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TIME-DEPENDENCE OF DISTAL-TO-PROXIMAL HIPPOCAMPAL NEURODEGENERATION PRODUCED BY N-METHYL-D-ASPARTATE RECEPTOR ACTIVATION

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ABSTRACT OF THESIS

TIME-DEPENDENCE OF DISTAL-TO-PROXIMAL HIPPOCAMPAL NEURODEGENERATION PRODUCED BY *N*-METHYL-D-ASPARTATE RECEPTOR ACTIVATION

Excitotoxicity is the overexcitation of neurons due to the excessive activation of excitatory amino acid receptors and is thought to be involved in many neurodegenerative states. The manner in which the neuron breaks down during excitotoxicity is still unclear. The current study used the organotypic hippocampal slice culture model to examine the time-dependent loss of the synaptic vesicular protein synaptophysin and the loss of *N*-methyl-D-aspartate (NMDA) receptor NR1 subunit availability following an excitotoxic insult (20 μM NMDA) to provide a better understanding of the topographical nature of neuronal death following NMDA receptor activation. Significant NMDA-induced cytotoxicity in the CA1 region of the hippocampus (as measured by propidium iodide uptake) was evident early (15 minutes after exposure) while significant loss of the NR1 subunit and synaptophysin was found at later timepoints (72 and 24 hours, respectively), suggesting delayed downregulation or degradation in axons and dendrites as compared to the soma. The addition of the competitive NMDA receptor antagonist 2-amino-7-phosphonovaleric acid (APV) significantly attenuated all NMDA-induced effects. These results suggest that NR1 and synaptophysin levels as measured by immunoreactivity are not reliable indicators of early cell death.

KEYWORDS: NMDA Receptors, Excitotoxicity, NR1 subunit, Synaptophysin, Topographical nature of death following excitotoxicity

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THESIS

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The Graduate School

University of Kentucky

2010

TIME-DEPENDENCE OF DISTAL-TO-PROXIMAL HIPPOCAMPAL NEURODEGENERATION PRODUCED BY *N*-METHYL-D-ASPARTATE RECEPTOR ACTIVATION

THESIS

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

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Introduction

Background

Excitotoxicity is the overexcitation of neurons due to the excessive activation of excitatory amino acid receptors and is thought to be involved in many neurodegenerative states, including traumatic brain injury (TBI), ischemia, stroke, epilepsy, and amyotrophic lateral sclerosis, ALS (see Choi, 1992 for a review). In addition, it is thought to be a factor in mediating the degenerative effects of alcohol withdrawal. It is estimated that there were over 1.5 million cases of TBI in the United States in 2003, with more than 50,000 deaths (Rutland-Brown, Langlois, Thomas, & Xi, 2006). Recent advances in the medical practices of developed countries have lowered the rate of incidence for hypoxic/ischemic events to 1-2 per 1000 live births, with a mortality rate of 10-20% (Verklan, 2009). Likewise, although the number of hospitalizations for a stroke decreased from 833,000 in 1997 to 726,000 in 2004 (Fang, Alderman, Keenan, & Croft, 2007), stroke is still listed as the third leading cause of death in the United States. The 2007 Substance Abuse and Mental Health Services Administration (SAMHSA) National Survey of Drug Use and Health reported that over 50% of people age 12 and older admitted to drinking alcohol within the last 30 days. Furthermore, over 1/5 of Americans age 12 and older reported past month binge use of alcohol, defined as five or more drinks on one occasion (Substance Abuse and Mental Health Services Administration, 2008). Given the wide range of effects on cellular integrity associated with excitotoxicity and the large number of people affected each year, uncovering the mechanisms by which excitotoxicity causes damage is one of the goals of many researchers today, as it can have

implications in a host of various degenerative conditions. In particular, the amino acid glutamate and it's receptors are thought to be critical in mediating the damaging effects of excitotoxicity.

Glutamate Receptors: Structure and Function

Glutamate is the major excitatory neurotransmitter in the mammalian brain and activates both ionotropic and metabotropic receptors. While the glutamate metabotropic receptors produce actions via G-proteins (linked to these receptors) to possibly release intracellular stores of calcium (Ca^{2+}), glutamate ionotropic receptors act via ion channels to allow sodium (Na^{+}), potassium (K^{+}), and/or Ca^{2+} to flow into the cell. The glutamatergic ionotropic family of receptors includes the kainate receptor, the 2-amino-3(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptor, and the *N*-methyl-*D*-aspartate (NMDA) receptor.

AMPA receptors are heteromeric structures composed of four different subunits, GluR1-4, which usually combine to form heterotetramers. Each subunit consists of a membrane region with three transmembrane domains (TM1, 3, and 4) and a second domain which loops back within the membrane and comes out the intracellular side (M2). Thus, each subunit has an extracellular N-terminal domain and an intracellular C-terminal domain. Of the four subunits, it is only the GluR2 subunit that can undergo RNA editing in the second membrane to produce a positively charged arginine (R) residue as opposed to the neutral glutamine (Q) residue that is present on unedited GluR2 subunits (Jonas & Burnashev, 1995). This editing of the GluR2 subunit mediates the receptor's permeability to Ca^{2+} and other ions (Jonas & Burnashev, 1995). In particular, receptors

missing the GluR2 subunit exhibit a high permeability to Ca^{2+} and other cations while receptors containing the edited GluR2 and therefore the arginine residue are impermeable to Ca^{2+} (Jonas & Burnashev, 1995). Previously it was thought that all AMPA receptors were impermeable to Ca^{2+}; however, it is now known that this is not the case as AMPA receptor antagonists have now been shown to be neuroprotective against excitotoxicity (for a review, see Gill & Lodge, 1997). The AMPA receptor is blocked by polyamines under resting conditions and is only opened when the membrane becomes depolarized to reach the threshold for an action potential. Once the polyamine blockade is removed, the ion channel opens to allow Na^{+} (and Ca^{2+}, if applicable) into the cell. This further depolarizes the cell and can lead to activation of the NMDA receptor as AMPA and NMDA receptors are often co-localized in the cell membrane. This close proximity between AMPA and NMDA receptors is important in memory processes such as long-term potentiation (LTP) and long-term depression (LTD; (for a review, see Wheal, et al., 1998).

The kainate receptors, although not yet well characterized, are thought to be made up of tetramers comprised of various subunits including GluR5-7 and KA1-2 (for a review, see Heath & Shaw, 2002). Structurally, each subunit is thought to be homologous to the subunits of the AMPA receptor (Sattler & Tymianski, 2001). The GluR5-7 subunits have a relatively low affinity for kainate compared to the high-affinity KA1 and KA2 subunits (reviewed in Lodge, 2009). The high-affinity KA1 and KA2 receptor subunits cannot form functional receptors alone but can combine with GluR5 or GluR6 to form functional receptors (Lodge, 2009). Similar to the GluR2 subunit of the AMPA receptor, GluR5 and GluR6 can undergo RNA (Q/R) editing in transmembrane

domain 2; this editing is thought to mediate the channel's permeability to Ca^{2+} (Pinheiro & Mulle, 2006). The kainate receptors have been shown to both facilitate and inhibit the synaptic release of glutamate when kainate receptors are found on the presynaptic nerve cell and thus serve as autoreceptors (see Jane, Lodge, & Collingridge, 2009 for a review).

NMDA receptors are ionotropic glutamate receptors which are tetramers made up of two essential NR1 subunits and two regulatory subunits composed of NR2A-D and/or NR3A-B subunits, although NR2A and NR2B are the most commonly expressed regulatory subunits (for a review, see Cull-Candy, Brickley, & Farrant, 2001). Structurally, the subunits are similar to AMPA receptor subunits, each possessing four membrane domains (3 transmembrane-spanning domains and one membrane domain that loops back around), an extracellular N-terminal domain, and an intracellular C-terminal domain (as reviewed in Paoletti & Neyton, 2007). The ion channel is blocked (voltage-dependently) by Mg^{2+}, which is removed via membrane depolarization to allow Ca^{2+} and a small amount of Na^{+} into the cell (Lynch & Guttmann, 2001). As noted earlier, AMPA receptor depolarization is a common mechanism by which the Mg^{2+} blockade is removed. Glycine serves as a co-agonist at the NMDA receptor and must be bound to the NR1 subunit before the receptor is fully activated; however, the glutamate binding site is located on the NR2 subunit and must also be bound (Paoletti & Neyton, 2007). Thus, the opening of the NMDA receptor ion channel requires three distinct events: removal of the Mg^{2+} blockade, glycine binding, and glutamate binding (Lynch & Guttmann, 2001). Polyamines, protons, and extracellular Zn^{2+} also act allosterically to modify receptor selectivity (Cull-Candy, et al., 2001). NMDA antagonists, such as MK-801, ketamine,

and phencyclidine (PCP), are ion channel blockers which are non-competitive with NMDA (Lynch & Guttmann, 2001).

The NR1 subunit has a total of eight functional splice variants, each of which has a different sensitivity to agonist, antagonists, Ca^{2+} and other ions, as well as one nonfunctional splice variant (see Zukin & Bennett, 1995 for a review). An asparagine residue (N598) found on the second membrane domain on both NR1 and NR2 subunits regulates the permeability to Ca^{2+}, Mg^{2+}, Na^{+}, polyamines, and Zn^{2+}, with contrasting effects on the NR1 and the NR2 subunit (Burnashev, et al., 1992; Schneggenburger & Ascher, 1997; Traynelis, Burgess, Zheng, Lyuboslavsky, & Powers, 1998). Specifically, replacing the asparagine residue with a glutamine residue on the NR1 was found to slightly reduce the Mg^{2+} blockade and decrease Ca^{2+} permeability, while the same replacement on the NR2 did not significantly affect Ca^{2+} permeability but did increase Mg^{2+} permeability and more strongly reduced the Mg^{2+} blockade (Burnashev, et al., 1992).

While the NR2 and NR3 subunits are regulatory, the NR1 subunit is essential for functional NMDA receptors. Genetic deletions of the NR1 subunit have proven to be lethal within the early postnatal period (Forrest, et al., 1994). In contrast, deletion of the NR2A subunit in mice resulted in survival and normal developmental appearance (Sakimura, et al., 1995). Previous research has also shown the NR1 subunit to be essential for immediate glutamate and NMDA-mediated toxicity (Tokita, et al., 1996). Alongside the various sensitivities of the NR1 receptor, each NR2 subunit also has distinctive sensitivities which give rise to the many combinations of various receptor subtypes. Specifically, the NR2 receptor subunits each exhibit different sensitivities to

the Mg^{2+} block, glycine, and glutamate affinity (Kutsuwada, et al., 1992; Monyer, et al., 1992; Stern, Behe, Schoepfer, & Colquhoun, 1992).

Each NMDA receptor subunit demonstrates differences in their intracellular C-terminal domain sequence, which is thought to mediate the interaction of the NMDA receptor and various intracellular cytoskeletal and synaptic proteins (for reviews, see Arundine & Tymianski, 2004; Forder & Tymianski, 2009). The NMDA receptor and related proteins make up a large multiprotein structure located beneath the postsynaptic membrane known as the postsynaptic density (PSD). Postsynaptic density proteins are involved in various functions, including regulation of receptor clustering, modulation of receptor function, and cell to cell bonding (Forder & Tymianski, 2009). Moreover, there are four key types of molecules within the PSD including cytoskeletal, membrane-bound, modulatory enzymes, and scaffolding proteins (Forder & Tymianski, 2009). The interactions between the NMDA receptor and PSD proteins are highly specific, indicating that these interactions connect NMDA receptor to particular downstream signaling molecules (Arundine & Tymianski, 2004).

Glutamate Receptors and Excitotoxicity

In early studies by Lucas and Newhouse (1957), glutamate injections into the mouse retina were found to be toxic, thus suggesting that glutamate may be a neurotoxin. Later experiments by Olney (1982) showed that excessive glutamate within the retina resulted in damage to the dendrosomal, but not the axonal, part of a neuron. Thus, Olney further postulated that since most neurons within the CNS contain one or more subtypes of excitatory amino acid (EAA) receptors on the dendrite or the soma, it is likely that

neurodegeneration due to overactivation of EAA receptors is often seen in the dendrites and the soma rather than in the axon (Olney, 1994). It was also Olney (1986) who first coined the term excitotoxicity, referring to neurodegeneration via overexcitation of postsynaptic ionotropic EAA receptors. Further, research has shown that magnesium, which attenuates synaptic activity, can reduce the damage associated with anoxic/hypoxic injury (Kass & Lipton, 1982; Rothman, 1983). More recently it has been shown that glutamate NMDA receptor antagonists such as 2-amino-7-phosphonovaleric acid or 2-amino-7-phosphonohepatonic acid (APV) and MK-801 given prior to the insult can protect against ischemic-induced damage (Ozyurt, Graham, Woodruff, & McCulloch, 1988; Simon, Swan, Griffiths, & Meldrum, 1984).

Choi and colleagues (1987) found that cortical cell cultures exposed briefly to glutamate resulted in rapid morphological changes. These changes were then followed by overall neuronal degeneration (Choi, et al., 1987). Indeed, it is the NMDA glutamate receptor, not the metabotropic, AMPA, or kainate receptors, that has been implicated as the main cause of excitotoxicity within the brain (Choi, 1992). Glutamate-mediated excitotoxicity results in an array of destructive changes within the cell, including swelling and neuronal degeneration (Choi, 1992). Complete blockade of the NMDA receptor is not a viable option to treat the effects of excitotoxicity, as this attempt failed during stroke trials (Davis, et al., 2000) and can be harmful to animals (Fix, et al., 1993). Recently, however, research has been focused on the downstream effects associated with excitotoxicity, such as the production of harmful reactive oxygen species (ROS) including superoxide, nitric oxide (NO) and peroxynitrite (for a review, see Forder & Tymianski, 2009). Nitric oxide is known to be an important messenger molecule in

several processes, but was also found to be generated following glutamate exposure via NMDA receptor activation in primary cortical cell cultures (Dawson, Dawson, London, Bredt, & Snyder, 1991). The same study found that NOS inhibitors completely blocked the excitotoxic effects of NMDA (Dawson, et al., 1991). Post-synaptic density proteins have also been investigated as possible mechanisms by which excitotoxicity can be reduced (Forder & Tymianski, 2009). Within the PSD domain, the protein PSD-95 plays an important structural role in connecting the NMDA receptor with signaling enzymes and intracellular proteins (Forder & Tymianski, 2009). A recent review suggested that PSD-95 inhibitors may be useful in the treatment of brain damage as a result of severe hyperthermia as well as ischemic insult, if given within hours of the insult, and could be useful in the treatment of stroke as well (Forder & Tymianski, 2009).

Glutamate-mediated Excitotoxicity: The Role of Calcium

One important ion intimately linked to the damaging effects of excitotoxicity is calcium (Ca^{2+}), which is also critical to many cellular processes, including cell growth and differentiation as well as synaptic movement. Neurons are able to control the intracellular Ca^{2+} concentration and the flow of Ca^{2+} ions via the interactions of Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} buffering, and internal Ca^{2+} storage (Sattler & Tymianski, 2000). A pioneer study by Schanne and colleagues discovered that hepatocyte survival was dependent on whether the membrane toxin contained Ca^{2+} (Schanne, Kane, Young, & Farber, 1979).

Although small, physiologically relevant amounts of Ca^{2+} are necessary for the cell to function properly, excessive Ca^{2+} influx can overcome regulatory mechanisms and

activate a number of enzymes, including proteases, phospholipases, and endonucleases, eventually leading to cell death (Choi, 1995; Sattler & Tymianski, 2000). Proteases, such as calpains, are Ca^{2+}-activated enzymes which have been shown to be mediated by glutamate receptor activation and can degrade several structural proteins, including microtubule-associated proteins, tubulin, and spectrin (Siman & Noszek, 1988). Thus, proteases destroy the cell via breakdown of the cytoskeleton. An earlier study by Siman and colleagues found that EAA-induced calpain I activation is closely associated with EAA-induced hippocampal damage since only those doses of EAAs which produce hippocampal damage also result in calpain I activation (Siman, Noszek, & Kegerise, 1989). Increased Ca^{2+} levels can also activate phospholipases, enzymes which hydrolyze phospholipids into fatty acids, which can produce arachidonic acid and lead to the production of free radicals (Sattler & Tymianski, 2000). Moreover, elevated Ca^{2+} levels can activate endonucleases, enzymes which cleave the phosphodiester bond, which can lead to DNA degradation (Sattler & Tymianski, 2000). Increased cytosolic Ca^{2+} can also work in conjunction with diacylglycerol to activate protein kinase C, which can lead to further Ca^{2+} release from intracellular stores such as the endoplasmic reticulum (Choi, 1988). Furthermore, elevated intracellular Ca^{2+} levels can also activate Ca^{2+}-binding proteins such as calmodulin to produce NO synthase (cytochrome P450-related enzymes), which can then produce NO, and eventually leads to the production of peroxynitrite ($ONOO^-$) and cell death (Nicotera & Orrenius, 1998; Sattler & Tymianski, 2000). In addition, the Ca^{2+}/calmodulin-regulated protein phosphotase calcineurin is activated by Ca^{2+} influx. Calcineurin inhibitors (i.e. cyclosporine A and FK-506) have been shown to be neuroprotective against the early necrotic and apoptotic effects of high

glutamate exposure (Ankarcrona, Dypbukt, Orrenius, & Nicotera, 1996). The same study also found that cyclosporine A prohibited the breakdown of the mitochondrial membrane potential which is normally associated with glutamate-induced necrosis (Ankarcrona, et al., 1996). Recently, it has been shown that the axonal damage induced by excessive glutamate is mediated by the cleavage or phosphorylation of the collapsin response mediator protein 2 (CRMP2) via activation of calpain or the Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), respectively (Hou, et al., 2009).

Interestingly, not all influxes of Ca^{2+} are damaging to the cell. The source-specificity hypothesis was established based on previous experiments showing that Ca^{2+}influx via L-type voltage-sensitive Ca^{2+} channels were not harmful to the cell while the same amount of influx via NMDA receptors resulted in cytotoxicity (Sattler, Charlton, Hafner, & Tymianski, 1998; Tymianski, Charlton, Carlen, & Tator, 1993). Thus, the location of Ca^{2+} entry and subsequent activation of signaling pathways mediates the toxicity associated with elevated Ca^{2+} levels. It has been postulated that Ca^{2+} influx via the NMDA receptor results in high Ca^{2+} concentrations in the vicinity of specific signaling molecules which have been linked to the NMDA receptor, thus initiating distinct cell death pathways (Sattler, et al., 1998). The destructive pathways have also been shown to be more easily activated when the Ca^{2+} influx is due to glutamate receptors which have specialized entry points (Arundine & Tymianski, 2004).

Berdichevsky and colleagues were the first to suggest that increased Ca^{2+} may be the mechanism mediating the damaging effects of both NMDA and glutamate exposure (Berdichevsky, Riveros, Sanchez-Armass, & Orrego, 1983). Choi (1985) expanded this theory by studying the effects of removing extracellular Na^{+} and extracellular Ca^{2+}

following a brief glutamate exposure in cortical cell cultures. Results showed that removing extracellular Na^+ produced an immediate reduction in neuronal loss, but only a minor reduction in loss measured 24 hours later (Choi, 1985). In contrast, removing extracellular Ca^{2+} produced little immediate changes in cell death but marked reductions in neuronal loss measured 24 hours later (Choi, 1985). However, not all researchers agreed that Ca^{2+} was the main mediator behind glutamate neurotoxicity. One early study found that acute glutamate toxicity in the chick embryo retina was dependent on Na^+ and Cl^-, but not Ca^{2+} (Olney, Price, Samson, & Labruyere, 1986). However, as the authors suggest, Ca^{2+} may play a role in mediating the more delayed effects of glutamate neurotoxicity (Olney, et al., 1986).

Choi (1987) first suggested that glutamate-mediated neurotoxicity can be divided into two components. The first stage involves neuronal swelling, depends on Na^+ and Cl^- extracellular concentrations, can be mimicked by high K^+, and occurs early. The second stage involves neuronal degeneration, depends on Ca^{2+}, and occurs later. Choi (1987) also suggested that while both components can produce damage, it is the second Ca^{2+}-dependent component which mediates the damage induced by low glutamate exposure.

Ca^{2+}-mediated Excitotoxicity in Ischemia

Calcium-mediated excitotoxicity is often implicated in ischemia-related injury, as the Ca^{2+}-mediated death pathways become overactive after ischemic injury. Initially following the ischemic injury, the cellular energy levels decline as a result of decreased oxygen and glucose, leading to decreased glutamate transporter activity and thus, a buildup of extracellular glutamate (for a review, see Arundine & Tymianski, 2004;

Camacho & Massieu, 2006). As discussed earlier, the accumulation of glutamate can lead to excessive activation of glutamate receptors, in particular the NMDA receptor, which is highly permeable to Ca^{2+}. Early studies revealed that cell death due to ischemic injuries was preventable by the addition of Mg^{2+} (Kass & Lipton, 1982; Rothman, 1983). It was later found that ischemia-induced cell death was mediated by the release of EAAs, which the author suggested was most likely glutamate or aspartate (Rothman, 1984). Indeed, the NMDA receptor has been repeatedly implicated in hypoxic/ischemic injury (Meldrum, Evans, Swan, & Simon, 1987; Simon, et al., 1984). Recent studies have shown that disrupting the NMDA receptor-PSD 95 interaction may be a useful treatment for ischemia-related injuries (Aarts, et al., 2002). Furthermore, calpain inhibitors and the removal of Ca^{2+} have been shown effective in reducing ischemia-induced cell death (Brana, Benham, & Sundstrom, 1999; Underhill & Goldberg, 2007).

Ca^{2+}-mediated Excitotoxicity in Traumatic Brain Injury

Calcium-induced excitotoxicity has also been shown to be involved in the effects elicited after a traumatic brain injury (TBI). Cell death can occur as a direct result of the central nervous system (CNS) tissue perturbation (primary injury) or as a result of the mechanisms triggered by the surviving cells (secondary injury), leading to further damage or death (for a review, see Arundine, et al., 2003). Recently, this theory was affirmed by studies which found that hippocampal neurons surviving the initial insult had increased intracellular Ca^{2+} levels as well as long-lasting abnormalities in mechanisms maintaining Ca^{2+} homeostasis (Sun, et al., 2008). Moreover, administration of the calpain inhibitor MDL28170 has been shown to improve both the locomotor function as

well as the pathological outcome following a spinal cord injury, suggesting that excitotoxic injury to the CNS may be in part mediated by Ca^{2+}-dependent pathways (Yu, Joshi, & Geddes, 2008).

Ca^{2+}-mediated Excitotoxicity in Alcohol Abuse & Withdrawal

Acutely, alcohol inhibits the excitatory glutamate NMDA receptors while facilitating the actions of chloride at the inhibitory γ-amino butyric acid (GABA) receptor (see Nagy, 2008 for a review). Conversely, chronic alcohol can lead to a compensatory upregulation of NMDA receptors. In particular, chronic ethanol resulted in an upregulation of the NMDA receptor subunits NR1, NR2A, and NR2B following a western blot analysis (Kalluri, Mehta, & Ticku, 1998). This increase in NMDA receptors attempts to counteract the effect of acute alcohol; however, the increase in NMDA receptors and subsequent overactivation during ethanol withdrawal is also thought to mediate the damaging effects associated with alcohol withdrawal (Allgaier, 2002). Rodent models investigating ethanol withdrawal lend support for the NMDA receptor hyperexcitability theory, as ethanol-dependent rats given NMDA while in the midst of ethanol withdrawal were more prone to handling-induced seizures; however, administration of the NMDA receptor antagonist MK-801 to ethanol-dependent rats during withdrawal resulted in decreased frequency and severity of handling-induced seizures (Grant, Valverius, Hudspith, & Tabakoff, 1990). Furthermore, NMDA receptor antagonists have been shown to block the toxicity and Ca^{2+} influx associated with ethanol withdrawal (Mayer, et al., 2002b).

Ca^{2+}-mediated Excitotoxicity: Role of the NMDA Receptor

Seemingly, since the NMDA receptor is more permeable to Ca^{2+} than the AMPA or kainate receptor and Ca^{2+} is thought to be a key mediator of excitotoxicity, it has been the focus of much research over the past two decades. An earlier study found that the addition of the competitive NMDA receptor antagonist APV dose dependently blocked NMDA-induced excitotoxicity and reduced glutamate-induced excitotoxicity (Choi, Koh, & Peters, 1988). In the same study, it was found that other NMDA receptor antagonists reduced glutamate mediated excitotoxicity while AMPA or kainate receptor antagonists did not, suggesting that glutamate-induced excitotoxicity is likely mediated, at least in part, by NMDA receptors (Choi, et al., 1988).

The NMDA receptor has also been implicated in the Ca^{2+}-dependent pathways leading to necrosis and/or apoptosis. Early studies revealed that NMDA receptor activation leads to the activation of a neuronal protein phosphatase, presumably the Ca^{2+}-dependent phosphatase calcineurin, to dephosphorylate the protein dopamine and cyclic AMP –regulated phosphoprotein-32 (DARPP-32), an effect blocked by the addition of the competitive NMDA antagonist APV (Halpain, Girault, & Greengard, 1990). Another study found that stimulation of the NMDA receptor leads to activation of the Ca^{2+}-dependent protease calpain (Gellerman, Bi, & Baudry, 1997). One recent study found that both NMDA and non-NMDA glutamate receptor agonism activated calpain; however, only the NMDA antagonist was effective at reducing glutamate-induced toxicity (Del Rio, Montiel, & Massieu, 2008).

Brief NMDA exposure has also been shown to have effects on the dendrites of the neuron. More specifically, brief exposure to NMDA (as little as five minute exposure at

50 μM concentration) resulted in a loss of dendritic spines and filamentous actin staining at synapses (Halpain, Hipolito, & Saffer, 1998). The effect of NMDA on actin was significantly attenuated by pretreatment with an actin stabilizer or by a calcineurin inhibitor, ascomycin, suggesting that calcineurin inhibitors could be useful in attenuating glutamate-mediated effects on actin stability (Halpain, et al., 1998). Similarly, a brief NMDA exposure (30 μM for 10 minutes) resulted in dendritic swelling first evident in the most distal branches and later evident in the more proximal branches (Hoskison, Yanagawa, Obata, & Shuttleworth, 2007). The same study found that immunoreactivity for the microtubule-associated protein MAP2 was also lost progressively from the distal dendritic branches and that this loss in MAP2, as well as the damage seen in the dendrites, was Ca^{2+} dependent (Hoskison, et al., 2007).

Experimental Rationale

These studies examined the time-dependent loss of the synaptic vesicular protein synaptophysin and the loss of NMDA receptor NR1 subunit availability following an excitotoxic insult to provide a better understanding of the topographical nature of neuronal death following NMDA receptor activation. Thus, immunoreactivity of a synaptic vesicle protein (synaptophysin) present mainly in axons, a cell death/damage marker (propidium iodide) which binds nucleic acids within the cell body, and an antibody against the NMDA NR1 subunit present mainly on dendrites were used to assess the time-dependent cellular breakdown of NMDA-induced excitotoxicity. The topographical "mapping" of cellular injury or death following EAA receptor activation is important because previous studies indicate the cell may not die all at once (Hoskison, et

al., 2007) but that neuronal death may be dependent on the part of the neuron that is injured and the amount of time following the insult. For example, previous research has indicated that the dendrites may be sensitive to rapid glutamate-induced excitotoxicity (Hoskison, et al., 2007). Thus, if the manner in which the neuron breaks down can be elucidated, novel therapeutics can target that particular part of the neuron first.

Chapter Two

Methods

Organotypic Hippocampal Cell Culture

Eight day old male and female Spraque-Dawley rats (acquired from Harlan Laboratories; Indianapolis, IN) were humanely sacrificed and the brains aseptically removed. Following removal, brains were transferred to ice-cold dissecting media, composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 µM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were removed in whole and cleaned of extra tissue under a dissecting microscope. Hippocampi were then placed into culture media, which contains dissecting medium along with distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), and 0.05% streptomycin/penicillin (Invitrogen). Unilateral hippocampi were sectioned at 200 µm using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Following sectioning, three intact hippocampal slices were plated onto Millicell-CM 0.4 µm biopore membrane inserts with 1 mL of pre-incubated culture media added to the bottom of each well of a six well plate, yielding a total of 18 intact slices per plate. Excess culture medium was aspirated off of the top of each well and the plates were then incubated at 37°C with a gas composition of 5% CO_2/95% air for 5 days to allow tissue to affix to the Teflon membrane. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Drug Exposure

At 5 days in vitro, slices were randomly transferred to new culture plates containing either 1 mL of culture media or either NMDA (20 μM; Sigma), APV (50 μM; Sigma) or NDMA + APV (same concentrations as above) dissolved in 1 mL of culture media. The concentration of NMDA chosen has been shown to be effective in producing cytotoxicity in previous studies (Mulholland & Prendergast, 2003). The concentration of APV was chosen based on preliminary data indicating that 20 μM was not able to significantly overcome the neurotoxic effects of 20 μM NMDA (not shown). Propidium Iodide (PI; 3.74 μM; Molecular Probes Eugene, OR) was also added to each well. PI is a nucleic stain used to detect cell damage and was used to measure cytotoxicity as described below.

At 24, 72, and 120 hours following the initial NMDA and PI exposure, tissue slices were imaged for cytotoxicity and subsequently fixed to assess NMDA NR1 subunit and synaptophysin immunoreactivity by transferring the hippocampal slice insert to a plate containing 1 mL of 10% formalin solution on the bottom of each well. One mL of formalin was also placed on top of the insert and the plates were allowed to sit for 30 minutes. The slices were then washed carefully (1 mL on bottom of the well and 1 mL on top of the well) with 1% phosphate buffered saline (PBS) twice and stored with 1 mL of 1% PBS on the bottom of the well overnight at 4°C. All experiments were replicated at least twice, with each experimental condition (treatment by exposure time by sex within region) containing 18-27 slices.

In a final series of experiments, slices (5 DIV) were exposed to either NMDA alone (20 μM) or control conditions to investigate the rapid timecourse of NMDA-

induced cytotoxicity. This experiment was conducted to determine if less than 24 hours of exposure to NMDA produced changes in cytotoxicity or synaptophysin immunoreactivity. At 15 or 120 minutes following the initial NMDA and PI exposure, slices were imaged for cytotoxicity and immediately fixed as described above for synaptophysin immunohistochemistry. This experiment was replicated twice, yielding a total of 18 slices per experimental condition (treatment by exposure time by sex within region).

Cytotoxicity assessment

Cytotoxicity (propidium iodide staining of neurons and glia with damaged membranes) was measured in the granule cells of the dentate gyrus (DG), the pyramidal cell layer of the cornu ammonis 3 (CA3) and the pyramidal cell layer of the cornu ammonis 1 (CA1) regions of the hippocampal formation using fluorescent microscopy. It has been shown previously that PI reliably correlates with other measures of cell death (for a review, see Zimmer, Kristensen, Jakobsen, & Noraberg, 2000). Slices were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) using a 5x objective with a Leica DMIRB microscope (w. Nuhsbahm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsburg). PI has an emission wavelength of 620 nm in the visual range and a peak excitation wavelength of 536 nm and was excited using a band-pass filter which emits a range of wavelengths (510-560 nm). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the PI

fluorescence. A background measurement was taken from the visual field surrounding each slice and was subsequently subtracted from the regional measurements of each slice. The fluorescent intensity was measured in each of the three regions of interest: the granule cell layer of the dentate gyrus and the pyramidal cell layers of the CA3 and CA1 regions of the hippocampus. Within each region, measurements of PI uptake were converted to percent control to limit potential differences between replications across weeks.

Immunohistochemistry

Following overnight storage, the inserts were transferred to a plate containing 1 mL of permeabilization buffer (200 mL 1% PBS [Invitrogen], 200 µL Triton X-100 [Sigma], 0.010 mg Bovine Serum [Sigma]) in each well and 1 mL of PBS buffer was also placed on top of each insert containing the slices. The slices were allowed to sit in buffer for 45 minutes to allow the buffer to penetrate the slice and were then washed twice with 1% PBS as described earlier. Inserts were then transferred to a plate containing 1 mL of 1% PBS on the bottom of each well and were treated with 1 mL of buffer containing either mouse anti-NR1 (1:100; BD Biosciences PharMingen, San Jose, CA) or mouse anti-synaptophysin (1:200; Sigma) on top of each well. Plates were then stored at 4°C for 24 hours. Following 24 hours, the slices were washed gently with 1% PBS twice and were again transferred to a plate containing 1 mL of 1% PBS on bottom. At this point, slices were treated with 1 mL of buffer containing the goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100; Sigma) on top of the insert and were stored at 4°C for 24 hours. After 24 hours, slices were washed twice with

1% PBS as described previously and placed into a plate containing 1 mL of 1% PBS on the bottom of each well. The slices were then imaged immediately. A range of concentrations was previously tested and the concentrations of NR1, synaptophysin, and FITC antibodies chosen were shown to have the least amount of background signal while maintaining a strong, specific signal within the regions of interest.

NMDA NR1 subunit density assessment

To assess the effects of NMDA-induced excitotoxicity on NR1 subunit expression, immunoreactivity was measured in the dentate gyrus, CA1 and CA3 regions of the hippocampus using FITC fluorescence. The slices were visualized as described previously above. The secondary antibody with FITC was excited using a band-pass filter at 495 nm (520 nm emission). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the FITC fluorescence. A background measurement of fluorescence was taken from the visual field surrounding each slice and was subsequently subtracted from the region measurements of each slice before analysis. The intensity was again measured in each of the three regions of interest: the granule cell layer of the dentate gyrus and the pyramidal cell layers of the CA3 and CA1 regions of the hippocampus. To minimize the variability between each replication, measurements of FITC immunoreactivity were converted to percent control for each region.

Synaptophysin assessment

To assess the effects of NMDA-induced cytotoxicity on the synaptic vesicular protein synaptophysin, measurement of synaptophysin in the dentate gyrus, CA1 and CA3 regions of the hippocampus were visualized using fluorescent microscopy. The slices were again visualized as described previously. The secondary antibody conjugated to FITC was excited using a band-pass filter at 495 nm (520 nm emission). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was again used to measure the intensity of the FITC fluorescence. A background measurement of fluorescence was taken from the visual field surrounding each slice and was subsequently subtracted from the region measurements of each slice before analysis. The intensity was measured in each of the three regions of interest: the granule cell layer of the dentate gyrus and the pyramidal cell layers of the CA3 and CA1 regions of the hippocampus. Within each region, measurements of FITC immunoreactivity were converted to percent control prior to analysis.

Statistical analyses

Each experiment was conducted a minimum of 2 times using different rat litters. Data from each replication were converted into percent control values and were subsequently combined since litter effects were absent, yielding 18-27 slices per condition (treatment by exposure time by sex within each region). Previous studies have shown a differential vulnerability between the regions of the hippocampus, with the CA1 region being the most vulnerable to excitotoxic insult (Butler, et al., 2010). Thus, a two-way analysis of variance test (ANOVA) was conducted (region x exposure time) on the raw control values for differences in regions across time for each measurement (PI, NR1

immunoreactivity, and synaptophysin immunoreactivity). For experiments examining the effects of NMDA and APV, a three-way ANOVA was conducted (treatment x exposure time x sex) for each region of the hippocampus (DG, CA1, CA3). The levels of treatment were NMDA alone, APV alone, or NDMA+APV exposure. The levels of time were 24, 72, and 120 hours following initial NMDA exposure. Thus, treatment groups were compared to the time-appropriate control condition. For experiments investigating the rapid timecourse of NMDA-induced toxicity (without APV), a three-way ANOVA was conducted (treatment x exposure time x sex) for each region of the hippocampus (DG, CA1, CA3). For this experiment, the levels of time were 15 and 120 minutes; the treatment (NMDA alone) groups were compared to the time-appropriate control condition. Outliers were identified using Grubbs' test for outliers and were subsequently removed from all further analyses. When appropriate, post-hoc tests were conducted using Fisher's LSD to examine further effects. Statistical significance was set at $p<0.05$.

Chapter Three

Results

Raw control values

The raw values from control-treated cultures were examined for regional differences in all markers across time. PI fluorescence is measured in arbitrary optical units, reflecting pixel intensity in the defined region. With regard to control levels of PI uptake, main effects for both region (CA1/CA3/DG; $F(2, 315)=56.464$, $p<0.001$) and time (24/72/120 hour exposure; $F(2, 315)=15.264$, $p<0.001$) were found. A significant interaction between region and time was also found (Figure 3.1A; $F(4, 315)=17.571$, $p<0.001$). Within the CA1, PI uptake following 120 hour exposure was significantly more from that seen following either 72 hour exposure (21.426 vs. 16.789 arbitrary units, respectively; Fisher's LSD post-hoc, $p < 0.05$) or 24 hour exposure (13.915; Fisher's LSD post-hoc, $p < 0.05$). The arbitrary level of PI uptake in the CA1 following 72 hour exposure was also significantly more than that following 24 hour exposure (Fisher's LSD post-hoc, $p < 0.05$). In contrast, within the CA3, PI uptake in cultures exposed to control treatment for 120 hour was significantly less than that seen following 24 hour exposure (18.072 vs. 21.454 arbitrary units, respectively; Fisher's LSD post-hoc, $p < 0.05$). The arbitrary units of PI uptake in the DG region following 120 hour exposure was significantly greater than that seen following either 72 hour exposure (16.508 vs. 13.723, respectively; Fisher's LSD post-hoc, $p < 0.05$) or 24 hour exposure (11.432; Fisher's LSD post-hoc, $p < 0.05$). Further, the control raw level of PI uptake in the DG following 72 hour exposure was significantly greater as compared to 24 hour exposure (Fisher's LSD post-hoc, $p < 0.05$).

In regard to region within time, in the 120 hour exposed group, the CA1 was found to have the greatest raw value of PI uptake as compared to either the CA3 (Figure 3.1A; 21.426 vs. 18.072 arbitrary units, respectively; Fisher's LSD post-hoc, $p < 0.05$) or the DG (16.508; Fisher's LSD post-hoc, $p < 0.05$). Conversely, following 72 hour exposure, the CA3 region of the hippocampus had the greatest arbitrary level of PI uptake as compared to either the CA1 (19.886 vs. 16.789, respectively; Fisher's LSD post-hoc, $p < 0.05$) or the DG region (13.723; Fisher's LSD post-hoc, $p < 0.05$). Also following 72 hour exposure time, the CA1 had significantly greater raw levels of PI uptake compared to the DG region (Fisher's LSD post-hoc, $p < 0.05$). Similarly, at the 24 hour timepoint, the CA3 region had the greatest level of raw PI uptake as compared to either the CA1 (21.454 vs. 13.915, respectively; Fisher's LSD post-hoc, $p < 0.05$) or the DG (11.432; Fisher's LSD post-hoc, $p < 0.05$) regions of the hippocampus and the CA1 also had a significantly greater level of raw PI uptake as compared to the DG region (Fisher's LSD post-hoc, $p < 0.05$).

The raw control values of NR1 immunoreactivity were also examined for differences among regions across the timepoints. The two-way (region by exposure time) ANOVA revealed a main effect of region, $F(2, 315)=176.967$, $p<0.001$ as well as a significant interaction between region and exposure time, (Figure 3.1B; $F(4, 315)=4.541$, $p<0.001$). Further analysis of the interaction revealed that within the CA3 region, raw NR1 immunoreactivity levels were significantly lower following 120 exposure than either 72 hour exposure (11.139 vs. 13.467 arbitrary units, respectively; Fisher's LSD post-hoc, $p < 0.05$) or 24 hour exposure (13.943; Fisher's LSD post-hoc, $p < 0.05$).

There were no significant differences in NR1 immunoreactivity among timepoints within the CA1 or the DG.

Within each timepoint, the CA1 region consistently had greater raw levels of NR1 immunoreactivity as compared to either the CA3 (Fisher's LSD post-hoc, $p < 0.05$) or DG regions of the hippocampus. Further, following either 24 or 72 hour exposure, raw NR1 immunoreactivity levels in the CA3 were significantly higher (13.943 and 13.467, respectively) than that seen in the DG from the same timepoints (12.548 and 12.099, respectively; Fisher's LSD post-hoc, $p < 0.05$). Conversely, following 120 hour exposure, the DG had greater levels of raw NR1 immunoreactivity as compared to the CA3 within the same exposure time (12.506 vs. 11.139, respectively; Fisher's LSD post-hoc, $p < 0.05$).

The raw control values of synaptophysin immunoreactivity were also examined for differences among regions across time. The two-way ANOVA revealed significant main effects of both region ($F(2, 315)=185.558$, $p<0.001$) and exposure time ($F(2, 315)=49.244$, $p<0.001$). A significant interaction between region and exposure time was also revealed (Figure 3.1C; $F(4, 315)=10.161$, $p<0.001$). Within each region of the hippocampus, the raw value of synaptophysin immunoreactivity following 120 hour exposure was significantly greater than that seen following either 72 hour exposure (Fisher's LSD post-hoc, $p < 0.05$) or 24 hour exposure (Fisher's LSD post-hoc, $p < 0.05$). Further, within the CA1 region only, the raw value of synaptophysin immunoreactivity following 72 hour exposure was significantly greater than that seen following 24 hour exposure (26.727 vs. 20.128, respectively; Fisher's LSD post-hoc, $p < 0.05$).

Within each timepoint, the CA1 region of the hippocampus had significantly greater values of raw synaptophysin immunoreactivity compared to either the CA3 (Figure 3.1C; Fisher's LSD post-hoc, $p < 0.05$) or DG (Fisher's LSD post-hoc, $p < 0.05$) regions. There were no significant region differences between the CA3 and DG at any of the timepoints examined.

NMDA-induced cytotoxicity

Studies were conducted to assess the effect of the glutamate NMDA receptor agonist NMDA alone, the NMDA competitive antagonist APV alone, and NMDA with APV at various timepoints (24, 72, and 120 hours) on PI uptake, a measure of cell death or damage. As there was no difference in PI uptake between sexes within each treatment group, data was collapsed over the sex variable. Thus, a two-way (treatment by exposure time) within each region was conducted. Within the CA1 region of the hippocampus, significant main effects were found for both treatment (CTRL/NMDA/APV/NMDA+APV; $F(3, 420)=399.33$, $p<0.001$) and time (24/72/120 hour exposure; $F(2, 420)=35.586$, $p<0.001$). Interestingly, a significant interaction between treatment and time was also observed (Figure 3.2; $F(6, 420=30.842$, $p<0.001$). Thus, within each timepoint, NMDA exposure resulted in a significant increase in PI uptake compared to control and APV treated tissue, and the addition of the competitive NMDA antagonist APV significantly attenuated this increase (Fisher's LSD post-hoc, $p < 0.05$). Co-exposure of NMDA and APV for 24 or 72 hours resulted in a small (~130% control), but significant, increase in PI uptake compared to control- and APV-treated cultures (Fisher's LSD post-hoc, $p < 0.05$); however, this effect was not seen following

120 hour exposure. Hence, the addition of APV significantly decreased NMDA-induced toxicity, though not to control levels before 120 hours of exposure.

When looking at the effects of time within treatment groups, the effect of NMDA exposure on PI uptake within the CA1 was time dependent, such that the most robust NMDA-induced PI uptake was seen at the 24 hour timepoint (~375% control) while a smaller, yet still significant, increase in PI uptake was seen at the 72 and 120 hour timepoints (~290% and 198 % control, respectively; Fisher's LSD post-hoc, $p < 0.05$). Thus, PI uptake following NMDA exposure for 72 and 120 hours was significantly different from that seen following 24 hour NMDA exposure (Figure 3.2; Fisher's LSD post-hoc, $p < 0.05$). Similarly, PI uptake after 120 hour NMDA exposure was significantly different from that seen at the 72 hour timepoint (Fisher's LSD post-hoc, $p < 0.05$).

A significant main effect of treatment ($F(3, 420=14.80$, $p<0.001$) and time ($F(2, 420=3.173$, $p<0.05$) was observed in the CA3 while no significant interaction was found (Figure 3.2). Collapsed across time, significant increases in PI uptake were found in cultures exposed to NMDA or co-exposed to NMDA+APV (~115% control) compared to control and APV alone treated cultures (Fisher's LSD post-hoc, $p < 0.05$). Further, data collapsed across treatment groups revealed a significant difference among timepoints, such that PI uptake following either 24 or 72 hour exposure was significantly greater than PI uptake following 120 hour exposure (110% control and 109% control vs. 102% control, respectively; Fisher's LSD post-hoc, $p < 0.05$).

Within the DG, a significant main effect of treatment was found ($F(3, 420)=23.367$, $p<0.001$); however, no significant main effect of time or significant

interaction between time and treatment was observed (Figure 3.2). Collapsed across time, both NMDA and NMDA+APV treated cultures resulted in a significant increase (~120% control) in PI uptake as compared to control and APV alone treated cultures (Fisher's LSD post-hoc, $p < 0.05$); the effect of NMDA was also significantly attenuated by co-exposure with APV (~125% control vs. ~116% control, respectively; Fisher's LSD post-hoc, $p < 0.05$). Representative images of these effects are presented in Figure 3.3.

NMDA-induced loss of NR1 Subunit Immunoreactivity

Further studies were conducted to assess the effects of NMDA, APV, and NMDA+APV exposure on the NMDA receptor subunit 1 immunoreactivity. As with the PI measurement, sex differences were not observed measuring NR1 subunit expression and the data were combined. Within the CA1 region of the hippocampus, a significant main effect of treatment was found ($F(3, 420)=20.286$, $p<0.001$). Also in the CA1, there was no significant main effect of time; however, a significant interaction of treatment and time was observed (Figure 3.4; $F(6, 420)=4.392$, $p<0.001$). In regard to the interaction of treatment within time, exposure to NMDA caused a time-dependent decrease in NR1 immunoreactivity in the CA1 region such that 24 hour exposure to NMDA resulted in no significant change in NR1 immunoreactivity compared to control- or APV-treated cultures, while 72 and 120 hour exposure to NMDA resulted in a significant decrease (88% control and 80% control, respectively) below that seen in control- or APV-treated cultures (Fisher's LSD post-hoc, $p < 0.05$). This effect of NMDA in the CA1 was blocked by the co-exposure of NMDA with APV, as NR1 immunoreactivity levels of NMDA+APV treated cultures at 72 and 120 hour timepoints were significantly higher

(109% control and 107% control, respectively) than NR1 immunoreactivity levels seen in NMDA treated cultures (88% control and 80% control, respectively; Fisher's LSD post-hoc, $p < 0.05$). At the 72 hour timepoint, APV co-exposure prevented NMDA-induced decreases in NR1 immunoreactivity to levels above control-treated cultures (107% control for APV-treated cultures; Fisher's LSD post-hoc, $p < 0.05$). At the 120 hour timepoint, exposure to APV alone resulted in a significant increase in NR1 immunoreactivity in the CA1 region as compared to control cultures (109% control; Fisher's LSD post-hoc, $p < 0.05$).

Further, in regard to the factor of time within treatment groups, NR1 subunit immunoreactivity in the CA1 following NMDA exposure for 72 and 120 hours was significantly less than that seen following 24 hour NMDA exposure (88% control and 80% control vs. 98% control, respectively; Fisher's LSD post-hoc, $p < 0.05$). In fact, following 120 hour exposure to NMDA alone, a significant decrease in NR1 immunoreactivity in the CA1 was found as compared to 72 hour exposure to NMDA alone (Fisher's LSD post-hoc, $p < 0.05$). Thus, each timepoint was significantly different than the others when treated with NMDA alone.

As seen with the PI data, no significant interaction was discovered within the CA3 region; however, a significant main effect of time on NR1 immunoreactivity within the CA3 was observed (Figure 3.4; $F(2, 420)=4.084$, $p<0.05$). Collapsed across treatment, cultures exposed for 24 or 72 hours resulted in significant decreases in NR1 immunoreactivity in the CA3 as compared to cultures exposed for 120 hours.

No significant main effects of treatment or exposure time were observed in the DG region of the hippocampus (Figure 3.4). Results also showed no significant

interaction between treatment and time within the DG. Representative images of hippocampal slices are shown in Figure 3.5.

NMDA-induced loss of Synaptophysin

Additional studies were conducted to assess the effects of NMDA, APV, and NMDA+APV exposure on the immunoreactivity of the synaptic vesicle protein, synaptophysin. A three-way ANOVA (treatment x exposure time x sex) was initially conducted, but as no sex differences were observed, the data were later combined for simplicity. Thus, a two-way ANOVA (treatment x exposure time) within each region was conducted. Within the CA1, a significant main effect of treatment was found ($F(3, 420)=75.442$, $p<0.001$). While no significant main effect of time within the CA1 was observed, a significant interaction of treatment by time in the CA1 was uncovered (Figure 3.6; $F(6, 420)=5.347$, $p<0.001$). Within each timepoint examined, NMDA exposure resulted in a time-dependent decrease in synaptophysin immunoreactivity and the addition of APV blocked this NMDA-induced effect, such that exposure to NMDA+APV resulted in synaptophysin immunoreactivity levels at or above those seen with control slices (Fisher's LSD post-hoc, $p < 0.05$).

In regard to the effect of time within treatment groups, the NMDA-induced decrease in synaptophysin immunoreactivity within the CA1 was shown to be time-dependent such that longer exposure (i.e. 72 or 120 hour) produced a significantly greater deficit (42% control and 32% control, respectively) in synaptophysin immunoreactivity compared to shorter exposure (i.e. 24 hour; 72% control); however, the NMDA-induced

loss of synaptophysin immunoreactivity was significantly different from control or APV treated cultures at all exposure times measured (Fisher's LSD post-hoc, $p < 0.05$).

While significant main effects of both time ($F(3,420)=5.681$, $p<0.001$) and treatment ($F(2, 420)=7.396$, $p<0.001$) were found within the CA3, no significant interaction was observed (Figure 3.6). Collapsed across time, treatment with NMDA resulted in a significant decrease (84% control) in synaptophysin immunoreactivity in the CA3 as compared to control or APV treated cultures (Fisher's LSD post-hoc, $p < 0.05$). The loss of synaptophysin immunoreactivity in the CA3 following NMDA exposure was significantly attenuated by co-exposure of NMDA and APV (Fisher's LSD post-hoc, $p < 0.05$). Further, data collapsed across treatment revealed that cultures exposed for 24 or 72 hours had significantly higher synaptophysin immunoreactivity levels (both ~98% control) from cultures treated 120 hours (86% control; Fisher's LSD post-hoc, $p < 0.05$).

A significant main effect of treatment ($F(3,420)=16.169$, $p<0.001$) was observed within the DG; however, no further significant effects were discovered (Figure 3.6). Collapsed across time, treatment with NMDA resulted in a significant decrease (73% control) in synaptophysin immunoreactivity in the DG region of the hippocampus as compared to control or APV treated cultures (Fisher's LSD post-hoc, $p < 0.05$). The loss of synaptophysin immunoreactivity in the DG following NMDA exposure was significantly attenuated by co-exposure of NMDA and APV (Fisher's LSD post-hoc, $p < 0.05$). Representative images of hippocampal slices labeled with synaptophysin are shown in Figure 3.7.

Rapid timecourse of NMDA-induced toxicity

Subsequent studies were conducted to determine if NMDA (20 μM) produced rapid changes in either cytotoxicity as measured by PI uptake or synaptophysin immunoreactivity prior to 24 hours (at 15 or 120 minutes following initial exposure) as compared to control conditions. Thus, a three-way ANOVA (treatment x exposure time x sex) was initially conducted, but as no sex differences were observed, the data were later combined for simplicity. A two-way ANOVA (treatment x exposure time) within each region was then conducted. Regarding PI uptake within the CA1 region, a significant main effect of treatment was observed (Figure 3.8; $F(1, 136)=39.183$, $p<0.001$), such that exposure to NMDA resulted in an increase in PI uptake (~272% control); however, there no further significant main effects or interactions within the CA1. Similarly, there were no significant main effects or interactions in the CA3 or DG when examining the effects of NMDA on rapid PI uptake (Figure 3.8).

The rapid effects of NMDA seen prior to 24 hours were also investigated by examining differences in synaptophysin immmunoreactivity. As with the rapid changes in PI uptake, no sex differences were observed with synaptophysin immunoreactivity. No significant main effects of interactions of treatment and exposure time were observed in any of the regions examined (Figure 3.9).

Figure 3.1. Effects of region and exposure time on the raw control values for PI uptake (A), NR1 immunoreactivity (B), and synaptophysin immunoreactivity (C). * = p<0.05 vs. 24 hour within region; ** = p<0.05 vs. 72 hour within region; # = p<0.05 vs. CA1 region within time; + = p<0.05 vs. CA3 region within time

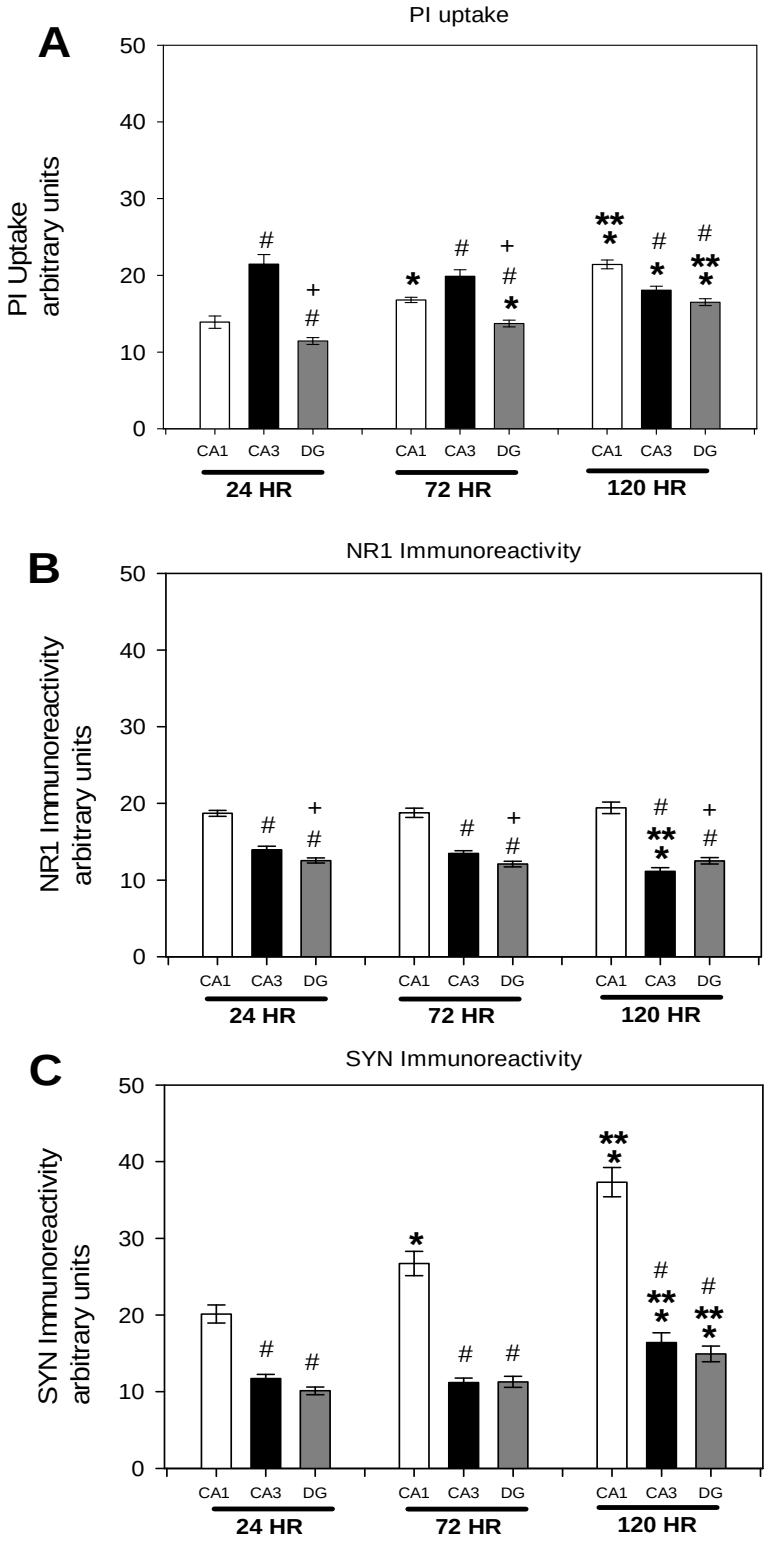


Figure 3.2. Effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on toxicity observed in the organotypic hippocampal slice cultures. Exposure to NMDA resulted in significant time-dependent toxicity within the CA1 region of the hippocampus, an effect which was prevented by co-exposure to APV. * = $p<0.05$ vs. control levels; # = $p<0.05$ vs. NMDA alone

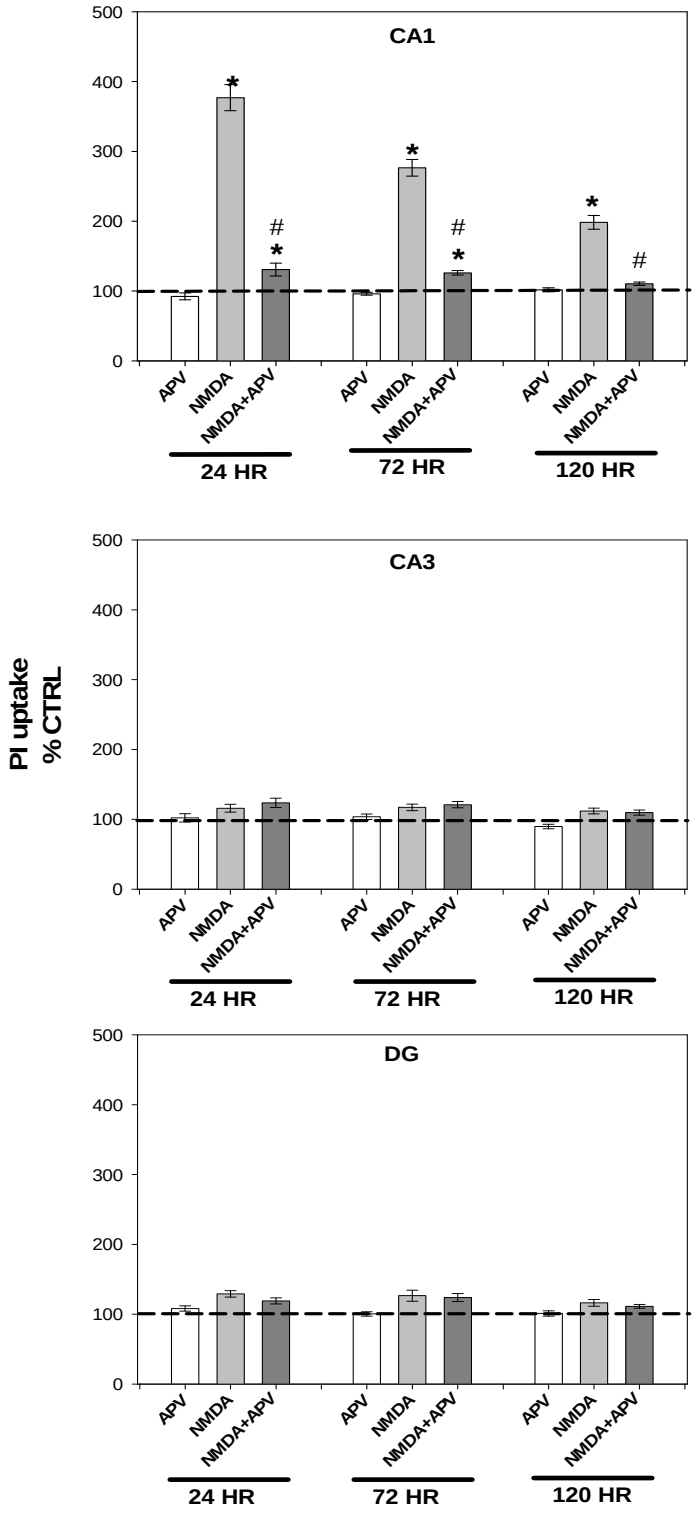


Figure 3.3. Representative images of the effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on toxicity observed in the organotypic hippocampal slice cultures. A. 24 HR control; B. 24 HR APV alone; C. 24 HR NMDA alone; D. 24 HR NMDA+APV; E. 72 HR control; F. 72 HR APV alone; G. 72 HR NMDA alone; H. 72 HR NMDA+APV; I. 120 HR control; J. 120 HR APV alone; K. 120 HR NMDA alone; L. 120 HR NMDA+APV

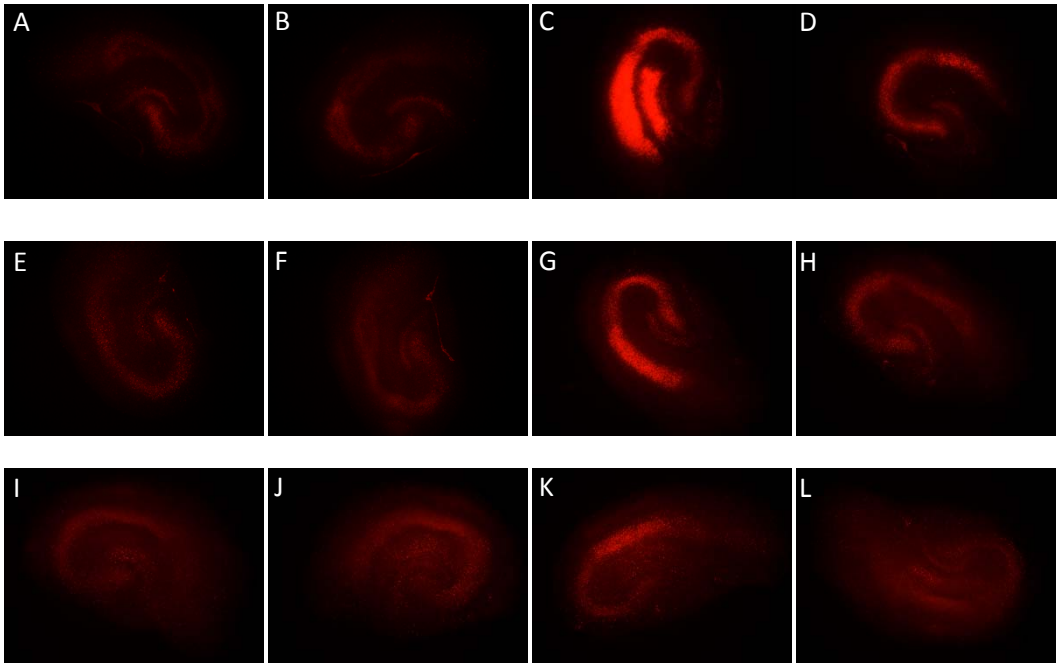


Figure 3.4. Effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on NR1 subunit immunoreactivity observed in the organotypic hippocampal slice cultures. Exposure to NMDA resulted in significant time-dependent NR1 loss within the CA1 region of the hippocampus, an effect which was prevented by co-exposure to APV. * = $p<0.05$ vs. control levels; # = $p<0.05$ vs. NMDA alone

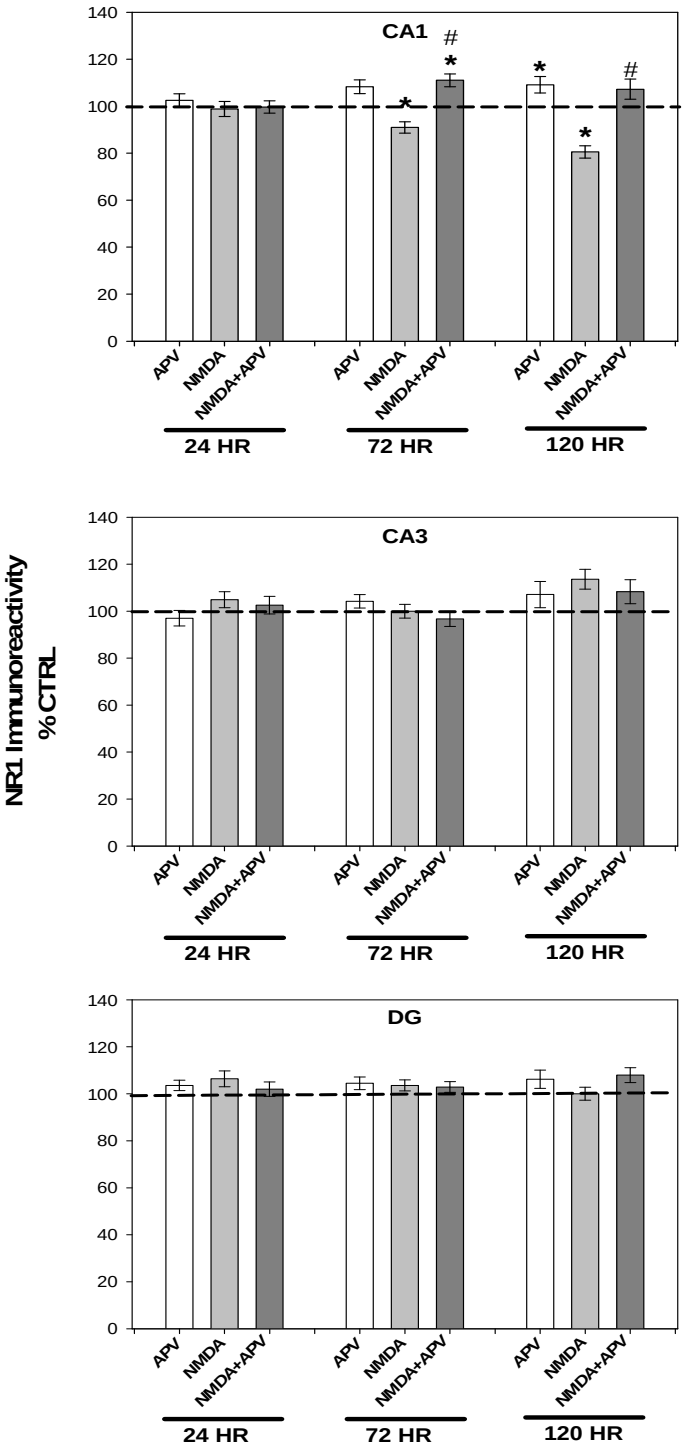


Figure 3.5. Representative images of the effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on NR1 subunit immunoreactivity observed in the organotypic hippocampal slice cultures. A. 24 HR control; B. 24 HR APV alone; C. 24 HR NMDA alone; D. 24 HR NMDA+APV; E. 72 HR control; F. 72 HR APV alone; G. 72 HR NMDA alone; H. 72 HR NMDA+APV; I. 120 HR control; J. 120 HR APV alone; K. 120 HR NMDA alone; L. 120 HR NMDA+APV

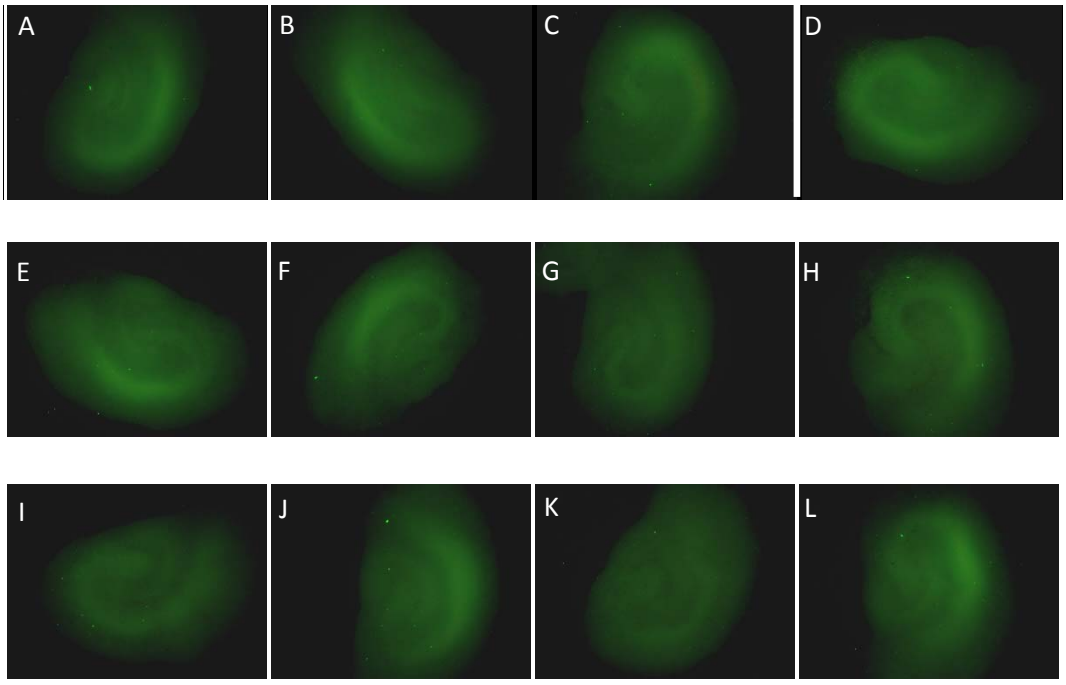


Figure 3.6. Effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on the synaptic vesicle protein synaptophysin immunoreactivity observed in the organotypic hippocampal slice cultures. Exposure to NMDA resulted in significant time-dependent synaptophysin loss within the CA1 region of the hippocampus, an effect which was prevented by co-exposure to APV. * = $p<0.05$ vs. control levels; # = $p<0.05$ vs. NMDA alone

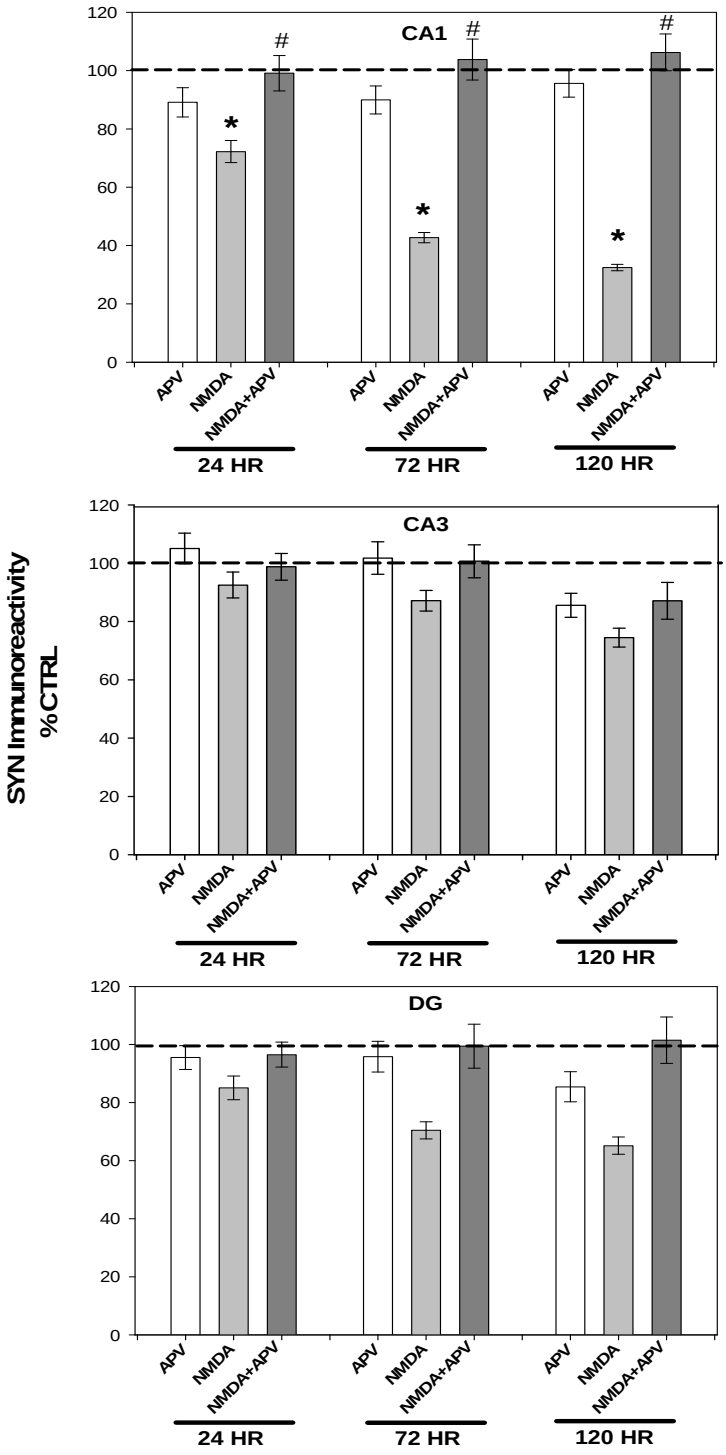
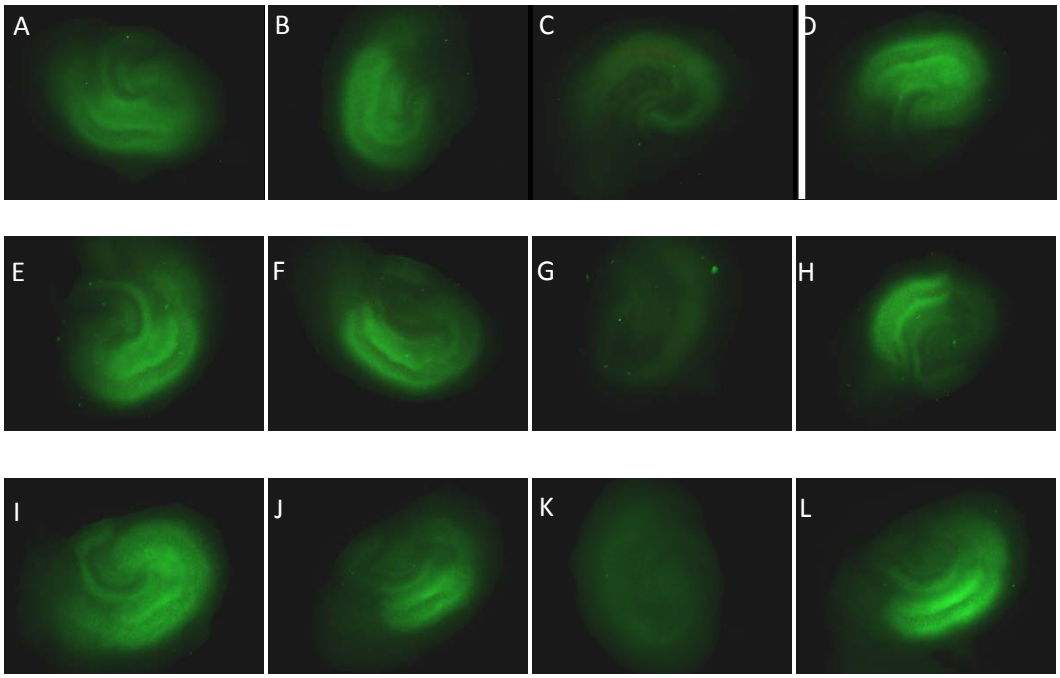


Figure 3.7. Representative images of the effects of exposure to the glutamate NMDA receptor agonist NMDA, the competitive antagonist APV, or NMDA+APV on synaptophysin immunoreactivity observed in the organotypic hippocampal slice cultures. A. 24 HR control; B. 24 HR APV alone; C. 24 HR NMDA alone; D. 24 HR NMDA+APV; E. 72 HR control; F. 72 HR APV alone; G. 72 HR NMDA alone; H. 72 HR NMDA+APV; I. 120 HR control; J. 120 HR APV alone; K. 120 HR NMDA alone; L. 120 HR NMDA+APV; M. control image labeling the regions of the hippocampus



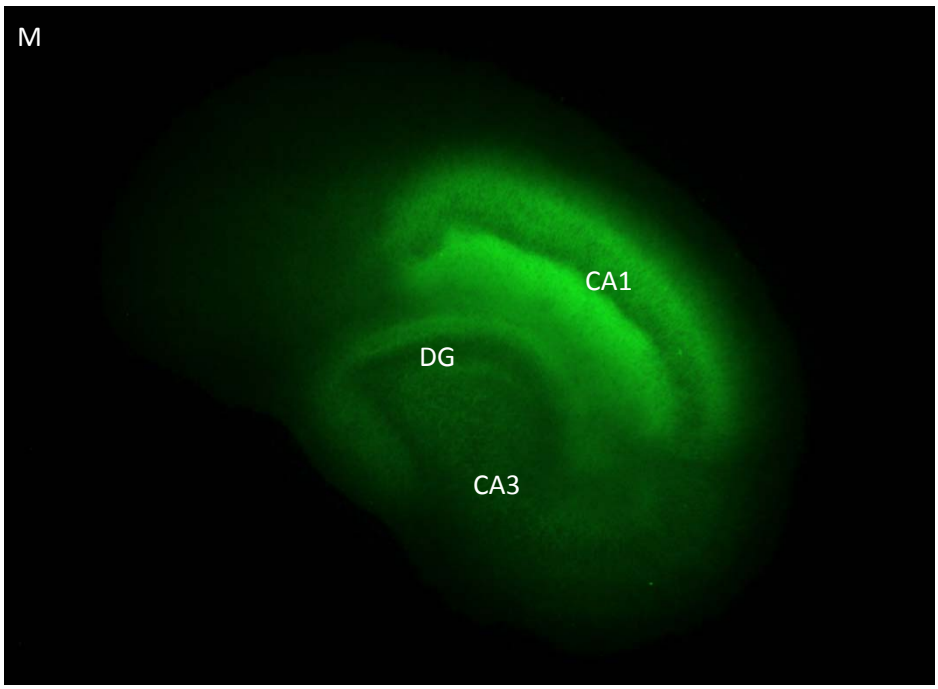


Figure 3.8. Effects of exposure to the glutamate NMDA receptor agonist NMDA on rapid changes in cytotoxicity as measured by PI uptake prior to 24 hours (at 15 and 120 minutes). Exposure to NMDA produced significant toxicity in the CA1 region when collapsed over time. * = $p<0.05$ vs. control levels.

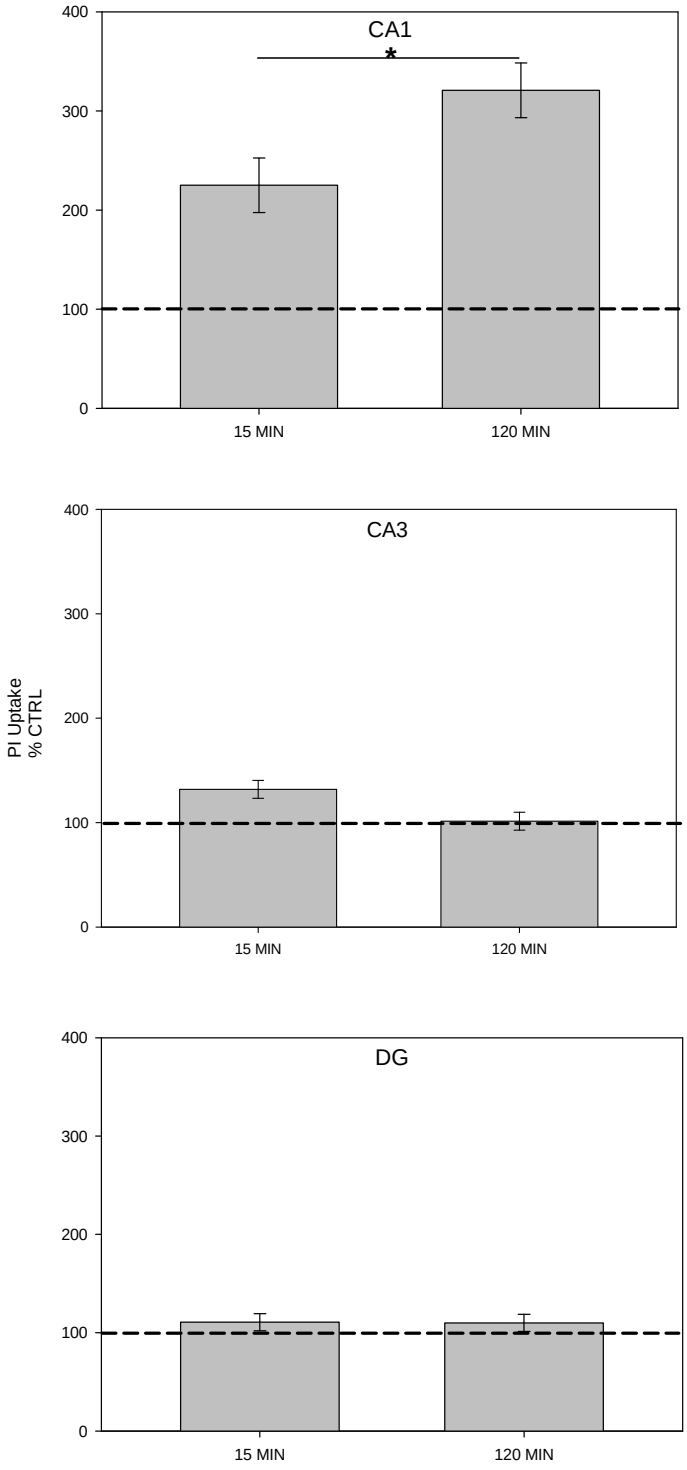
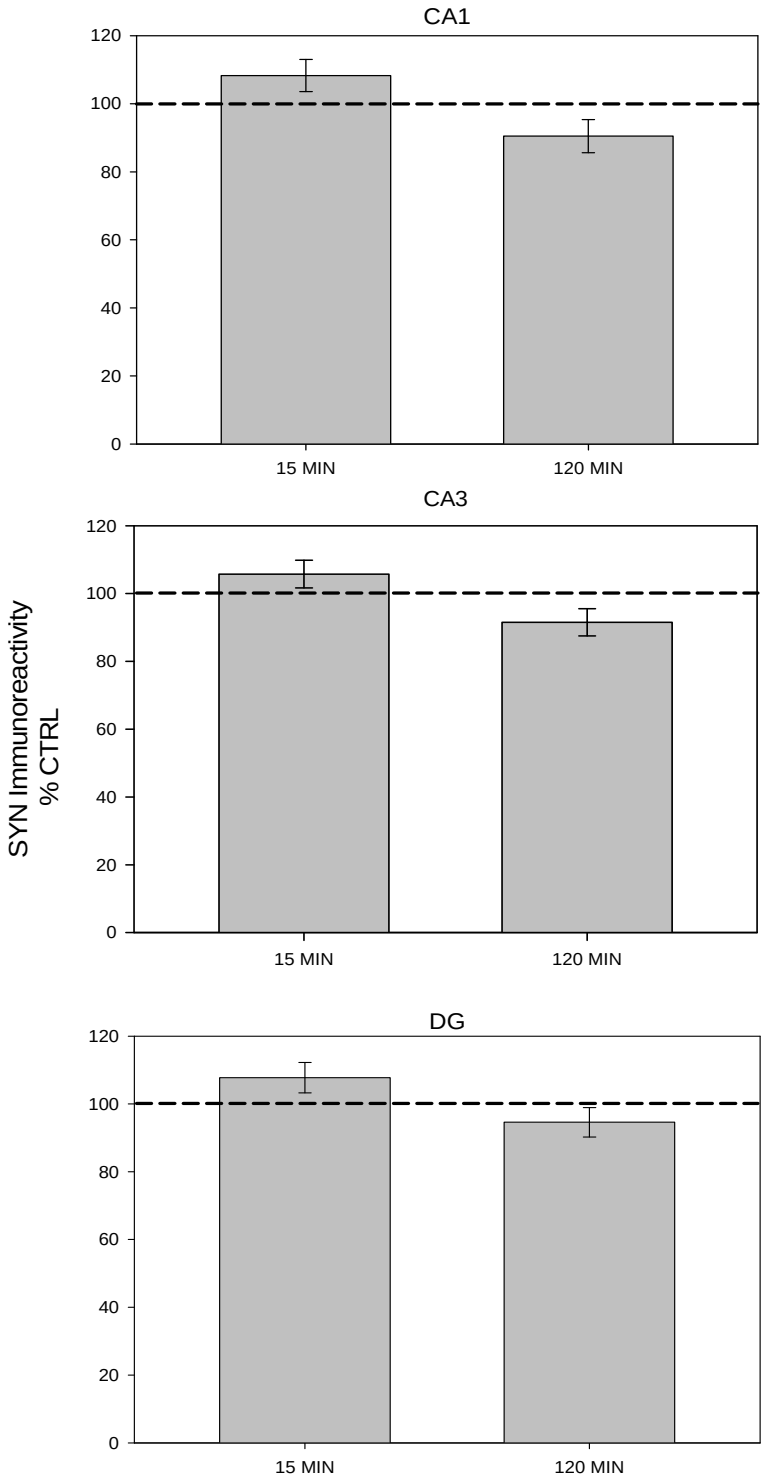


Figure 3.9. Rapid effects of exposure to the glutamate NMDA receptor agonist NMDA on synaptophysin immunoreactivity as measured prior to 24 hours (at 15 and 120 minutes). Exposure to NMDA for 15 or 120 minutes did not produce significant changes in synaptophysin levels.



Chapter Four

Discussion

Excitotoxicity & the NMDA Receptor

Excitotoxicity has been linked to many neurodegenerative states, including traumatic brain injury, ischemia/hypoxia, ALS, and is also thought to be involved in the degenerative effects of ethanol withdrawal. The NMDA receptor has been the focus of much research in recent years and is thought to be a key factor in excitotoxicity, in part due to the receptor's permeability to Ca^{2+}. An influx of Ca^{2+} can lead to the activation of many cell death enzymes, including endonucleases, proteases, and phospholipases (Sattler & Tymianski, 2000). Recent research has also shown a propensity for dendritic degradation. Following a short exposure to NMDA, dendritic swellings were found first in the distal branches and later in the more proximal dendritic branches (Hoskison, et al., 2007). The dendritic swelling was also accompanied by a time-dependent loss of immunoreactivity of the microtubule-associated protein MAP-2 in the dendrites of pyramidal cells (Hoskison, et al., 2007). Also, decreased dendritic spines and a loss of filamentous actin has also been observed following a brief NMDA exposure, while over the same exposure period, staining for the presynaptic marker synapsin 1 as well as staining for PSD-95 and the NR1 subunit of the NMDA receptor remained stable (Halpain, et al., 1998). In particular, the Ca^{2+}-dependent protease calpain has been shown to cleave the cytoskeletal protein spectrin, resulting in two large protein breakdown products (BDPs; Vanderklish & Bahr, 2000). These BDPs have been detected *in vitro* following as little as a 5 minute exposure to NMDA (Bahr, Tiriveedhi, Park, & Lynch, 1995), while *in vivo* work has suggested that BDPs take many hours and even days to be

observed following NMDA-induced degeneration (Siman, et al., 1989). However, there has been confusion regarding which markers might reflect calpain-mediated cell death and the timecourse associated with the expression of these markers remains unclear. For example, previous research has indicated that calpain-dependent axonal varicosities appear prior to neuronal damage following glutamate-induced excitotoxic insult (Hou, et al., 2009). Hence, previous research has produced many discrepancies regarding the manner in which neuronal degeneration occurs following NMDA-induced excitotoxic insult. The present studies were designed to examine the time-dependent neuronal degradation produced by excessive NMDA receptor activation to elucidate the possible differences in the time course of dendritic and axonal injury as reflected by the magnitude of dendritic (NR1) or synaptic (synaptophysin) markers.

Raw Control Values

The raw control levels of cytotoxicity as measured by PI uptake across time revealed that levels of PI uptake increase over time in the pyramidal cell layer of the CA1 region and the granule cell layer of the DG. This was in contrast to the decrease in PI uptake across time seen in the pyramidal cell layer of the CA3 region of the hippocampus. This was unexpected as previous research has indicated that the CA1 region contains a greater density of NMDA receptors as compared to the CA3 or DG (Butler, et al., 2010). The lowest levels of PI uptake were seen in the DG region of the hippocampus across all timepoints examined. This is in agreement with previous research indicating that the DG region has the greatest immunoreactivity of calbindin-D28K compared to either the CA1 or CA3 regions (Prendergast, et al., 2001). Calbindin-

D28K is a Ca^{2+} binding protein which sequesters cytosolic Ca^{2+} for a period of time, protecting the cell from Ca^{2+}-mediated cell death (Prendergast, et al., 2001). The increase in arbitrary PI uptake over time in the CA1 seen in control-treated slices is possibly due to increased NMDA receptor activation caused by "medium-change" toxicity and an increase in glutamine, leading to increased glutamate concentrations (Mayer, et al., 2002a). Glutamine present in the culture media has been shown to be contaminated with glutamate and also produce glutamate; both events are sufficient to produce toxicity associated with the medium change (Driscoll, Deibler, Law, & Crane, 1993). The increase in glutamate as a result of media change could also be responsible for the high levels of arbitrary PI uptake seen within the CA3 region of the hippocampus. The pyramidal call layer of the CA3 is thought to contain the greatest number of kainite receptors as compared to either the CA1 or DG regions (Martens & Wree, 2001). Further, treatment of kainite resulted in greater levels of toxicity in the CA3 compared to the CA1 or DG in organotypic hippocampal cultures (Holopainen, Jarvela, Lopez-Picon, Pelliniemi, & Kukko-Lukjanov, 2004). Thus, it is possible that the influx of glutamate induced by the medium change first activated kainite receptors in the CA3 region and subsequently activated NMDA receptors in the CA1 region. The very low levels of PI uptake seen in control-treated cultures is not unexpected as small amounts of toxicity may be attributed to the trauma induced by hippocampal removal, chopping the hippocampus into slices, as well as "medium-change" toxicity.

Examining the raw control data for differences in region and exposure time revealed significantly greater immunoreactivity of the NR1 subunit of the NMDA receptor and the synaptic vesicle protein synaptophysin within the pyramidal cell layer of

the CA1 region of the hippocampus as compared to either the CA3 or DG regions. Previous research has indicated that the CA1 region may have a select vulnerability to excitotoxic insult as a result of increased mature neurons and functional NMDA receptors (Butler, et al., 2010). Furthermore, recent research also suggests that exposure to NMDA results in higher Ca^{2+} concentrations and increased mitochondrial Ca^{2+} accumulation in the CA1 region of the hippocampus compared to the CA3 region (Stanika, Winters, Pivovarova, & Andrews, 2010). Thus, the results of the present study may also suggest that more functional NMDA receptors are present in the CA1 region as opposed to the CA3 or DG regions of the hippocampus. These results are consistent with previous research indicating decreased expression of NR2B in the CA3 and DG regions as compared to the CA1 region of the hippocampus (Coultrap, Nixon, Alvestad, Valenzuela, & Browning, 2005). The expression of NR1 within the CA3 was also decreased in comparison to the CA1 (Coultrap, et al., 2005). Previous research has also indicated that NR1 and NR2A, but not NR2B, subunit expression increases with age in culture (Brewer, et al., 2007); however, this effect was not observed in the present study. Further, earlier research also revealed that protein levels as well as receptor surface expression for NR1, NR2A, and NR2B increased with age in cultures (Fogal, Trettel, Uliasz, Levine, & Hewett, 2005; Mizuta, Katayama, Watanabe, Mishina, & Ishii, 1998). Though significant methodological differences between the studies could explain the discrepancy in NMDA receptor expression (i.e. primary vs. organotypic cultures, different DIV), it is nevertheless surprising that NR1 immunoreactivity within the CA1 region was not time-dependent in the present study.

Immunoreactivity of the synaptic vesicle protein synaptophysin was also increased in the CA1 in comparison to the CA3 and DG and this effect was significantly potentiated with elevated exposure time. Levels of synaptophysin immunoreactivity were similar between the DG and CA3, and immunoreactivity was increased similarly in both regions following 120 hour exposure to control conditions. The perforant pathway in the brain provides largely unidirectional projections from the entorhinal cortex to the granule cell layer of the dentate gyrus, although the entorhinal cortex also projects directly to the other subfields of the hippocampal formation (i.e. CA1-CA3, subiculum). Mossy fibers connect the dentate gyrus to the CA3 pyramidal cell layer while projections from the CA3 (termed Schaffer collaterals) synapse onto CA1 pyramidal cells (for a review, see Knowles, 1992). Fibers from the CA1 then project to the subiculum and finally out of the hippocampus and back to the entorhinal cortex. The major input (perforant pathway) is clearly interrupted by use of the organotypic hippocampal cell culture model as hippocampal input from the entorhinal cortex is interrupted. Previous research has suggested that a synaptic reorganization occurs in the organotypic hippocampal slice culture model used in the present set of experiments and that this reorganization lead to the production of new synaptic pathways between the CA1 and DG (Gutierrez & Heinemann, 1999; Mulholland & Prendergast, 2003). Thus, the time-dependent increase in synaptophysin immunoreactivity in all regions, and in particular the CA1 region, likely demonstrates enhanced synaptogensis following disruption of the afferent innervations to the dentate gyrus. The increase in pre- and post-synaptic markers over time seen in the present study is consistent with previous *in vitro* work demonstrating increased expression of two synaptic marker proteins synaptophysin (pre-

synaptic marker) and PSD-95 (post-synaptic marker) over the developmental period seen in the organotypic hippocampal slice culture model (Buckby, Mummery, Crompton, Beesley, & Empson, 2004; Mielke, et al., 2005).

NMDA-induced cytotoxicity

A multitude of studies have revealed that the glutamate receptor agonist NMDA produces reliable toxicity as measured by propidium iodide uptake, neuron-specific nuclear protein (NeuN) immunoreacitivity (a measure of mature neurons), and fluoro-jade B staining (a marker of neuronal loss; Butler, et al., 2010; Gibson, et al., 2003; Kim, Chang, Chen, Rapoport, & Rao, 2009; Mulholland, Self, Harris, Littleton, & Prendergast, 2004; Wilkins, et al., 2006; Zimmer, et al., 2000). The present results reveal a time-dependent NMDA-induced increase in PI fluorescence within the CA1 region which peaked 24 hours following NMDA exposure. Even though NMDA-induced PI fluorescence decreased at later timepoints, the level of PI uptake never returned to baseline levels seen with control-treated cultures. These results are in partial agreement with previous research, which revealed significant increases in PI uptake assessed 1 day or 3 days following exposure, but not when PI was measured 5 or more days following NMDA exposure (Wilkins, et al., 2006). A smaller, but still significant, increase in PI uptake (~200% control) was seen following 120 hour exposure to NMDA in the present study, which is in disagreement with the previously mentioned study (Wilkins, et al., 2006). However, significant methodological differences may be accountable for the differences observed. For example, the cultures in the current study were exposed to NMDA continuously for 120 hours while other cultures were only exposed to NMDA for

1 hour and then transferred into fresh culture media (Wilkins, et al., 2006). The cytotoxic effect of NMDA was significantly attenuated by the addition of the competitive NMDA receptor antagonist APV, suggesting that the toxicity seen is indeed dependent on the NMDA receptor activation. Though significant interactions in the CA3 or DG regions were not found, cultures exposed to NMDA, collapsed across time in both regions, showed significant increases in PI fluorescence compared to control-treated cultures. A significant effect of time was also found in the CA3 such that, collapsed across treatment groups, PI uptake was greater following 24 or 72 hour exposure compared to 120 hour exposure, agreeing with results found for the CA1 suggesting a time-dependent decrease of PI fluorescence.

The present set of experiments used PI fluorescence as a measure of cytotoxicity. PI is an intercalating agent which binds to DNA by attaching between the bonds of various base pairs. PI has been shown to reliably correlate with other markers of neuronal death and has been used as a dependable indicator of cell damage in the organotypic slice culture model (Noraberg, 2004; Zimmer, et al., 2000). The current study investigated PI fluorescence over three timepoints, including 24, 72, and 120 hours. The decrease in NMDA-induced PI fluorescence over time may suggest that damaged or dead cells were engulfed by macrophages prior to the time-appropriate measurement of PI fluorescence. Thus, a loss of PI uptake following extended exposure to NMDA may not suggest less initial death, but less fluorescence due to fewer visible dead or damaged cells. Alternatively, an excitatory-induced increase in deoxyribonuclease (DNase), which cleaves the phosphodiester links in the DNA backbone, could also lead to a loss of PI signal as a result of the loss of base pairs (Marti & Fleck, 2004). Furthermore,

endonuclease G (endoG) is a caspase-independent nuclease found in mitochondria (Li, Luo, & Wang, 2001). During apoptosis, endoG is released from the mitochondria and cleaves chromatin DNA within the nucleus (Li, et al., 2001). Previous research has indicated that levels of endoG rise following AMPA-induced excitotoxicity (Henne, et al., 2006), an effect which may generalize to glutamate-induced excitotoxicity. Thus, there are several possible explanations for the loss of PI signal. Additional markers of neuronal degradation used previously with the organotypic cell culture model may be of use in future studies to illuminate the results found in the present study. For example, histological staining by Fluoro-Jade B labeling degenerating neurons, lactate dehydrogenase release in the culture medium labeling cell lysis, and Nissl stain labeling ribosomal RNA all correlate well with PI activity and are, alone, good measures of cell damage (Zimmer, et al., 2000). Further, previous studies have also looked at the immunoreactivity of NeuN to measure healthy mature neurons and iummunoreactivity of the Ca^{2+}-binding protein calbindin D28k (Calb) to quantify neuronal loss (Wilkins, et al., 2006).

NMDA-induced loss of NR1 immunoreactivity

Exposure to NMDA produced a significant time-dependent loss of NR1 immunoreactivity in the CA1 region of the hippocampus. Previous research with primary hippocampal cultured neurons has indicated that transcription of the NR1 subunit is downregulated following excitotoxic insult (Gascon, et al., 2005). The results of the current study support the hypothesis that overactivation of the NMDA receptor produces a downregulation in NMDA receptor subunits. It has previously been shown that NMDA

receptors desensitize very quickly; in fact, the inhibition of synaptic NMDA receptors in cultured hippocampal neurons is thought to be mediated by Ca^{2+} (Rosenmund, Feltz, & Westbrook, 1995; Rosenmund & Westbrook, 1993). Thus, it is possible that initial overactivation of the NMDA receptor followed by desensitization and downregulation resulted in the time-dependent loss of NR1 subunit immunoreactivity.

Alternatively, the NMDA-induced loss of NR1 immunoreactivity following 72 or 120 hour exposure could signify the degradation of the dendrites of the cell. This is supported by data from previous studies indicating that NMDA receptors are primarily found on the dendrites of post-synaptic receptors (Jones & Baughman, 1991). In addition, exposure to glutamate induced a redistribution of the AMPA receptor subunit GluR1 but not the NMDA receptor subunit NR1 in primary hippocampal cultures, suggesting that a brief exposure to glutamate (100μM for 15 minutes) does not result in downregulation of the NMDA receptor (Lissin, Carroll, Nicoll, Malenka, & von Zastrow, 1999). Further, early (5-10 minute exposure) Ca^{2+}-dependent NMDA-induced dendritic injury has been shown previously (Halpain, et al., 1998; Hoskison, et al., 2007). However, loss of dendrites as measured by NR1 immunoreactivity was not seen following 24 hour exposure to NMDA in the present study, suggesting a possible delay in NMDA-induced dendritic injury in organotypic hippocampal slice cultures. Although several methodological differences exist between the previous studies and the current study, it is possible that the marker of dendritic injury used in the present study was not as sensitive to rapid NMDA-induced injury as markers used in prior experiments (i.e. use of fluorescent dyes to label the plasma membrane to look at branched dendrites, labeling of F-actin, and high-power magnification of MAP2 labeling).

A significant loss of NR1 immunoreactivity was not found in cultures treated for 24 hours, but rather in cultures exposed for 72 or 120 hours. The thickness of the cultured hippocampal slices could play a part in activating NMDA receptors at various depths, leading to an initial downregulation of NR1 subunit immunoreactivity seen only at certain depths. The immunohistochemistry method used in the present study utilized an overall quantification of fluorescence within each region, not taking into account the thickness of the slice and the many layers of tissue. Thus, while overall NR1 immunoreactivity was not decreased following 24 hour exposure, it is possible that there was an undetectable reduction in NR1 immunoreactivity present at certain depths (i.e. the layers closer to the membrane insert) following 24 hour exposure. One method to determine the extent to which the layers of tissue influence the overall fluorescence is confocal microscopy. Confocal microscopy offers an advantage over the current method in that the fluorescence can be measured at various depths of the tissue, allowing for a more precise measurement of fluorescence.

Past studies have also found a differential effect of synaptic versus extrasynaptic NMDA receptors (for a review, see Groc, Bard, & Choquet, 2009). Importantly, synaptic NMDA receptors are tethered to the membrane by the scaffolding protein PSD-95, which interacts with the C terminal of NR2-containing receptors (Dong, Waxman, & Lynch, 2004; Husi, Ward, Choudhary, Blackstock, & Grant, 2000). NMDA receptors found at the synaptic level are thought to have a prosurvival effect when activated while extrasynaptic NMDA receptors may activate cell death pathways (Levine, Cepeda, & Andre, 2010). Prosurvival synaptic NMDA receptors aid in survival via the induction of the activity of cyclic adenosine monophosphate (cAMP) response element-binding

protein (CREB) and brain-derived neurotrophic factor (BDNF) gene expression (Hardingham, Fukunaga, & Bading, 2002). On the contrary, Ca^{2+} entry through extrasynaptic NMDA receptors promotes death by blocking BDNF expression via activation of a dominant CREB shut-off pathway (Hardingham, et al., 2002). NMDA-induced excitotoxicity has been shown to produce calpain-dependent cleavage of PSD-95 as well as calpain-dependent cleavage of the C-terminal region in NR2A-containing receptors and subsequently uncouple the synaptic NMDA receptor from downstream survival pathways (Gascon, Sobrado, Roda, Rodriguez-Pena, & Diaz-Guerra, 2008). Calpain-dependent cleavage of NR2B-containing subunits has also been recognized (Simpkins, et al., 2003). Thus, the present results may also indicate an uncoupling of synaptic NMDA receptors via NMDA-induced cleavage of PSD-95.

While the majority of NMDA receptors have been found at the synapse (Jones & Baughman, 1991; Luo, et al., 2002; Mattson, Wang, & Michaelis, 1991), previous research has indicated that the NR2A subunit is found primarily within mature synaptic neurons (Thomas, Miller, & Westbrook, 2006) while NR2B-containing receptors are primarily extrasynaptic (Tovar & Westbrook, 1999). Since the present studies use hippocampal slice cultures taken from immature rat pups, it is important to note that the NR2B subunit is the prominent subunit during early development, whereas NR2A predominates later in development (Tovar & Westbrook, 1999). However, recent research suggests that both NR2A- and NR2B-containing receptors may be localized either synaptically or extrasynaptically (Thomas, et al., 2006).

NMDA-induced loss of synaptophysin

Exposure to NMDA also produced a time-dependent loss in the immunoreactivity of the synaptic vesicle protein synaptophysin in the CA1 region of the hippocampus. Although the NMDA-induced loss was significant at every timepoint examined, the loss was more pronounced following 72 or 120 hour exposure as compared to 24 hour exposure. The NMDA-induced, time-dependent loss of synaptophysin immunoreactivity suggests axonal degradation that was exacerbated with increased exposure to NMDA. Specifically, activation of the Ca^{2+}-dependent protease calpain is thought to produce cell death via breakdown of the cytoskeleton (Sattler & Tymianski, 2000; Siman & Noszek, 1988). Calpain-mediated cleavage of the membrane cytoskeletal protein spectrin produces BDPs, which are produced following NMDA receptor-mediated exctiotoxicity (Bahr, et al., 1995; Vanderklish & Bahr, 2000). Thus, the results of the present study may suggest calpain-mediated breakdown of the cytoskeleton following excessive NMDA receptor activation.

Activation of the NMDA receptor can also lead to activation of necrotic or apoptotic neuronal death, depending on the intensity of the insult (Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton, 1995). The protease family of caspases, and in particular caspase-3, is thought to be involved in apoptotic cell death (for a review, see Chan & Mattson, 1999). Activation of the executor protease caspase-3 has been shown to be dependent on the early activation of the Ca^{2+}-dependent protease calpain following neonatal hypoxia-ischemia (Blomgren, et al., 2001). However, intrastriatal injections of glutamate were shown to result in no caspase-3 activity but significant calpain activation relating to NMDA or non-NMDA receptor-mediated excitotoxicity (Del Rio, et al., 2008). In addition, activation of calpain but not caspase-3 was found in cerebellar

granule cells exposed to excitotoxic injury produced by nitric oxide (Volbracht, et al., 2005). Kainate acid-induced status epilepticus was also shown to produce spectrin BDPs in the hippocampus, while there was no evidence for caspase activation under the same conditions (Araujo, et al., 2008). Thus, although caspase-dependent apoptotic neuronal death cannot be ruled out as a possibility, it is unlikely that caspase-3 is involved in the early loss of synaptophysin immunoreactivity.

Timecourse of NMDA-induced neuronal death

In order to determine whether somatic or axonal injury (i.e. PI uptake or loss of synaptophysin immunoreactivity, respectively) occurs first, subsequent studies were conducted on cultures exposed to NMDA for less than 24 hours. Results show that while PI uptake is increased following as little as 15 minute NMDA exposure, loss of synaptophsin was not found at the early timepoints examined. This suggests that the somatic injury may precede axonal degeneration as well as the loss of NR1 subunit immunoreactivity. However, given the previous research indicating early changes in axonal and dendritic swellings as well as early loss of dendritic spines following excitotoxic insult (Halpain, et al., 1998; Hoskison, et al., 2007; Hou, et al., 2009; Ikegaya, et al., 2001; Mizielinska, Greenwood, Tummala, & Connolly, 2009), it is possible that the measures used in the present study were not sensitive enough to detect changes at the level of the individual neuron. Thus, confocal microscopy might provide a useful tool in determining the sensitivity of the current measurements and aid in further characterizing the topographical nature of NMDA-induced neuronal death.

Conclusions and Future Directions

The current data suggest that axonal injury may precede dendritic degradation (or NR1 downregulation) in response to excessive NMDA receptor activation. The loss of NR1 subunit immunoreactivity does not appear to be a useful marker of early cell damage. Similarly, the loss of synaptophysin immunoreactivity appeared earlier than the NMDA-induced loss of NR1 subunits, but neither marker appeared prior to PI uptake. Thus, neither NR1 nor synaptophysin immunoreactivity appear to be a reliable indicator of early neuronal death or damage. Confocal microscopy and various dendritic/axonal markers will be used to further assess the effects of NMDA-induced insult at the level of the individual neuron to determine which part of the cell is more vulnerable to early excitotoxic injury. Future studies will also investigate the significance of calpain activation in the time-dependent neuronal death following excitotoxic NMDA injury using the calpain inhibitor MDL-28170. Additional live animal studies will be conducted to determine if the results of the present *in vitro* study generalize to *in vivo* experiments.

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