harris, et al., 2002). Activation of the NMDA receptor directly changes membrane permeability by opening a membrane channel pore, which allows the entry of Ca^{2+} as well as Na^{+} (Cooper et al. 2003). Physiological activation of the NMDA receptor is integral to learning and the formation of memories. However, pathological activation of this receptor is sufficient to bring about excitotoxicity; such is the case that its activation is dependent upon two events occurring simultaneously. First, the receptor must be bound by both glycine and glutamate and, while bound, a localized membrane depolarization, which removes Mg^{+} from the receptor channel, must also occur. Depolarization of the membrane is carried out by glutamatergic AMPA/KA receptors and is opposed by GABA receptors; both are co-localized with NMDA receptors. The receptors and exchangers discussed here are all crucial to maintaining Ca^{2+} homeostasis. However, once homeostasis is disrupted, they are also the means by which rises in cytosolic Ca^{2+} concentrations occur. Due to its ability to contribute to an excitotoxic intracellular environment, the NMDA receptor has been the focus of numerous investigations, including the current research.

Once NMDA receptors are activated, ionic membrane permeability is determined by the stoichiometry of the receptor, with NR2B containing NMDA receptors able to conduct the greatest concentrations of Ca^{2+} due to longer channel opening time (Erreger et al., 2005). Additionally, the NR2B receptor is unique among the receptor subunit types in its excitatory reaction to polyamines. Polyamines are expressed during excitotoxic events such as alcohol withdrawal and TBI (Williams, 1994). This unique

consequence increases the ability of the NR2B subunit to confer Ca^{2+} across the membrane. Further, recent evidence indicates that NR2B containing NMDA receptors that are located extrasynaptically may inhibit activation of protective mechanisms such as BDNF (Vanhouk & Badding, 2003), CREB (Hardingham et al., 2003), and ERK (Ivanov et al., 2009). Therefore, activation of these receptors could worsen damage by inhibiting the cells naturally occurring repair mechanisms. The pyramidal cells of the CA1 express a high density of NR2B subunits as compared to the CA3 and DG (Butler et al., 2010). Additionally, this subregion has a greater density of mature neurons and greater expression of NMDA receptors (Butler et al., 2010). It is therefore not surprising that, as hypothesized, this subregion also incurred the greatest NMDA induced damage as compared to the CA3 and DG. Importantly, the pyramidal cells of the CA1 project to cortical regions of the brain and are crucial to learning and the formation of memory. It is therefore understandable that those neurodegenerative disorders associated with excitotoxicity are also associated with memory dysfunction.

Given the central role of the NMDA receptor to the current research, it was important to demonstrate that the treatment effects observed could be attributed to activation of this receptor and not due to generalized activity. This was achieved by co-exposing cultures to APV and NMDA, which effectively reduced excitotoxicity to control values (as measured by PI and NeuN) at all time points investigated. Therefore, it can be said that damage related to the NMDA treatment was mediated selectively by the NMDA receptor. Previously stated hypotheses indicated that NMDA treatment would have significant effects on day one and greatest effects on day five (NeuN and NR2B). PI fluorescence was predicted to be greatest on day one and to decline by day five, as the

signal was degraded. This occurrence would imply that, upon excitotoxic stimulation of the NMDA receptor, a mass damage was affected, which destroyed the cell soma, membrane, and dendrites all following a 24 hour time lapse and escalate uniformly until day five. However, this did not occur. The greatest membrane damage (PI) *was* observed on day one, consistent with the hypothesis. This loss was maintained through day three, and did not decline back to raw control values (data not shown) until day five. Declines in the density of mature neurons were significant on day one and peaked on day *three*, which was not hypothesized. It was the pattern of NR2B subunit protein loss that was quite different than expected. Decreases in these protein levels did not vary from controls until day three and did not significantly vary on day five. Together, these results imply that damage begins in the soma, where neuronal markers were affected by NMDA exposure first. This damage then cascades to affect NR2B protein density in the distal projections of the neuron, the dendrites. These effects are elucidated in the visual timeline provided in Figure 8.

The most likely reason for NR2B protein loss can be explained by a compensatory down regulation and subsequent reabsorption of the subunit protein. In response to excessive influxes of Ca^{2+}, the cell would attempt to regain homeostasis by internalizing and reabsorbing the receptor proteins, thus limiting the influx of the cation. It is important to note that, because the cell membrane is permeabilized prior to immunohistochemistry, NR2B staining in these experiments represents both surface

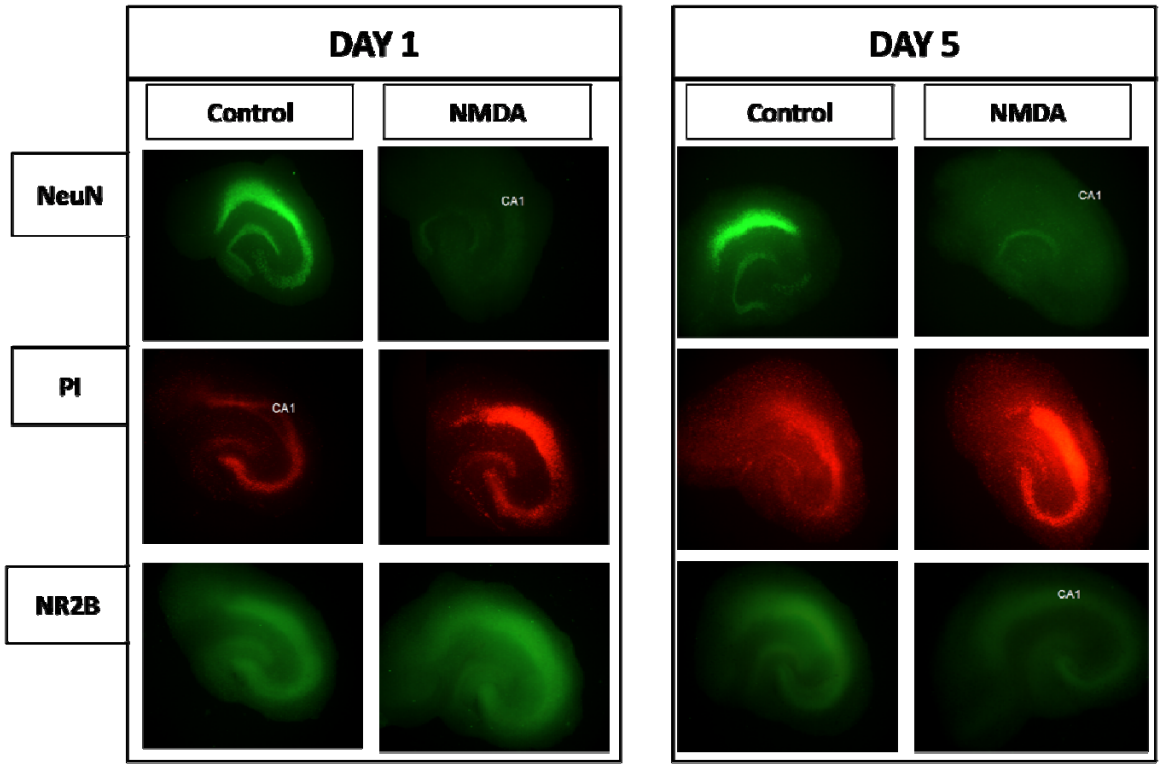

Figure 7: Visual timeline. Representative images of control and 20μM NMDA exposed hippocampal slices during days one and five on NeuN immunofluorescence, PI fluorescence, and NR2B immunofluorescence.

receptors as well as those that are intact but that have been internalized. Therefore, reductions seen in the NR2B marker would represent a total loss of the protein. Research conducted in cortical neurons by Bretin et al.(2002), found that transient exposure to 50 μM concentration of NMDA led to decreases in NR2B surface receptors but not total receptor density. To obtain a loss in total receptor density, 200 μM concentrations of NMDA were necessary. Importantly, this receptor trafficking was mediated by the enzyme calpain, which is dependent upon increased levels of intracellular Ca^{2+} concentrations for activation. Although the concentration of NMDA necessary to decrease NR2B proteins in the Bretin et al.(2006), data is 10 times that used in the current research, exposure time was measure in seconds, whereas tissue in the current research was exposed for days. Therefore, it is feasible that lengthening exposure time would

produce increases in Ca^{2+} concentrations sufficient to activate calpain, leading to the down regulation and reabsorption of NR2B subunit proteins observed. Calpain activation is most often associated with detrimental effects resulting from excitotoxicity due to is capacity to perpetuate increased intracellular Ca^{2+} concentrations by disrupting the mechanisms crucial in the maintenance of Ca^{2+} homeostasis. For example, known substrates for calpain include the PMCA transporter and Na^{+}/Ca^{2+} exchanger. As discussed previously, these plasma membrane proteins work to transport Ca^{2+} from the cytosol. Once activated, calpain is able to degrade these proteins, leaving the cell without a high or low affinity Ca^{2+} transporter (Magnusson et. al., 1993). Additional substrates for calpain include, the IP_3 and ryanodine receptors, both act to sequester Ca^{2+} in the ER; degradation of these receptors terminate their ability to function. The protective and deleterious activity of calpain elucidates the complexity and diversity of enzymes and signaling molecules activated in response to excitotoxic insult. Beneficially, however, this complexity offers several opportunities for intervention before cell death becomes inevitable.

These data indicate the search for such opportunities to block cell death mechanisms should focus on the soma. The ER and mitochondria, which are resident in the soma, are likely candidates for such research. Under physiological conditions, the ER maintains high Ca^{2+} concentrations within its membrane and expresses two Ca^{2+} mediating receptors, the IP_3 and ryanodine receptors. The IP_3 receptor is activated by IP_3, which is bound intracellularly to the plasma membrane and is co-localized with mGluRs. When mGluRs are activated by extracellular glutamate, IP_3 is released from the plasma membrane and is then free to migrate to the ER membrane. There, it can bind to

and activate IP_3 receptors, changing ER membrane permeability to Ca^{2+}, which will give rise to cytosolic Ca^{2+} levels. The ryanodine receptor, also located on the ER membrane, is activated by cytosolic Ca^{2+}. Therefore, increases in local cytosolic Ca^{2+} provided by activation of IP_3 receptors can also provide the ligand for ryanodine receptor activation, leading to a further release of ER sequestered Ca^{2+}. These increased cytosolic levels of Ca^{2+} additively affect an already excitotoxic environment. Similar to the ER, mitochondria also sequester cytosolic Ca^{2+}. This is done in response to elevated cytosolic Ca^{2+} concentrations. However, during excitotoxic conditions when mitochondrial Ca^{2+} accumulation is prolonged, the mitochondria membrane becomes depolarized and mPTPs can form; conditions that will release Ca^{2+} stores and destroy the organelle. In addition to releasing Ca^{2+} stores and further elevating cytosolic Ca^{2+} levels, compromise of the mitochondrial membrane can also lead to the release of membrane bound cytochrome c and AIF, both are initiators of apoptosis. It is possible that, by blocking the IP3 receptor on the ER membrane, the Ca^{2+} mediated Ca^{2+} release that would normally follow could be prevented. This could halt or slow the activation of death pathways, reducing overall neurodegeneration.

It is important to note that, as these data have been presented thus far, they represent one of several possible interpretations. Additionally, there has been no discussion of limitations concerning the methods employed during these experiments. As these aspects are important to all research, limitations and alternate interpretations regarding the current research project will follow.

One limitation in methodology concerns the PI marker. As explained previously, PI binds to nucleic acids. However, upon excitotoxic insult, glia cells can become

damaged and can expose nucleic acids to which PI can bind. The resulting PI signal is therefore increased from what would be evidenced from neuronal injury alone. Importantly, NeuN, the marker for mature neurons, is specific to neuronal cells. Because both markers are represented and additionally, tell the same story regarding excitotoxic damage, the increased fluorescence provided by glial contents is not believed to significantly affect the data. A second issue regarding the methods concerns the age of the animals used in this study. Because the rats are PND 8, there are certain limitations concerning the translation of data. It is important to understand that the organotypic hippocampal model is just that, a model. By definition, models are representative and data derived from them should be translated with this fact in mind. While it is likely that the mechanisms behind excitotoxicity are similar in early development and maturity, there are cellular molecules whose expression changes with age. For example, calapain 1 expression increases during develop in the rat CNS (Li, et al., 2009). Because this enzyme is implicated in mediating events associated with excitotoxicity, knowledge of how its expression changes during development is important when interpreting and translating data to more developed systems.

The method of organotypic hippocampal slice culture provides researchers many benefits including the ability to mirror in vivo circuitry. However, an important limitation especially pertinent to the current research concerns this circuitry. It has been shown that over time the CA1 subregion undergoes synaptic reorganization over time and will develop projections to the DG that are unique to in vitro cultures (Gutiérrez & Heinemann, 1999). Therefore, the excitotoxicity seen in the CA1 is perhaps increased

from what would be seen in vivo, as these projections have the ability to confer increased excitability from the DG to the CA1.

Perhaps the most important caveat to interpreting this data set revolves around the NR2B subunit. Throughout this document, it has been stated that NR2B subunit proteins are expressed in the dendrites of the pyramidal cells of the CA1. While this is true there are also populations found in the membrane of the soma. In the current interpretation, the decrease of NR2B subunits on day three is said to occur on dendritic processes. However, it is possible that the declines are occurring in those receptors populating the soma or declines may be representative of the two locations. However, because the data show that injury to the neuron begins 24 hours after exposure to NMDA and this initial injury is occurring in the soma, it is likely that the more distal dendrites would experience delayed degeneration seen on day three. Additionally, because methods used in this study have measured only protein levels, no statement can be made regarding the functionality of the remaining receptor subunits. To definitively identify what area is incurring the subunit proteins loss or to investigate the functionality of the remaining receptors will require subsequent investigation. These studies could include staining the cells with MAP-2, which is a marker for proteins that make up the structure of membranes. This marker would allow the topography of the entire neuron to be visualized using confocal microscopy. By co-labeling with MAP-2 and NR2B, it would be evident where on the neuronal membrane loss of the subunit proteins was occurring. The functionality of the receptors could be investigated using Calcium Orange. This marker carries a fluorescent tag that is activated once it passes into the membrane of a cell. By comparing Calcium Orange fluorescent values between control and NMDA

exposed slices, NMDA receptor activity could be determined, although it would not be selective for NR2B containing receptors.

In conclusion, the current research provides a temporal and topographical map of the progression of excitotoxic neurodegeneration. The data reveal that initial injury is evident in the soma. However, even after the occurrence of this significant damage, distal regions of the cell are still engaging regulatory mechanisms in an effort to stop the excitatory signal. This information could aid future research in developing therapies to diminish neurodegeneration resulting from excitotoxicity.

References

Albert B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. (2002). *The cell*. New York: Garland Science.

Al-Hallaq RA, Conrads TP, Veenstra TD, Wenthold RJ. (2007). NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. J Neurosci;27(31):8334-43.

Arndt-Jovin DJ & Jovin TM. (1989). Fluorescence Microscopy of Living Cells in Culture Part B. Quantitative Fluorescence Microscopy—Imaging and Spectroscopy. Methods in Cell Biology;30 417-48.

Arundin M & Tymianski M. (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity.Cell Calcium, 34;325–337

Arundine M & Tymianski M. (2004). Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cell. Mol. Life Sci. 61,657–668.

Albensi BC. 2007. The NMDA receptor/ion channel complex: a drug target for modulatinsynaptic plasticity and excitotoxicity. Curr Pharm Des 13(31):3185-94.

Barria A, Malinow R. (2002). Subunit-specific NMDA receptor trafficking to synapses. Neuron 35(2):345-53.

Bernardi P & Rasola A. (2007). Calcium and Cell Death: The Mitochondrial Connection. *Carafoli and M. Brini (eds.), Calcium Signalling and Disease*, 481–506.

Bretin S, Rogemond V, Marin P, Maus M, Torrens Y, Honnorat J, Glowinski J, Prémont J, Gauchy C.(2006). Calpain product of WT-CRMP2 reduces the amount of surface NR2B NMDA receptor subunit. J Neurochem 98(4):1252-65.

Butler TR, Self RL, Smith KJ, Sharrett-Field LJ, Berry JN, Littleton JM, Pauly JR, Mulholland PJ, Prendergast MA.(2001). Selective vulnerability of hippocampal cornu ammonis 1 pyramidal cells to excitotoxic insult is associated with the expression of polyamine-sensitive N-methyl-D-asparate-type glutamate receptors. Neuroscience 20;165(2):525-34.

Cater HL, Gitterman D, Davis SM, Benham CD, Morrison B III, Sundstrom LE (2007) Stretch-induced injury in organotypic hippocampal slice cultures reproduces in vivo post-traumatic neurodegeneration: role of glutamate receptors and voltage-dependent calcium channels. J Neurochem 101:434–447.

Choi DW, Jae-young Koh J, Peters S. (1988). Pharmacology of Glutamate Neurotoxicity inCortical Cell Culture: Attenuation by NMDA Antagonists. J Neuroscience 8(l): 165-I 96.

Choi DW. (1992). Excitotoxic cell death. J Neurobiol (9):1261-76.

Choi DW, Maulucci-Gedde M, and Kriegstein AR. (1987). Glutamate Neurotoxicity in Cortical Cell Culture. J Neuroscience 7(2): 357-388.

Crews CT, Newsom H, Gerber M, Sumners C, Chandler LJ, Freund G. (1993). *Alcohol, cell membranes, and signaling transduction in brain.* (C. Alling et al., Ed.) New York, NY: Plenum Press.

Cooper JR, Bloom FE, Roth RH. (2003). *The biochemical basis of neurohparmacology*. Oxford: University Press.

Crompton M. (1999). The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233-49.

Dingledine R, Borges K, Bowie D, Traynelis SF. (1999). The glutamate receptor ion channels Pharmacol Rev 51, 1.

Dong XX, Wang Y, Qin ZH. (2009). Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin 30(4):379-87.

Erreger K, Dravid SM, Banke TG, Wyllie DJ, Traynelis SF. (2005). Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. J Physiol 1;563(Pt 2):345-58.

Feldman RS, Meyer JS, Quenzer LF. (1997). *Principles of neuropsychopharmacology*. Sunderland, MA: Sinauer Associates, Inc.

Forder JP, Tymianski M.Neuroscience. (2009). Postsynaptic mechanisms of excitotoxicity: Involvement of postsynaptic density proteins, radicals, and oxidant molecules. Neuroscience 158;293–300 .

Frederickson CJ, Maret, W, Cuajungco, MP. (2004). Zinc and Excitotoxic Brain Injury: A New Model. Neuroscientist 10(1):18-25.

Fujikawa DG. (2005). Prolonged seizures and cellular injury: understanding the connection. Epilepsy Behav;7 Suppl 3:S3-11.

Gennarelli TA.(1996). The spectrum of traumatic axonal injury. Neuropathol Appl Neurobio.;22(6):509-13.

Graham D. I., McIntosh T. K., Maxwell W. L. and Nicoll J. A. (2000) Recent advances in neurotrauma. J. Neuropathol. Exp. Neurol. 59, 641–651.

Greenamyre JT, Penney JB, Young AB, D'Amato CJ, Hicks SP, Shoulson I. (1985). Alterations in L-glutamate binding in Alzheimer's and Huntington's diseases. Science.;227(4693):1496-9.

Gutiérrez R, Heinemann U. (1999). Synaptic reorganization in explanted cultures of rat hippocampus. Brain Res;815(2):304-16.

Hardingham GE, Bading H. (2003). The Yin and Yang of NMDA receptor signalling. Trends Neurosci 26(2):81-9.

Hardingham GE, Fukunaga Y, Bading H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways; 5(5):405-14.

Harris BR, Prendergast MA, Gibson DA, Rogers DT, Blanchard JA, Holley RC, Fu MC, HartSR, Pedigo NW, Littleton JM. (2002). Acamprosate inhibits the binding and neurotoxic effects of trans-ACPD, suggesting a novel site of action at metabotropic glutamate receptors. Alcohol Clin Exp Res;26(12):1779-93.

Ivanov A, Pellegrino C, Rama S, Dumalska I, Salyha Y, Ben-Ari Y, Medina I. (2006). Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. J Physiol.;572(Pt 3):789-98.

John W. Olney JW, Degubareff T, Sloviter RS. (1983). “Epileptic” brain damage in rats induced by sustained electrical stimulation of the perforant path. II. Ultrastructural analysis of acute hippocampal pathology Brain Res Bull;10 (5) 699-712.

Koch HJ, Szecsey A, Haen E. (2004). NMDA-antagonism (memantine): an alternative pharmacological therapeutic principle in Alzheimer's and vascular dementia. Curr Pharm Des.;10(3):253-9.

Köhr G. (2006). NMDA receptor function: subunit composition versus spatial distribution. Cell Tissue Res. 2006; 326(2):439-46.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL. (2002). Calcineurin acts Via the C-terminus of NR2A to modulate desensitization of NMDA receptors. Neuropharm; 42(5):593-602.

Kumari M, Ticku MK. (2000). Regulation of NMDA receptors by ethanol. Prog Drug Res.54:152-89.

Leif Hertz L. (2008). Bioenergetics of cerebral ischemia: A cellular perspective Neuropharmacology. 55;289–309.

Li J, Doyle KM, Tatlisumak T. (2007). Polyamines in the brain: distribution, biological interactions, and their potential therapeutic role in brain ischaemia. Curr Med Chem14(17):1807-13.

Magnusson A, Haug LS, Walaas SI, Ostvold AC. (1993). Calcium-induced degradation of the inositol (1,4,5)-trisphosphate receptor/Ca(2+)-channel. FEBS Lett. 1;323(3):229-32.

Marcoux FW & Choi DW. (Eds.). (1991) CNS Neuroprotection. Heidelberg, Germany: Springer.

Maulucci-Gedde, M. & Choi, DW. (1987). Cortical neurons exposed to glutamate rapidlyleak preloaded 51chromium. Exp Neurology 96,420-429.

McIlhinney RA, Philipps E, Le Bourdelles B, Grimwood S, Wafford K, Sandhu S, Whiting P. (2003). Assembly of n-methyl-f-aspartate (NMDA) receptors. Biochem Soc Trans.;31(Pt 4):865-8.

McNamara JO. (1999). Emerging insights into the genesis of epilepsy. Nature. 24;399(6738 Suppl):A15-22.

Miguel-Hidalgo JJ, Alvarez XA, Cacabelos R, Quack G. (2002). Neuroprotection by memantine against neurodegeneration induced by β-amyloid(1–40) Brain Research; 958(1), 210-21.

Mitani A, Yanase H, Sakai K, Wake Y & Kataoka K. (1993) Origin of intracellular Ca2 + elevation induced by in vitro ischemia-like condition in hippocampal slices. *Brain Research,* 601 103-110.

Mony L, Kew JN, Gunthorpe MJ, Paoletti P. (2009). Allosteric modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic potential. Br J Pharmacol.; 157(8):1301-17.

Mullen RJ, Buck CR, Smith AM. (1992). NeuN, a neuronal specific nuclear protein in vertebrates Development 116, 201-211.

Olney JW. (1986). Inciting excitotoxic cytocide among central neurons. Adv Exp Med Biol 203:63 1-645.

Ommaya AK. (1995). Head injury mechanisms and the concept of preventive management: a review and critical synthesis. J Neurotrauma;12(4):527-46.

Onodera H, Sato G, Kogure K. (1986). Lesions to Schaffer collaterals prevent ischemic death of CA1 pyramidal cells. Neurosci Lett. 68(2):169-74.

Paoletti P, Neyton J. (2006). NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol;7(1):39-47.

Prendergast MA, Harris BR, Blanchard JA 2nd, Mayer S, Gibson DA, Littleton JM. (2004). In vitro effects of ethanol withdrawal and spermidine on viability of hippocampus from male and female rat. Alcohol Clin Exp Res.;24(12):1855-61.

Renolleau S, Fau S, Charriaut-Marlangue C. (2007). Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. Neuroscientist. 14(1):46-52.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ. (2001). Molecular determinants of NMDA receptor internalization. Nat Neurosci;4(8):794-802.

Sahuquillo J, Poca1 MA, Amorós S. (2001). Current aspects of pathophysiology and cell dysfunction after severe head. Injury. *Current Pharmaceutical Design;7,*1475-1503.

Sattler R, Xiong Z, Lu W, MacDonald JF, Tymianski M. (200). Distinct Roles of Synaptic and Extrasynaptic NMDA Receptors in Excitotoxicity. Journal Neuroscience 20(1):22–33.

Stoppini L, Buchs PA, Muller D (1991). A simple method for organotypic cultures of nervous tissue. J Neurosci Methods;37(2):173-82.

Vanderklish PW, Bahr BA. (2000). The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol. 81(5):323-39.

Vanhoutte P, Bading H. (2003). Opposing roles of synaptic and extrasynaptic NMDA receptors in neuronal calcium signalling and BDNF gene regulation. Curr Opin Neurobiol.;13(3):366-71.

Vicini S, Wang JF, Hongli J, Zhu WJ, Wang YH, Lou JH, Wolfe BB, Grayson DR. (1998). Functional and Pharmacological Differences Between Recombinant *N*-Methyl-D-Aspartate Receptors. J Neurophysiol 79:555-566.

Vosler PS, Brennan CS, Chen J. (2008). Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. Mol Neurobiol. 38(1):78-100.

Wasterlain CG, Chen JW. (2008). Mechanistic and pharmacologic aspects of status epilepticus and its treatment with new antiepileptic drugs. Epilepsia. 49 Suppl 9:63-73.

Williams K, Zappia AM, Pritcheit DB, Shen YM, Molinoff PB. (1994) Sensitivity of the N-methyl-D-aspartate receptor to polyamines is controlled by NR2 subunits. Mol Pharmacol. 45(5):803-9.

Yashiro K, Philpot BD. (2008). Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology;55(7):1081-94.

Yi JH, Hazell AS. (2006). Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury.Neurochem Int;48(5):394-403.

Zimmer J, Kristensen BW, Jakobsen B, Noraberg J.(2009). Excitatory amino acid Neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures. Amino Acids.;19(1):7-21.

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PUBLICATIONS

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2. Butler TR, Smith KJ, Berry JN, **Sharrett-Field LJ**, & Prendergast MA. (2009). Sex differences in caffeine neurotoxicity are exacerbated by prior ethanol exposure and withdrawal. Alcohol Alcoholism, 44(6):567-74.

3. **Sharrett-Field LJ**, Berry JN, Butler TR, Prendergast MA (In Preparation). Somatic injury precedes distal atrophy following excitotoxic hippocampus insult.

4. Berry, JN, **Sharrett-Field LJ**, Butler TR, Prendergast MA (In Preparation). Time-dependence of distal-to-proximal hippocampal neurodegeneration produced by *N*-methyl-D-aspartate receptor activation.

PRESENTATIONS

Sharrett-Field LJ, Berry JN, Butler TR, Prendergast MA (In Preparation). Somatic injury precedes distal atrophy following excitotoxic hippocampus insult. Poster presentation, Bluegrass Neuroscience. Lexington, KY. (2010). Award Winner.

Sharrett-Field LJ, Palladino J, Bloom C. SDIQ-R; Reevaluating popular misconceptions concerning sleep and dreams. Poster presentation, annual meeting of Mid-America Undergraduate Psychology Research Conference at Thomas Moore College. (2008).

Bloom C, Harden M, Venard J, Slevin R, Fritz C & **Sharrett-Field LJ**. Obsessive-compulsive symptoms and superstitious conditioning in a non-clinical sample. Presentation, the annual meeting of American Psychological Society. Washington, D.C. (2007).

SERVICE

Bluegrass Chapter of the Society for Neuroscience Community Outreach 2009-2010

AWARDS

Graduate School Academic Non-Service Fellowship (UK)	2008-2009
Palladino/Assante Memorial Scholarship (USI)	2007
Dean's List, University of Southern Indiana	2005-2008

MEMBERSHIPS

Society for Neuroscience	2009-Current
Research Society on Alcoholism	2009-Current
Association for Psychological Science	2007-Current

TEACHING ASSISTANT LECTURES

An introduction to memory. Psychology 201. University of Southern Indiana. Evansville, IN (2007).

An introduction to Learning. Psychology 201. University of Southern Indiana. Evansville, IN (2007).