THE INDUCTION OF LONG-TERM POTENTIATION ATTENUATES KAINIC ACID-INDUCED EXCITOTOXICITY

by

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Abstract

The activation of N-methyl-D-aspartate glutamate receptors (NMDARs) is required for the long term potentiation (LTP) and long term depression (LTD) of excitatory synaptic transmission at hippocampal CA1 synapses, and plays an important role in learning and memory. In addition, it is accepted that the over-activation of NMDARs leads to the neurotoxicity associated with stroke and other neurodegenerative disorders. Thus, the NMDAR provides a logical starting point to investigate a possible relationship between synaptic plasticity and the cell-signalling pathways which ultimately determine neuronal fate. Research in our lab has indicated that NR2A-containing NMDARs are essential for LTP induction whereas NR2B-containing NMDARs are crucial for the production of LTD *in vitro*, and the results of this study support these findings in the anaesthetized rat. Furthermore, using the kainic acid (KA) model of neurotoxicity, this research has explored the opposing roles that activity-dependent synaptic plasticity, through different NMDAR subtypes, can play in determining neuronal outcome in an excitotoxic environment. In these experiments, it is shown that (1) the induction of LTP using high-frequency stimulation (HFS) promotes the phosphorylation of Akt, which plays a critical role in controlling cell survival and apoptosis, (2) the induction of LTP using HFS attenuates kainic acid (KA) induced neurodegeneration while the induction of LTD using low-frequency stimulation (LFS) has no incremental effect on the degree of cell death resulting from exposure to KA, (3) the blockade of NR2B-containing NMDARs using Ro25-6981 attenuates KA-induced neurodegeneration while the blockade of NR2A-containing NMDARs using NVP-AAM077 does not influence KA-induced neurotoxicity, (4) pre-treatment with NR2A antagonists blocks both the induction of LTP and its neuroprotective effect against KA while NR2B antagonists neither block the induction of LTP nor the neuroprotection that this can provide against KA, (5) the administration of NR2A antagonists after the induction of LTP has no effect on the expression of LTP or its neuroprotective effect against KA, and (6) pre-treatment with a high dose (2.4mg/kg) of NVP-AAM077 leads to the induction of LTD rather than LTP as a result of HFS. Altogether this research supports the hypothesis that the production of LTP via the activation of NR2A-containing NMDARs protects neurons against excitotoxic neuronal death by promoting cell survival signalling. Furthermore, because NR2A antagonists applied after the production of LTP do not block neuroprotection, it can be concluded that LTP itself, and not NR2A activation, is responsible for this neuroprotective effect.

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Abbreviations

APV	2-amino-5-phosphonovalerate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CA1	Cornu ammon's region 1
CA2	Cornu ammon's region 2
CA3	Cornu ammon's region 3
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CREB	cAMP response element binding protein
dlBNST	Dorsolateral bed nucleus of the stria terminalis
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
EPSC	Excitatory post-synaptic current
EPSP	Excitatory post-synaptic potential
FJ	Fluorojade B
HFS	High frequency stimulation
KA	Kainic acid
kg	Kilogram
LFS	Low frequency stimulation
LTP	Long term potentiation
LTD	Long term depression
mg	Milligram
Mg^{2+}	Magnesium
ml	Milliliter
mV	Millivolt
NMDAR	N-methyl-D-aspartate receptor
PB	Paired burst stimulation
Hz	Hertz
IHC	Immunohistochemistry
i.p.	Intraperitoneal
PKC	Protein kinase C
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling
μA	Microamp

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1. INTRODUCTION

1.1 Synaptic plasticity and its connection to neuronal survival/death

Synaptic plasticity, the plastic change in strength of communication between neurons in the brain, is a fundamental process that is intimately involved with various normal physiological brain functions including learning and memory, neuronal circuit development, as well as with many neuropathological disorders such as the neurotoxicity associated with stroke (Albensi, 2001). At most excitatory synapses, normal synaptic transmission is mediated via the neurotransmitter glutamate, which acts primarily upon two pharmacologically distinct subtypes of glutamate receptor: the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and the N-methyl-D-aspartate (NMDA) receptor (Bliss and Collingridge, 1993). Glutamate receptor-mediated synaptic transmission exhibits a high level of plasticity, and one of the best examples of such plasticity is the long-term changes in synaptic efficacy observed at the glutamatergic synapses in Cornu ammon's region 1(CA1) of the hippocampus (Malenka, 2003). These include high-frequency-stimulation (HSF) induced long-term potentiation (LTP) and low-frequency-stimulation (LFS) induced long-term depression (LTD). Both LTP and LTD in the hippocampus have long been considered to be candidates for the cellular mechanisms underlying learning and memory (Lynch, 2004); however, these forms of synaptic plasticity may also play an important role in physiological processes such as the apoptotic elimination of cells during early development and under pathological conditions that include the neurotoxic cell death that results from neurodegenerative diseases. This is a likely possibility because, in addition to participating in synaptic plasticity through physiological excitation of the NMDA receptor, glutamate can also cause neurodegeneration via excessive activation of these same receptors. For instance, the drug clausenamide has been shown to both facilitate LTP and attenuate apoptosis in animal models of central nervous system (CNS) dysfunction (Tang and Zhang, 2003) suggesting a relationship between the pathways governing these distinct cellular processes. Furthermore, a new class of drugs, AMPA receptor potentiators, has been shown to enhance LTP while also providing a neuroprotective effect through increased neurotrophin expression (O'Neill et al., 2004). These neurotrophins, which have themselves been identified as important for long-term potentiation, promote neuronal survival, and regulate synaptic transmission, stability, and efficiency at both developing and mature synapses (Schuman, 1999). Moreover, cytokines have been linked to synaptic plasticity and have

also been shown to be neuroprotective *in vivo* (Mattson and Scheff, 1994). Sanna et al. (2002) have demonstrated *in vitro* that the induction of LTP in the CA1 consistently promotes the activation of the phosphoinositide 3-kinase (PI3K)/Akt signalling cascade which has classically been implicated in stimulating cell survival via its antiapoptotic activity. Akt has also been shown to be necessary for the expression of LTP as the administration of Akt inhibitors 1 hour after LTP induction significantly reduces the observed level of fEPSP-potentiation (Karpova et al., 2006). On the other hand, it has been reported that the induction of LTP can be blocked by the administration of β-amyloid peptides, which are known to cause the neurotoxicity and decline in cognition found in Alzheimer's disease (Freir et al., 2000), and it has also been demonstrated that the β-amyloid peptides promote long-lasting reductions (LTD) in synaptic strength (Kim et al., 2001). Together, these findings suggest that there exists a link between synaptic plasticity and neuronal fate, however, there is still no conclusive evidence directly linking these cellular mechanisms with one another. A major aim of this research project is to test the hypothesis that LTP is associated with a cell survival pathway while LTD is associated with a signalling pathway that leads to cell death.

1.11 LTP in CA1 region of the hippocampus

The hippocampus was the first area of the brain in which LTP, a cellular mechanism that seemed applicable to the initial storage of memories, was reported (Bliss and Lomo, 1973). A brief high-frequency train of action potentials in the perforant path was shown to induce an increase in the excitatory synaptic potential of granule cells that, in some conditions, would persist for days. Subsequent research determined that high-frequency induced LTP reliably occurs at all three major synaptic pathways that comprise the trisynaptic cytoarchitecture of the hippocampus: the Schaffer collateral fiber pathway, the mossy fiber pathway, and the aforementioned perforant fiber pathway (Figure 1). The potentiation seen at each of these synaptic pathways has, however, shown to have different properties and is therefore hypothesized to involve different mechanisms (Zalutsky and Nicoll, 1990) The research outlined in this project will focus specifically upon LTP in the CA1 region of the hippocampus resulting from HFS to the Schaffer collateral fiber pathway, and this form of LTP has three defined characteristics. First, this type of LTP has pathway specificity, which means that the increase in excitatory synaptic potentials is restricted to synapses that are a part of the tetanized pathway and LTP generated at one synapse does not propagate to all others in the

same cell or in nearby cells (Anderson et al., 1977). Second, there is a threshold level of stimulus intensity that is required to induce long-term potentiation using a high-frequency tetanus (McNaughton et al., 1978). This property is referred to as cooperativity. Finally, long-term potentiation in the CA1 region is associative; when weak, sub-threshold, stimulation of one afferent pathway is temporarily yoked with strong stimulation of another input pathway; the weak pathway also manifests LTP (Levy and Steward, 1979). These three properties of LTP in the hippocampus that have been described all agree with the hypotheses regarding synaptic plasticity that were formulated by Donald Hebb in 1949: coincident activity in both the postsynaptic pyramidal neuron and presynaptic neurons is necessary and sufficient for the induction of LTP. Manilow and Miller (1986) found that blocking postsynaptic firing reversibly prevents the induction of LTP, and Kelso et al. (1986) showed that intracellularly induced postsynaptic firing can induce long-term potentiation when it is paired with weak, otherwise sub-threshold, presynaptic stimulation. These Hebbian characteristics of LTP have been attributed to the activity properties of the NMDAR and its channel. Glutamate is the predominant excitatory neurotransmitter in the hippocampus, and it exerts its action through two major classes of receptors: NMDARs and nonNMDARs (i.e. AMPA receptors). Furthermore, the ion channels associated with the NMDARs permit the flow of calcium ions (Ca^{2+}) across the cell membrane, and it is this influx of Ca^{2+} that has been proposed to be an integral component of the mechanism associated with LTP induction in the CA1 (Malenka et al., 1992). The NMDAR channel is blocked at the resting membrane potential by the presence of bound extracellular magnesium ions (Mg^{2+}) and when the membrane is sufficiently depolarized, the Mg^{2+} is expelled from the channel. This voltage dependent Mg^{2+} block ensures that Ca^{2+} influx through the NMDA channel requires both postsynaptic depolarization and the activation of the NMDARs through the binding of presynaptically released glutamate. As already mentioned, these two events can be produced artificially by applying strong, high-frequency stimulation to the presynaptic fibers that run along the Schaffer collateral pathway. This results in the activation of AMPAR channels, which sufficiently depolarize the postsynaptic neuron thereby removing the Mg^{2+} blockage of NMDAR channels and thus allowing Ca^{2+} influx. LTP in the CA1 can be blocked by preventing the activation of the NMDA receptor with selective antagonists (Collingridge et al., 1983). In addition, the infusion of NMDA antagonists into the hippocampus blocks spatial learning, and this supports the notion that it is an NMDA receptor mediated mechanism such as LTP that is involved

in this form of learning (Davis et al., 1992). There is also experimental evidence that suggests the induction of LTP in the CA1 involves protein phosphorylation that is triggered by the entry of Ca^{2+} into the postsynaptic neuron (Malenka et al., 1989). This activity is thought to occur via Ca^{2+}-calmodulin kinase II, protein kinase C (PKC), and tyrosine kinase. Finally, Lu et al. (2001) have shown NMDA-dependant LTP to be accompanied by AMPAR insertion into excitatory synapses with cultured hippocampal neurons. In the CA1 region of the hippocampus, LTP is now believed to be induced via postsynaptic mechanisms, however; much less is understood about the maintenance of LTP and the site of expression remains controversial at best. Using quantal analysis, Bliss et al. (1986) have provided evidence for an increase in the amount of glutamate that is released by afferent neurons during LTP. But other subsequent research has established that the expression of LTP in the CA1 is the result of postsynaptic modulation (Kauer et al., 1988). All that can be said at this time is that there is evidence for both pre- and postsynaptic effects during the maintenance of LTP, and it is quite conceivable that these two possibilities are not mutually exclusive. While the precise origin of LTP maintenance remains to be elucidated, this phenomenon has received enormous attention and has been extremely well-characterized. This research employs this model of increased synaptic efficacy to test the hypothesis that electrically induced LTP in the CA1 region of the hippocampus can be neuroprotective by attenuating the degree of neuronal loss resulting from excitotoxic insult.

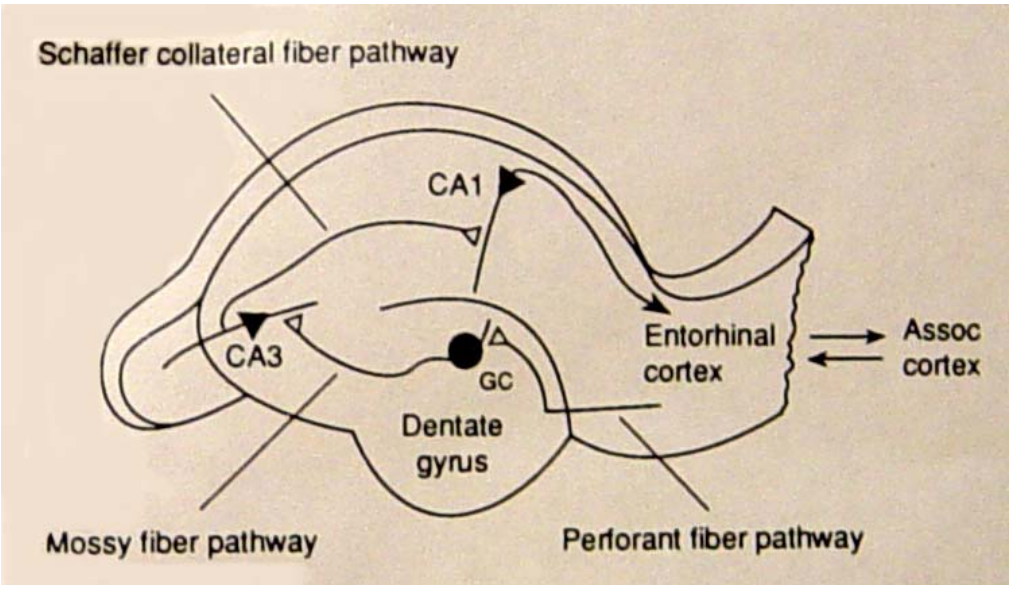


Figure 1. The main neural circuit connections in the hippocampus. Fibers in the perforant path originating in the entorhinal cortex synapse on granule cells located in the dentate gyrus. The mossy fiber efferents of the granule cells synapse on pyramidal cells within the CA3 hippocampal region. The Schaffer collateral axons of the CA3 pyramidal cells project onto the pyramidal cells in the CA1 region, which then project back to the entorhinal cortex. In turn, the entorhinal cortex is reciprocally connected to many areas of the association cortex. Adapted from Hawkins et al. (1994).

1.12 LTD in CA1 region of the hippocampus

LTD is defined as a persistent decrease in the slope and amplitude of the excitatory post-synaptic potential (EPSP) in naïve (unpotentiated) pathways, and it is typically induced by the application of LFS tetanus protocols. This effect has been shown to be more reliably induced and robust in hippocampal slices prepared from immature rats than in slices collected from adult animals (Dudek and Bear, 1993), suggesting that there is a process occurring in the developing brain, but not in the adult brain, that permits a greater capacity for the manifestation of LTD. This research focuses specifically upon long-term depression in the CA1 region of the hippocampus resulting from low-frequency stimulation to the Schaffer collateral fiber pathway *in vivo* where Heynen et al. (1996) showed that the administration of 900 pulses at 1 Hz. can reliably produce robust input-specific LTD in anesthetized rats. In addition, the application of the NMDAR antagonist 2-amino-5-phosphonovalerate (APV) prior to LFS has been shown to completely block LTD induced with this protocol, suggesting that the initiation of LTD in the hippocampal CA1 region *in vivo* is dependent upon the activation of NMDARs (Manahan-Vaughan, 1997). LTD, like LTP, is

triggered by postsynaptic Ca^{2+} entry through the NMDARs, with the difference being that large influxes of Ca^{2+} result in the induction of LTP while small increases in post-synaptic intracellular Ca^{2+} result in the expression of LTD (Cummings et al., 1996; Yang et al., 1999). Moreover, activity-dependent synaptic depression in the adult hippocampus *in vivo* involves a decrease in PKC activity that is mediated, at least in part, by dephosphorylation of the catalytic domain of PKC by protein phosphatases activated after LTD-inducing stimulation (Thiels et al., 2000). However, the persistence of LTD, lasting for days in area CA1 *in vivo* suggests that the underlying molecular mechanisms involve altered gene expression where signalling cascades are recruited to transduce synaptic signals into transcriptional signals (Kauderer and Kandel, 2000). In addition, the induction of hippocampal LTD has been associated with the internalization of AMPA receptors at the synapse (Man et al., 2001). The research conducted during this study will utilize this established model of activity-dependent synaptic depression in an effort to confirm the hypothesis that LTD in the CA1 region of the hippocampus facilitates apoptosis.

1.2 Kainic acid model of neurodegeneration

It is widely accepted that the excitotoxic over-activation of receptors for the neurotransmitter glutamate ultimately results in the tissue destruction seen in many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. However, the numerous deleterious processes triggered by this excitoxicity that eventually lead towards a form of programmed cell death called apoptosis are poorly understood and remain to be delineated. Therefore, the modeling of excitotoxic cell death in animal models is a useful tool for investigating the molecular basis of this type of neurodegeneration. Kainic acid (KA) is a glutamate analogue with excitotoxic properties. It activates both the kainate and AMPA class of ionotropic glutamate receptors where it increases synaptic activity resulting in seizures, neurodegeneration, and remodelling primarily in limbic structures such as the hippocampus (Bortolotto et al., 2003; Sperk, 1994). It is AMPA receptors which mediate this excitotoxicity because, while they desensitize rapidly when activated by AMPA, they desensitize slowly while being activated by kainate (Yoshioka et al., 1995). Furthermore, the AMPAR antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) has been shown to inhibit KA-induced excitotoxicity (Lopez-Picon et al., 2006). KA treatment, therefore, can be employed to assess a variety of different signalling mechanisms in which the brain attempts to remain plastic and protect itself following excessive excitotoxic stimulation. KA-induced excitotoxicity is known to be associated

with the excessive release of glutamate that underlies the pathogenesis of neuronal injury. Accordingly, KA can be used as a model agent for the study of neurotoxicity among various excitatory amino acids, since its administration produces a highly specific pattern of neuronal loss in CA1 and CA3 regions of the hippocampus (Arias et al., 1990). Cell death following KA is thought to be partly apoptotic (Pollard et al., 1994), which is characterized by nuclear condensation, membrane blebbing and caspase-3 activation (Simonian et al., 1996; Korhonen et al., 2001). Hunsberger et al. (2005) used caspase-3 immunohistochemistry (IHC) to demonstrate increased caspase-3 protein expression in the CA1, CA2, and CA3 hippocampal regions of severe seizure rats, further supporting the idea that KA administration activates a cell signalling pathway that leads towards apoptosis. Moreover, the neuronal death which results from KA administration can be detected by deoxyribonucleic acid (DNA) fragmentation in hippocampal pyrimadal cells (Tooyama et al., 2002), and this neuronal loss has been shown to be progressive, dose-dependent and subfield specific (Humphrey et al., 2002; Sato et al., 2001). It has been speculated that there exists a balance between the relative levels of anti- and pro-apoptotic proteins that determines cell fate towards death or survival. For instance, Korhonen et al. (2003) have demonstrated that the administration of KA induces specific alterations in the levels of pro- and anti-apoptotic Bcl-2 family proteins in vulnerable regions of the hippocampus. This being said, the roles played by different downstream signals and gene products causing cell death and degeneration after excitotoxic neuronal damage are so far not fully understood. This research uses KA to induce neurodegeneration in the CA1 region of the hippocampus and aims, in part, to shed some light on possible mechanisms by which excitotoxic insults ultimately result in neurodegeneration. Furthermore, this project seeks to determine whether the electrophysiological induction of synaptic plasticity can influence the neuronal loss caused by KA administration.

1.3 Fluorojade B labelling of degenerating neurons

Fluorojade B (FJ) is a fluorescent anionic marker of neuronal degeneration that can be used to specifically label injured neurons. Schmued & Hopkins (2000) have shown that FJ stains neurons undergoing degeneration from a variety of insults which include injury by KA and domoic acid, manganese and iron compounds, phencyclidine and MK-801, ibogaine, and enucleation. The diverse range of mechanisms manifesting the neurotoxicity of these agents suggests that FJ is a specific marker of general neuronal cell

death. Furthermore, Sato et al. (2001) concluded that FJ is a marker of irreversible neuronal injury because the appearance of FJ labelled cells in the cortex, hippocampus, and thalamus after traumatic brain injury corresponded closely to the initial pattern of silver and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) while the distribution of FJ-labelled cells coincided with typical regions of overt neuronal loss. Moreover, Poirier et al. (2000) used an experimental model of pilocarpine-induced status epilepticus to demonstrate that increases in FJ labelling correspond in time to neuronal loss. FJ is capable of labelling both the degenerating neuronal cell body as well as its processes. Thus, at any selected time point, FJ offers a comprehensive approach to evaluating regional neuronal vulnerability by providing a 'snapshot' of neuronal injury and defining the patterns of degeneration within both the cell bodies and processes of particular neurons. Although the exact mechanism by which FJ stains injured neurons is not known, it presumably acts as an acidic dye with a high affinity to strongly basic molecules expressed during neuronal degeneration (Ye et al., 2001). This research uses FJ to assess the degree of neuronal damage in the CA1 region of the hippocampus at various time points following the administration of KA under a number of different experimental conditions.

1.4 NMDARs play a critical role in both synaptic plasticity and neurodegeneration

The NMDA subtype of glutamate receptor is a key receptor involved in the regulation of multiple processes related to synaptic plasticity including learning and memory, neuronal development, spine formation, LTP and LTD (Collingridge & Bliss, 1995; Neuhoff et. al., 1999). In addition, irreversible neuronal damage associated with the over-stimulation of NMDARs leads to acute detrimental effects such as hypoxic/ischemic neuronal death as well as to chronic neurodegenerative disorders such as Parkinson's disease (Zhang et al., 1997; Steece-Collier et al., 2000). To this date, the mechanisms underlying such a diversity of neuronal responses to the activation of a single receptor are not yet fully understood. NMDARs are abundant, widely distributed throughout the brain, fundamental to excitatory neurotransmission and necessary for normal CNS function. The NMDAR is a complex heteromeric protein with an obligatory subunit, NR1, essential for channel function, which is potentially associated with four other subunits, NR2A-D (Laube et al., 1997). Activation of the NMDAR is sophisticated as both glutamate and glycine binding are necessary to open the ion channel that permits Ca^{2+} entry. Glutamate is the neurotransmitter as it is presynaptically released in an activity-dependent manner, whereas glycine acts as a

modulator. The ion channel integral to the NMDAR is voltage-dependently blocked by Mg^{2+}, and depolarization is required to remove this block. Thus, the NMDAR acts as a coincidence detector, linking neurotransmitter release activation with the electrical state of the neuron. The relative representation of members of the NR2 family modifies the channel properties and relative binding specificities, along with other functional characteristics (Hollman and Heinemann, 1994). For instance, NR2B-containing NMDARs have a longer phase of Ca^{2+} permeability when activated than NR2A-containing NMDARs (Monyer et al., 1994), whereas the peak current density is higher in NR1/NR2A receptors than in NR1/NR2B receptors (Chen et al., 1999). Thus, different NMDAR subtypes may trigger the separate signalling cascades required for a variety of distinct cellular processes. The precise signalling cascades still remain to be determined, however, data suggesting that NR2A and NR2B subunits couple variously to different intracellular substrates has emerged (Leonard et al., 2002; Li et al., 2002). Such fundamental differences in channel properties based on subunit representation clearly have the capacity to influence circuit characteristics and plasticity, should they be multifunctional and evidence is accumulating in support of this theory. This may provide clues as to how NR2A and NR2B-containing NMDAR subtypes have opposing roles in influencing the direction of synaptic plasticity. Recently, Liu et al. (2004) have demonstrated that selectively blocking NMDAR's that contain the NR2B subunit abolishes the induction of LTD but not LTP whilst the preferential inhibition of NR2A-containing NMDAR's prevents the induction of LTP without effecting LTD production, and similar results have also independently been reported (Mallon et al., 2005). Furthermore, synaptic NR2A receptors have been shown produce LTP while extrasynaptic NR2B receptors have been shown to produce LTD (Massey et al., 2004). On the other hand, a role of NR2B-type NMDARs in LTP has been demonstrated in genetically modified mice, with overexpression of NR2B or upregulated NR2B expression leading to enhanced LTP (Tang et al., 1999; Kohr et al., 2003) and Weitlauf et al. (2005) have reported that the NR2A subunit is not obligatory for the induction of LTP outside of the hippocampus as NR2A knock-out mice were able to exhibit LTP in the dorsolateral bed nucleus of the stria terminalis (dlBNST). While it is possible that compensatory mechanisms account for the contradictory results seen in genetically altered mice, further research will be required to resolve this matter. Nonetheless, recent experiments also suggest that synaptic and extrasynaptic NMDARs may have distinct roles in gene regulation, and cell death as well (Lu et al., 2001).

For example, the activity-dependent activation of synaptic NMDARs seen under normal physiological circumstances results in the release of brain-derived neurotrophic factor (BDNF) which ultimately leads to a neuroprotective state that protects all vulnerable neurons against the excitotoxic effects of glutamate (Marini et al., 2004). Additionally, the extracellular signal-regulated kinase (ERK) signalling cascade is a key pathway that mediates both synaptic plasticity and survival (Thomas & Huganir, 2004), and there is a differential restriction in the regulation of ERK activity that depends on the pool of NMDARs that is activated. This bidirectional control of the ERK signalling cascade by different pools of NMDARs is an early signalling event that enables a diverse number of physiological and/or pathological outcomes to occur (Chandler et al, 2001). Specifically, the synaptic pool of NMDARs (NR2A) activates ERK whereas the extrasynaptic (NR2B) pool triggers a pathway that results in the inactivation of ERK (Ivanov et al. 2006). This negative regulation of ERK activity modifies spine formation, long-term potentiation, and cell survival (Hardingham & Bading, 2002; Goldin & Segal, 2003) and therefore might be one of the first signalling events determining brain injury. Furthermore, it has been reported that Ca^{2+} flux through predominantly NR2A -containing synaptic NMDARs activates cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) whereas flux through predominantly NR2B-containing extrasynaptic NMDARs triggers a CREB shut-off signal (Hardingham & Bading, 2002). Since CREB regulates a number of pro-survival genes, this is a possible downstream mechanism whereby NR2A-containing NMDARs mediate cell-survival and NR2B-containing NMDARs mediate cell death (Liu et al., 2007). So while sustained NMDAR activation promotes signalling to the nucleus that culminates in CREB phosphorylation, multiple gene activation and long term synaptic plasticity, excess glutamate chronically overstimulates NMDARs, and the resultant excess intracellular Ca^{2+} leads to excitotoxicity – the glutamate-dependent mechanism by which neurons die in various CNS disorders. Overactivation of NMDARs plays a critical role in animal models of ischemic brain damage, and several different types of NMDAR blockers have attracted interest in recent years as neuroprotective compounds. Ro 25-6981 is a highly selective, activity-dependent blocker of NR2B-containing NMDARs that has demonstrated potent neuroprotective effects *in vitro* (Fischer et al., 1997), which can also be used to block the production of LTD (Liu et al., 2004) and possibly facilitate the induction of LTP (Mallon, 2005). Presumably, any CNS disorder in which neuronal loss is caused by glutamate-induced excitotoxicity has the potential to be treated by blocking

NMDARs. This includes cerebral ischemia, which occurs after stroke or brain trauma. In this case, dying neurons at the damaged core release glutamate to overactivate those in the penumbra and it is the activation of extrasynaptic NMDARs, in particular, that is attributed mainly to the global release of glutamate occurring under these pathological conditions, which eventually leads to cell death (Arundine & Tymianski, 2004). This NMDA blocking approach may also be applied to neurodegenerative disorders such as Parkinson's disease and Huntington's disease in which excess glutamate is not the primary problem, but compromised neurons become sensitized to excitotoxic damage. Unfortunately though, clinical trials using NMDAR antagonists in stroke and traumatic brain injury have so far failed (Kemp & McKernan, 2002).

In summary, the NMDARs role in excitotoxicity has driven the search for antagonists as neuroprotective agents and its role in synaptic plasticity has inspired research into receptor potentiators to treat cognitive dysfunction. It is quite conceivable, however, that synaptic plasticity and excitotoxicity are not mutually exclusive processes and that they are, in fact, closely related to one another through the NMDAR. Taken a step further, different forms of synaptic plasticity such as LTP and LTD may actually influence the balance that exists between cell survival and cell death in an excitotoxic environment. Through the electrophysiological induction of activity-dependent synaptic plasticity and the pharmacological production of neurodegeneration, it will be possible to experimentally test if this relationship does indeed exist. Additionally, NMDAR subtype-specific antagonists can be used to further characterize the hypothesized association between synaptic plasticity and neuronal survival or death. Ultimately, this research project aims to test the hypothesis that NR2A-containing NMDARs are required for the induction of LTP and this process is coupled to a postsynaptic cell survival pathway while NR2B-containing NMDARs are necessary for the production of LTD and this mechanism is linked to a downstream apoptotic pathway.

2. MATERIALS AND METHODS

Animals

400 male Sprague-Dawley rats (UBC Animal Care Center), aged 25-32 days were used in the present research. Rats were group housed in transparent polycarbonate cages (59 × 38 × 20 cm), with 4-6 rats per cage, post-weaning. Animals were maintained on a 12/12hr light/dark cycle and testing was carried out during the dark phase. The colony was maintained at 21 ± 1°C and animals were given *ad libatum* access to food and water. All experiments were performed in accordance with UBC and Canadian standards for animal care and careful consideration was made to reduce the number of animals used and to minimize animal suffering during the course of this research.

In vivo electrophysiology

Seven experimental groups participated in the electrophysiological study. The animals received either no HFS, HFS alone, LFS alone, HFS and LFS, NVP-AAM077 and HFS, Ro25-6981 and HFS, NVP-AAM077 and Ro25-6981 and HFS. The animals were anaesthetized using Somnotol (1ml/kg i.p.; MTC Pharmaceuticals), and placed in a stereotaxic instrument (Kopf Instruments). Rectal temperature was maintained at 37±0.5°C with the use of a homeothermic blanket (Harvard Apparatus). The depth of anesthesia was monitored during the course of each experiment via the foot-pinch withdrawal reflex and supplemental doses of anesthetic were administered as necessary. To gain access to the hippocampus, the scalp was cut and retracted to expose the skull surface. A stereotaxic drill unit (Kopf Instruments) was then used to drill holes in the appropriate locations and the dura was gently removed to permit the insertion of electrodes into the brain. The recording electrode was inserted -3.5mm posterior and 2.2mm lateral to bregma, and the stimulating electrode was inserted -4.0mm posterior and 2.7mm lateral to bregma. Both electrodes were lowered into the CA1 stratum radiatum region of the hippocampus and the correct depth was determined when a maximal CA1 EPSP could be obtained with minimal electrical stimulation (50-100μA). In addition, a hole was drilled anterior to bregma to accommodate a reference electrode for the recording electrode. The monopolar reference and recording electrodes were constructed from 75μm Teflon-coated, platinum-iridium wire (A-M Systems Inc.) and a bipolar stimulating electrode was used. Stimulation was generated with an A/D Analog Digital Converter (National Instruments), and a Digital Stimulus Isolation unit (Getting Instruments). Pyramidal cell responses to ipsilateral Schaeffer collateral

stimulation were recorded using a differential amplifier (Getting Instruments), and data was stored and analyzed with the respective software programs Stim & Record and Browse & Analyze (Getting Instruments). Initial baseline responses were obtained by delivering a single 0.12 ms square pulse once every 15 seconds. Once a stable baseline was established for at least 30 minutes, HFS was applied via the stimulating electrode in the form of 100 Hertz (Hz.) for 1 second (100 total pulses). LFS was applied via the stimulating electrode in the form of 1Hz for 15 minutes (900 total pulses) or paired-burst stimulation (PB) delivered at 1Hz for 300 trains (1200 total pulses) where a train consists of two bursts, with a 10ms interburst interval and each burst consists of two pulses, with a 2.5ms interpulse interval. Following the tetanus, stimulation was returned to baseline rates of 1 pulse every 15 seconds, and the decay was followed for up to two hours. All injections (NVP-AAM077, Ro25-6981, saline) were delivered i.p. 60 minutes prior to HFS.

Kainic Acid

Sixteen groups of animals participated in a kainic acid dosage study to determine the optimum intraperitoneal dosage for eliciting neurodegeneration in the CA1 region of the hippocampus: saline controls (n=3), 1mg/kg KA (n=6), 5mg/kg KA (n=6), 10mg/kg KA (n=12), 15mg/kg (n=20), 18mg/kg KA (n=20), 20mg/kg KA (n=20), and 30mg/kg (n=20) where 3 animals from each dosage were given a 24 hour, 48 hour or 1 week recovery period in their cages.

Pharmacology

Six groups participated in the pharmacological study: saline controls (n=5), saline pre-treatment with KA (n=6), NVP-AAM077 alone (n=4), NVP-AAM077 pre-treatment with KA (n=6), Ro25-6981 pre-treatment with KA (n=9), and NVP-AAM077 + Ro25-6981 with KA (n=5). All drugs were delivered i.p. and pre-treatments were given one hour prior to the administration of KA. To reduce stress, each animal was placed in plexiglass chamber and temporarily anaesthetized with halothane before receiving an injection. A saline solution (0.9% NaCl) was used as the vehicle solution and NVP-AAM077 was administered at 2.4mg/kg while Ro25-6981 was administered at 6.0mg/kg. The NVP-AAM077 and Ro-6981 were obtained from Novartis Chemicals Ltd., and were stored at -20C until used. Animals in each group were given a 24 hour recovery period following pharmacological treatment.

Histology

At the conclusion of each experiment, animals were euthanized and perfused intracardially with saline, followed by perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The perfused brains were then quickly removed, placed in a 30% sucrose solution for cryoprotection, and finally stored in a minus 80°C freezer. Post-fixed brains were then cut into 25 or 50μm coronal sections using a cryostat (Leica), laid onto slides and stained with 0.1% cresyl violet to determine the correct placement of the electrodes within the CA1 region of the hippocampus under a light microscope. In addition, sections from each brain were also stained with Fluorojade B (Chemicon) to assess neuronal damage in the hippocampus. Briefly, sections dried onto gelatinized slides were gradually re-hydrated in a series of ethanol baths. These sections were then placed in a potassium permanganate solution with a stir bar for 10 minutes before being moved to a Fluorojade B staining solution (0.001% in 0.1% acetic acid) for 30 minutes. The slides were then washed in three consecutive baths of distilled water for 1 minute each and allowed to air dry overnight in the dark. Finally, the slides were washed in three baths of xylene solution for 3 minutes each and cover-slipped with a xylene based mounting medium. Once dry, the slides were examined under a fluorescence microscope using a FITC filter to identify the presence of FJ-B positive neurons in the CA1 region of the hippocampus. Next, digital images were captured using Northern Eclipse (Empix) and 2000 pixel samples were selected from the cell body layer of the CA1 region using Photoshop 7.0 (Adobe) to determine a measure of mean fluorescent luminosity in each hemisphere. For immunofluorescence histochemistry, sections were produced in the aforementioned manner and then incubated overnight at 4°C in mouse monoclonal antibody to p-Akt (Cell Signaling; diluted 1:200) or mouse monoclonal antibody to neuronal nuclear antigen (NeuN, Chemicon; diluted 1:100). The sections were then incubated with Cy3-conjugated goat anti-mouse IgG (Jackson; diluted 1:500) for 2 hours at room temperature. The slides were viewed under a fluorescence microscope and captured images were converted to TIFF format, and contrast levels of images were adjusted using Photoshop 7.0.

3. RESULTS

3.1 Synaptic Plasticity in the CA1 region of the hippocampus *in vivo*

The first experiments conducted during this research were aimed at establishing reliable protocols for the induction of LTD and LTP in the CA1 region. Initially, a PB (Staubli & Scafidi, 1997) stimulation protocol was used in an attempt to reliably induce LTD *in vivo*. This tetanus protocol was found to be capable of inducing significant LTD in small groups of animals (-24.83 ± 0.96, $p<.05$) (Figure 2A), however, it was unreliable for the production of LTD on a consistent basis as only 54% of experiments (n=60) were considered to be successful where the EPSP slope (Figure 2B) was < -10% of the baseline 1-hour post-tetanus. Next, 1Hz. LFS (Heynen et al., 1996) was tested in a several groups of animals and was found to be even less effective than PB in producing LTD as only 37% of experiments (n=30) resulted in an acceptable level of LTD. On the other hand, LTP was found to be much more attainable *in vivo* as 100Hz HFS was effective in inducing significant levels of LTP (18.46 ± 1.07, $p<.05$) that persisted for at least one hour (Figure 3A) on a consistent basis. Due to the aforementioned inability to produce reliable LTD, this research primarily focuses on effect of LTP on cell survival and death

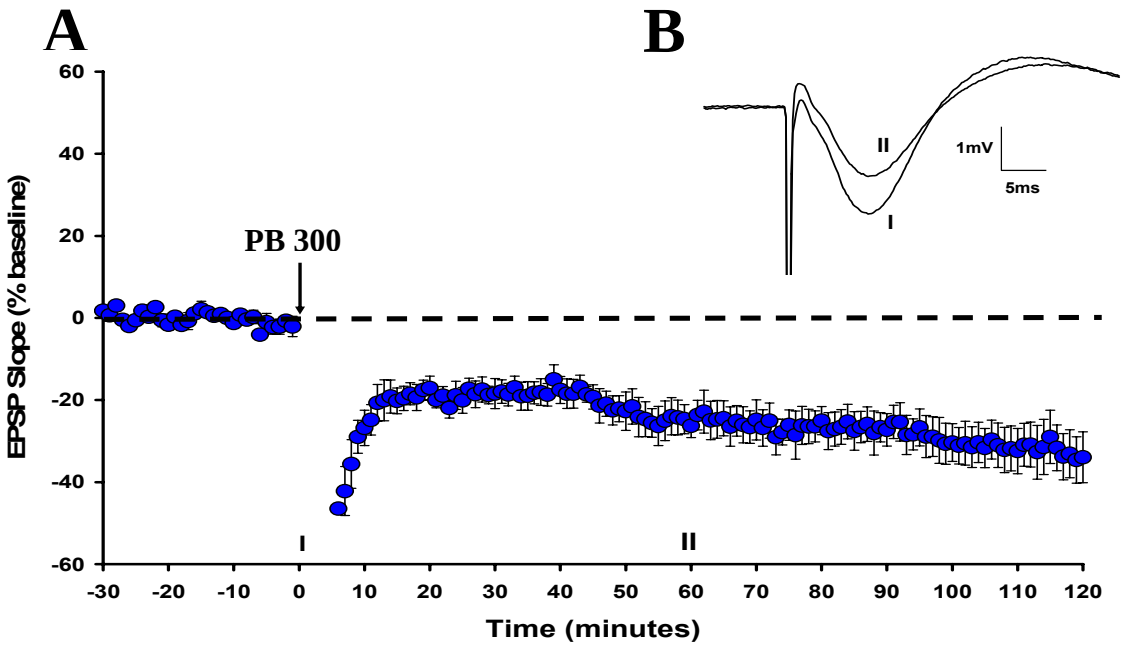


Fig. 2. (A) The induction of homosynaptic LTD using a 300 paired burst stimulus. **(B)** Representative fEPSCs averaged from 10 consecutive stimuli were taken at the corresponding time points (I and II) (n=5).

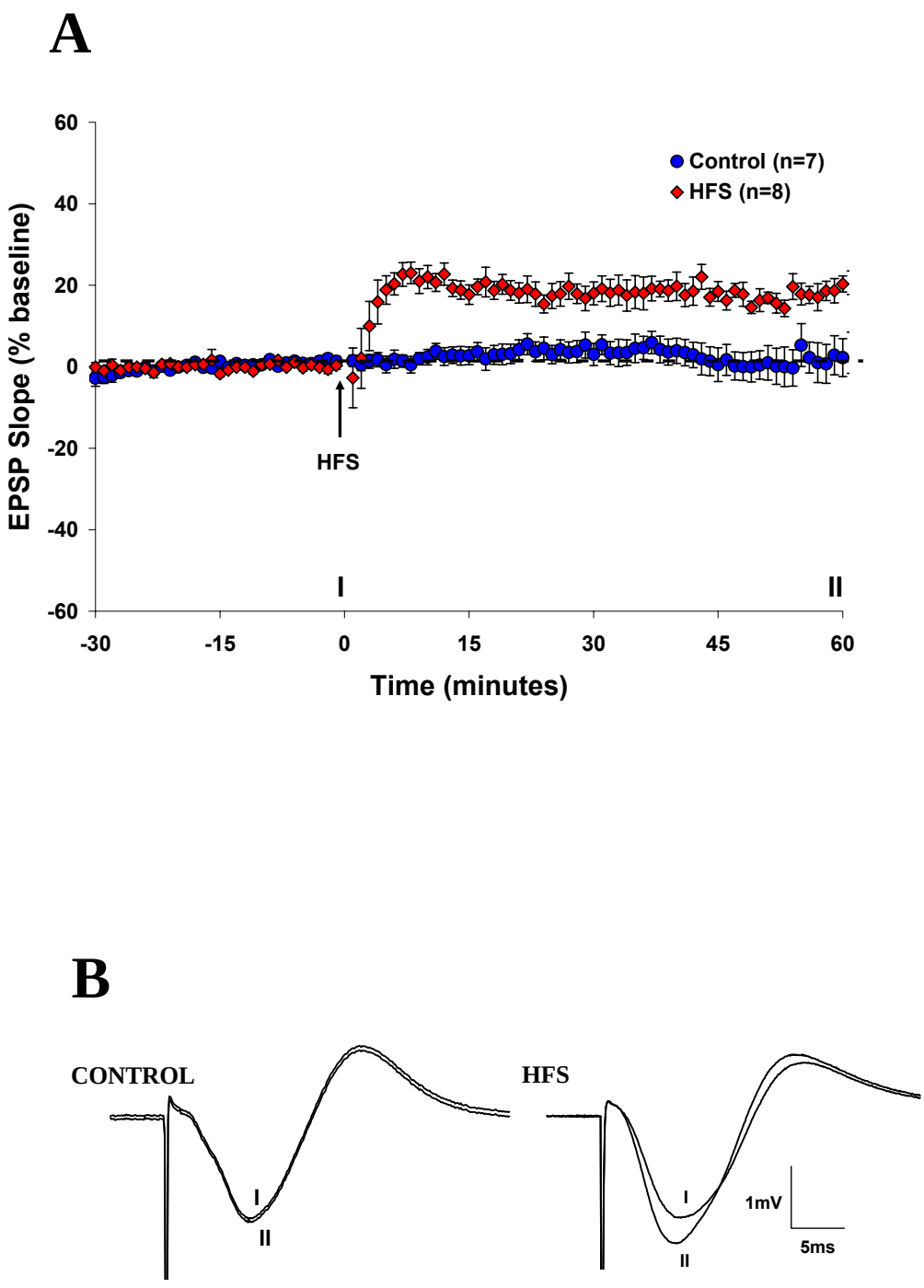


Fig. 3. (A) The induction of homosynaptic LTP using HFS compared to the stable baseline that is seen in the absence of HFS. **(B)** Representative fEPSCs averaged from 10 consecutive stimuli were taken at the corresponding time points (I and II).

3.2 The induction of LTP promotes the activation of Akt in the CA1 region of the hippocampus

To test the hypothesis that LTP is associated with a cell survival pathway, experiments were carried out to determine if there is a relationship between the induction of LTP and Akt in the CA1. This was a logical starting point, as the PI3/Akt pathway is a critical transducer for several major survival signals in CNS neurons (Datta et al., 1999). Immediately following the successful induction of LTP, animals were perfused intracardially and immunohistochemistry was performed on sections of the hippocampus. Immuno-reactivity for phospho-Akt (Ser473) was found to be significantly increased in the hemisphere that received LTP-inducing HFS (Left side, 20.15 ± 3.14; Right side, 33.92 ± 3.57; $p<.001$) while there was no significant difference in phospho-Akt immuno-reactivity between hemispheres of non-tetanized control animals (Figures 4+5). These data indicate that LTP induction appears to activate anti-apoptotic signaling by Akt, and therefore suggest that the induction of LTP could bestow a protective effect upon CA1 neurons in the hippocampus *in vivo*.

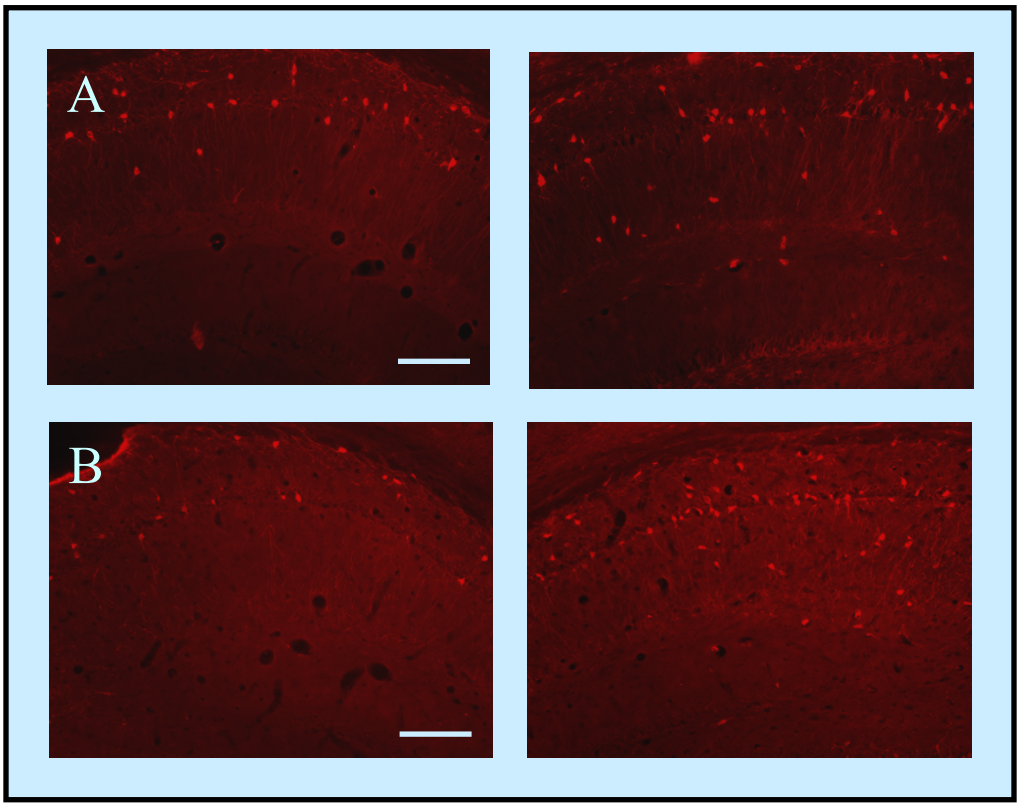


Fig. 4. Representative sections (25µm thick) of the rat hippocampus immunofluorescently labelled for p-Akt following the completion of electrophysiological experiments. **(A)** Photos of the hippocampus in the left and right hemisphere of a control animal. **(B)** Photos of the hippocampus in the left and right hemisphere of a rat receiving LTP-inducing HFS to the right side (Mag. = 10X). Scale bars are 200µm.

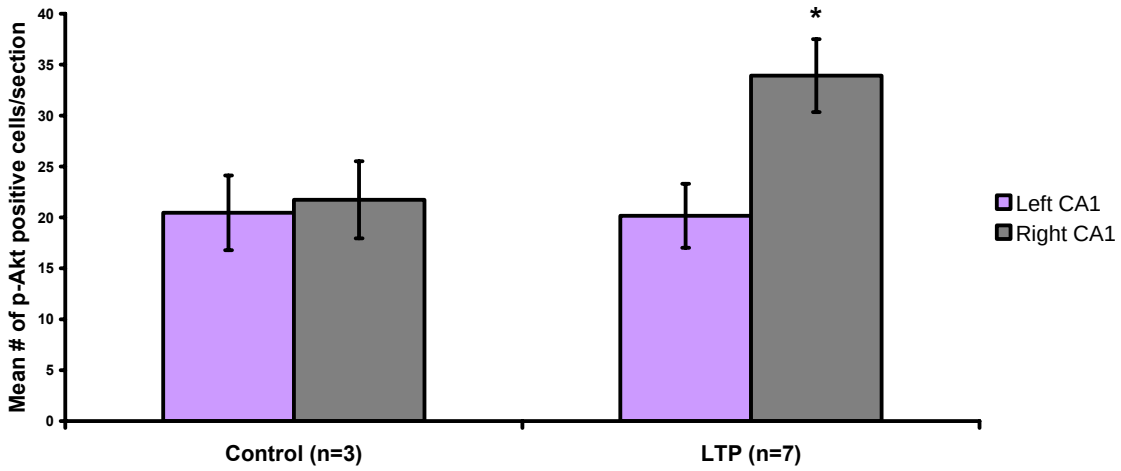
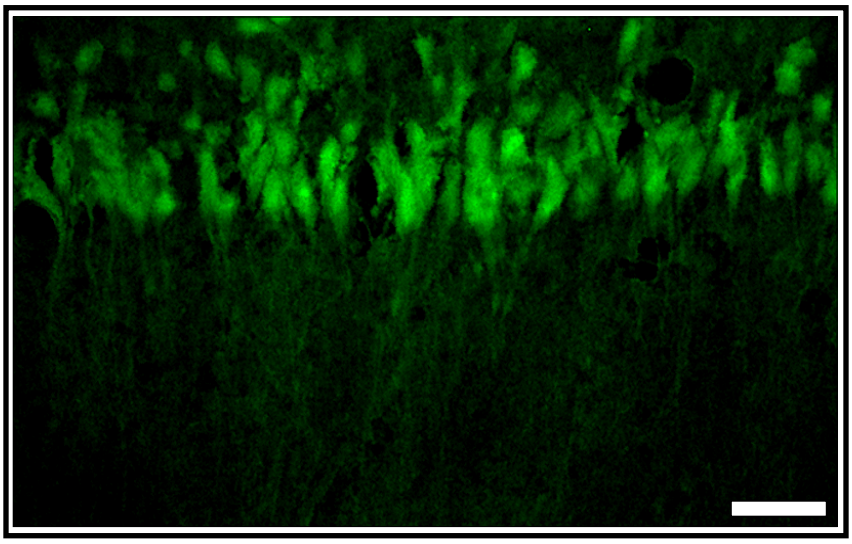


Fig. 5. p-Akt immunoreactivity is increased when LTP is induced in the CA1 region of the hippocampus.

3.3 Kainic acid induced excitotoxicity in the CA1 region of the hippocampus *in vivo*

The next step in this research project was to establish a reliable model of neurodegeneration in the hippocampus so a dosage study was employed to determine the optimum dose of KA required to induce neuronal death in the CA1 region. Fluorojade B was used as a sensitive and reliable indicator of degenerating neurons shortly after the administration of KA (Figure 6A+B), while cresyl violet staining was employed to assess KA-induced cell death over a longer time periods (Figure 7). It was found that a low dose of KA (10mk/kg i.p.) was capable of inducing CA1 death, detectable by FJ staining, in only a small proportion (16.67 ± 10.76%) of 30±3 day-old rats. On the other hand, a high dose (30mg/kg i.p.) was found to be lethal in a large percentage of animals (50.00 ± 11.18%), but effective in producing neurodegeneration in those that survived (80.00 ± 12.65%) (Figure 8). An ideal dosage that could produce maximal short- and long-term cell death in the CA1 while causing minimal fatalities appeared to lie in the 15-20mg/kg range, and in the end, 18mg/kg (i.p.) was chosen (Figure 6A).

A



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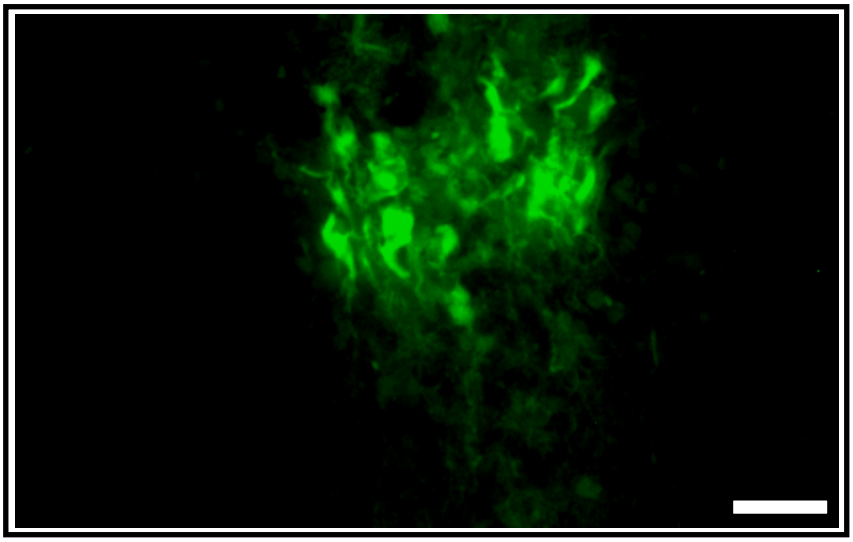


Fig. 6. (A) The systemic administration of KA (18mg/kg, i.p.) reliably produces bilateral neurodegeneration in the CA1 region of the hippocampus, and Fluorojade B sensitively and specifically binds to these degenerating neurons. Here, pyramidal neurons in the CA1 are labeled on the cell body and on some of the dendrites of the stratum radiatum. **(B)** Fluorojade B also stains neurons damaged by the insertion of a recording electrode into the CA1; however, there is absolutely no staining of the healthy adjacent neurons in the absence of KA administration (Mag. = 32X). Scale bar is 50μm

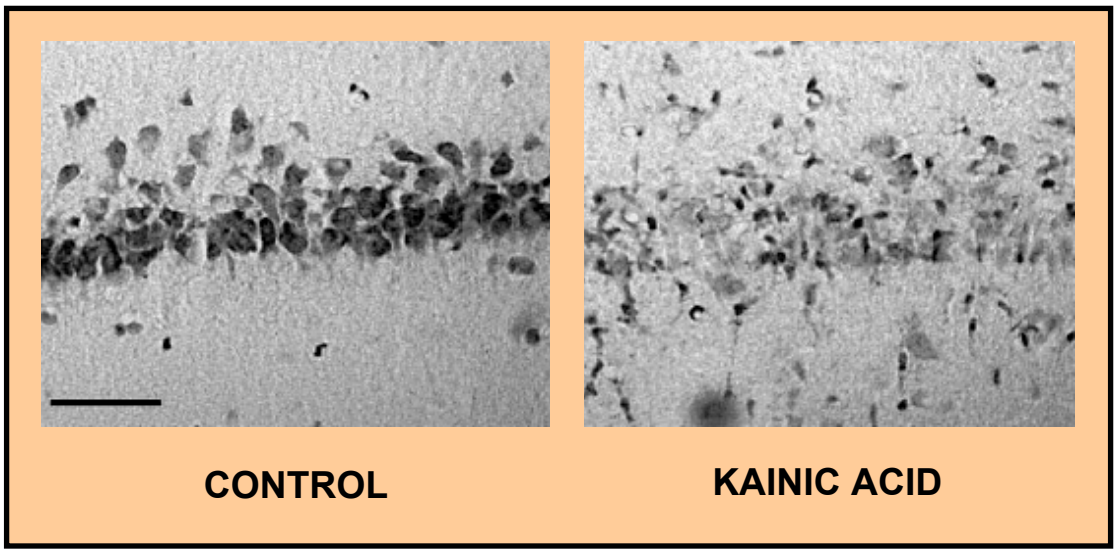


Fig. 7. Cresyl Violet-stained brain sections of the CA1 region of the hippocampus in a control and another rat two weeks after kainate (18mg/kg) treatment. The majority of the neuronal profiles have disappeared. (Mag. =40X). Scale bar=100µm.

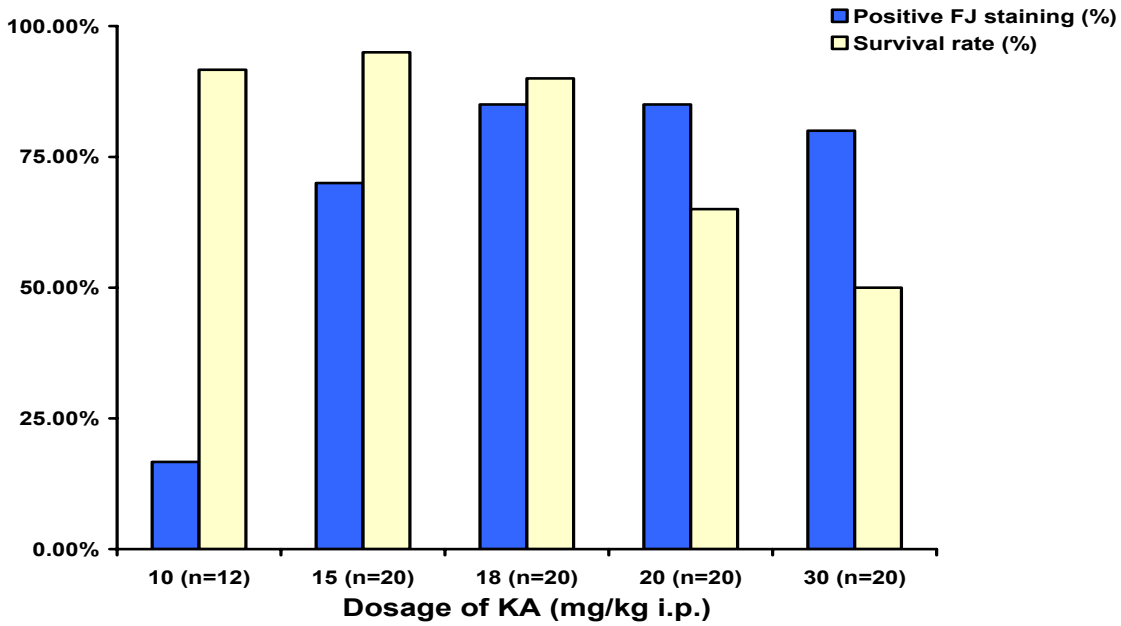


Fig. 8. Increasing doses of KA result in progressively improved levels of FJ labeling in the CA1 region of the hippocampus, however, the number of animals dying as a result of KA-induced excitotoxicity also increases in a dose-dependent manner. FJ staining was performed on 25µm sections prepared from surviving animal's brains 24 hours after KA injection.

3.4 The induction of LTP promotes neuronal survival 24 hours after KA-induced excitotoxicity

To determine if synaptic plasticity has any influence over the outcome resulting from KA-induced excitotoxicity, LTP was induced, and then KA was administered 24 hours following LTP production and recovery from anesthesia. The reason for this recovery period is because the anesthetic used during electrophysiology experiments, Somnotol, is a GABA modulator that was found to strongly attenuate the effects of KA during preliminary experiments. Fluorojade B labeling was used to assess neuronal damage and was quantified and compared by measuring Fluorojade B-dependent fluorescent luminosity in the cell body layer of the CA1 in each hemisphere (Figure 12). There was no significant difference in the degree of CA1 neurodegeneration between hemispheres in control animals (no tetanus, Figures 3A+9A+12), however, the induction of LTP in a group of animals (Figure 3A) was found to significantly attenuate the amount of CA1 neurodegeneration measured in the tetanized hemisphere compared to that found in the non-tetanized hemisphere (Left side, 47.80 ± 5.82; Right side, 17.78 ± 5.85; $p<.05$) (Figures 9B + 12). This would suggest that, in the short-term, the induction of LTP reduces KA-induced cell death.

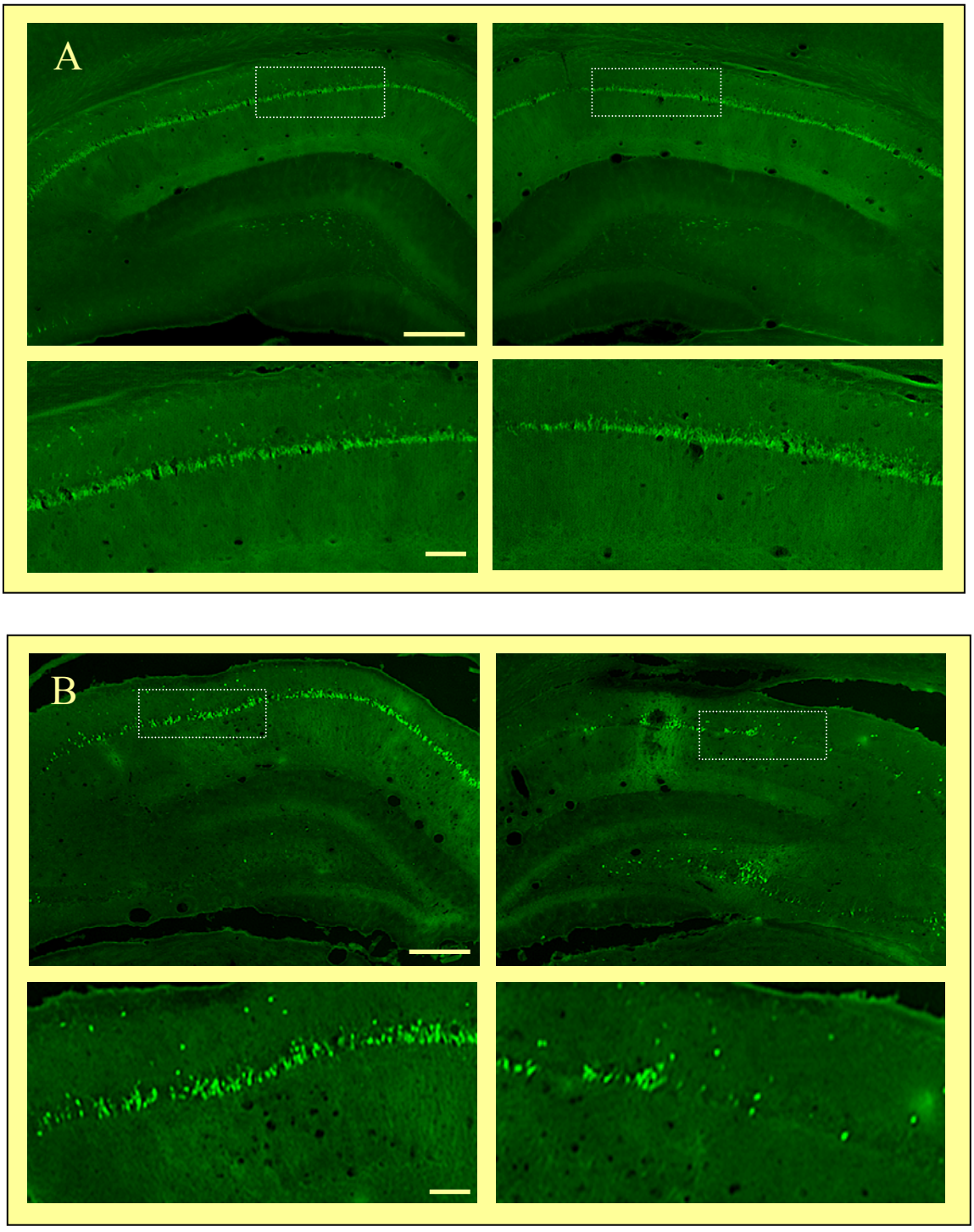


Fig. 9. Representative Fluorojade B stained sections (50µm thick) of the rat hippocampus following electrophysiology experiments and kainic acid administration. **(A)** Photos of the hippocampus in the left and right hemisphere of a control animal. **(B)** Photos of the hippocampus in the left and right hemisphere of a rat receiving HFS to the right side (Mag. = 5X). The pictures on the bottom row of each figure are blown-up images of the outlined areas in the pictures directly above them. Scale bars are 500µm on the top row and 90µm on the bottom row.

3.5 LTP induction promotes neuronal survival in the CA1 two weeks after KA-induced excitotoxicity

It has already been established that KA treatment leads to substantial hippocampal cell loss two weeks following injection (Figure 8). In this group, LTP was induced, KA was administered 24 hours following LTP production, and the animals were housed for a two week recovery period. To measure the degree of neuronal damage two weeks after the administration of KA (18mg/kg, i.p.), sections were stained with cresyl violet (Figure 11A) and CA1 neuronal profiles were then quantified (Figure 11B). Furthermore, immunohistochemistry using the neuronal marker NeuN was carried out on a number of sections, and a correlation between the patterns of cresyl violet and NeuN staining was seen (Figure 10). It was found that the number of CA1 neurons in the hemisphere receiving LTP-inducing HFS was significantly higher in comparison to the non-stimulated hemisphere. (Left side, 37.50 ± 2.35; Right side, 59.33 ± 2.57; $p<.0001$) (Figure 11B). These data support the hypothesis that LTP induction attenuates the neuronal loss resulting from kainate treatment, and further demonstrates that this effect is persistent over a long time period.

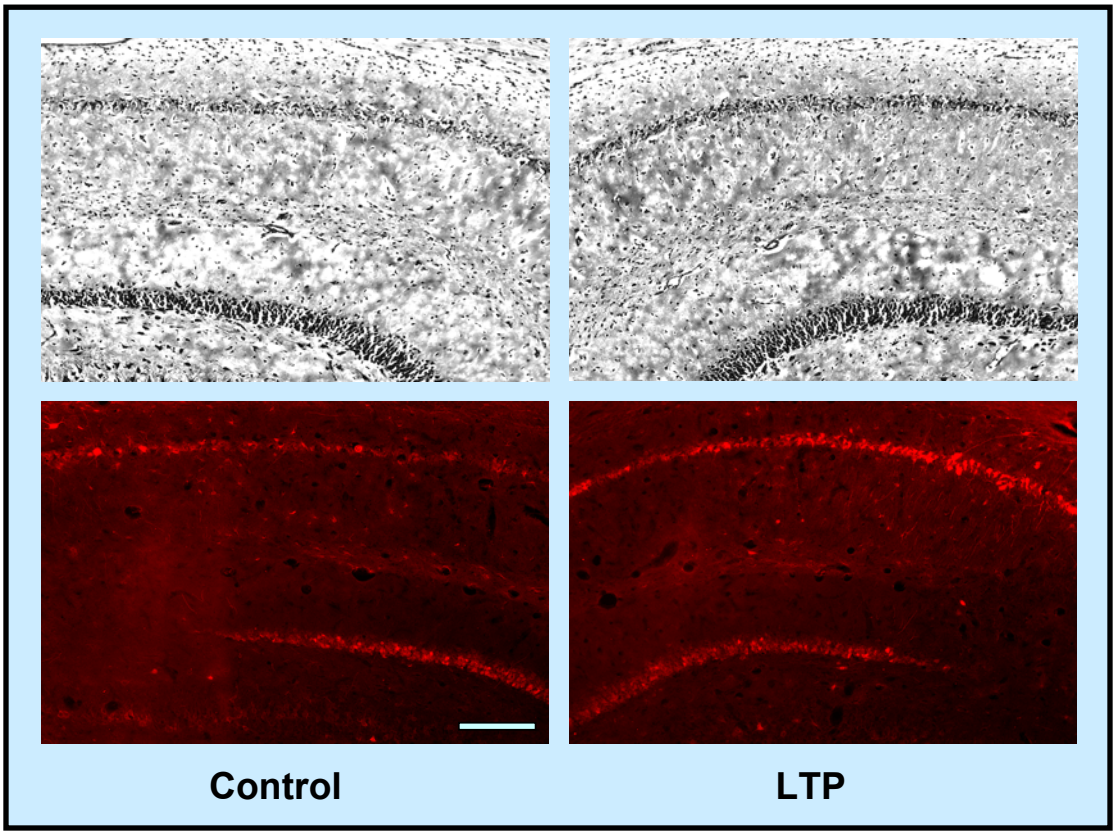
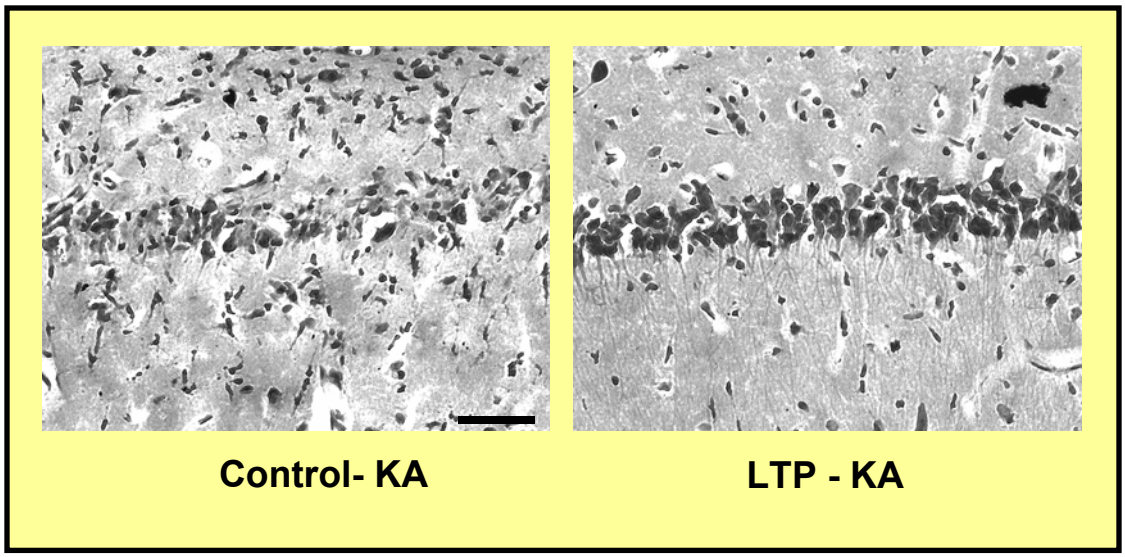


Fig. 10. Cresyl violet (top row) and NeuN (bottom row) staining in two sections from the same brain two weeks following LTP induction and KA administration (18mg/kg, i.p.)(Mag. =10X). Both stains indicate a higher number of CA1 neurons in the hemisphere that received LTP-inducing HFS. Scale bar is 400µm.

A



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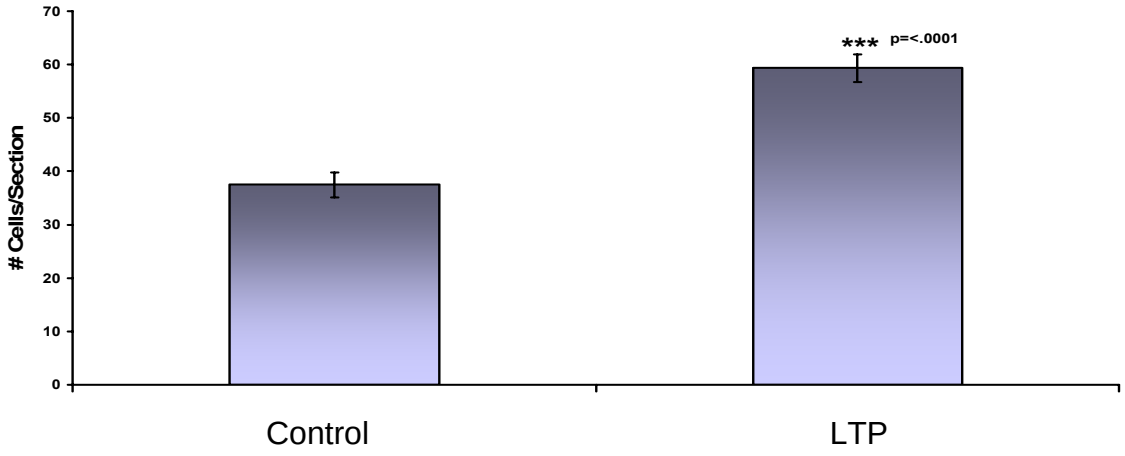

Fig. 11. (A) Representative cresyl violet stained sections (25µm) of the CA1 region of the rat hippocampus 2 weeks after the induction of LTP in the right hemisphere and subsequent administration of KA (18mg/kg, i.p., 24 hours post-HFS) (Mag.=40X). Scale bar is 100µm. **(B)** Neuronal survival is drastically enhanced in the tetanized region of the CA1 2 weeks following the induction of LTP and subsequent KA administration (18mg/kg, i.p. 24 hours post-HFS) (n=4).

3.6 The induction of LTD does not influence KA-induced excitotoxicity in hippocampal CA1 neurons

As previously mentioned, a protocol capable of reliably producing LTD *in vivo* was unable to be achieved during the course of this research. Nonetheless, the successful induction of LTD did occur in a number of experiments which were then selected to examine whether or not LTD has any influence over CA1 neuronal fate resulting from KA-induced excitotoxicity. LFS was applied, and then KA was administered to animals exhibiting LTD 24 hours following surgery. The induction of LTD in a group of animals (Figure 2) was not found to have any significant effect on the degree of cell death produced by KA in each hemisphere when compared with non-tetanized controls (Figure 12). Furthermore, the successful induction of LTD itself was not found to cause any measurable degree of CA1 neurodegeneration in the absence of KA administration.

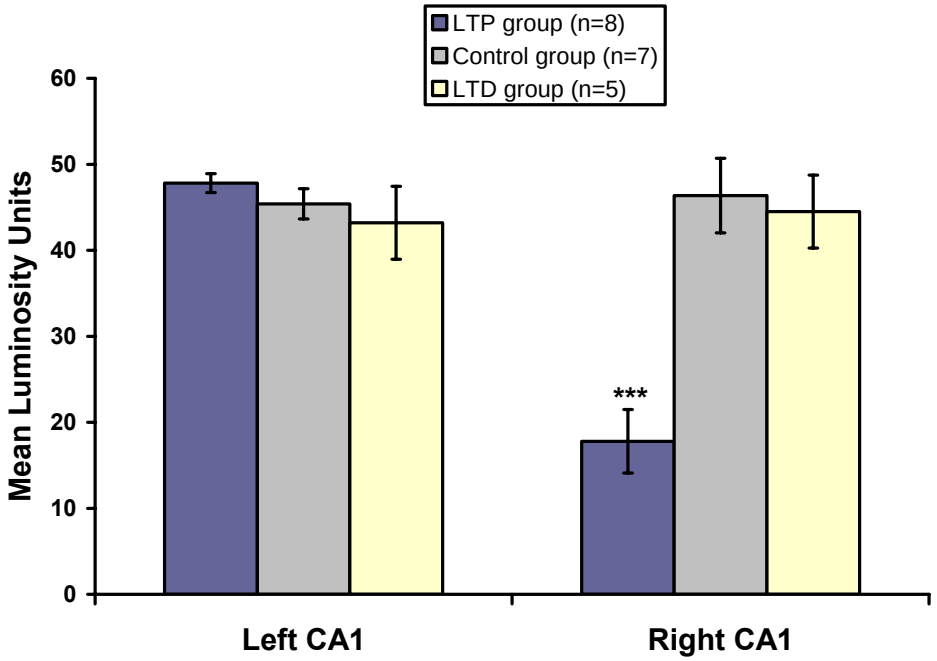

Fig. 12. Following the administration of KA (18mg/kg i.p.), hippocampal neurodegeneration was found to be significantly reduced in the tetanized hemisphere of the LTP group compared to the control and LTD groups. Neurodegeneration was quantified and compared by measuring Fluorojade B-dependent fluorescent luminosity in the cell body layer of the CA1 in each hemisphere.

3.7 The roles of NR2A- and NR2B-containing NMDARs in KA-induced excitotoxicity

To determine whether KA-induced excitotoxiciy is NMDAR subunit dependent, the NR2A specific antagonist NVP-AAM077 and the NR2B specific antagonist Ro-25-6981 were administered 1-hour prior to KA treatment (Figures 13+14). KA given alone results in a substantial degree of FJ staining in CA1 neurons which can be quantified by measuring the fluorescent luminosity in a fixed area of the cell body layer. It was found that both NVP-AAM077 (2.4mg/kg) and Ro-25-6981 (6.0 mg/kg), when given alone, did not result in any significant cell death in the CA1 region. Additionally, pre-treatment with NVP-AAM077 one hour prior to the administration of KA (18mg/kg, i.p.) had no measurable influence on the degree of neurodegeneration normally seen under this excitotoxic condition. On the other hand, pre-treatments with Ro-25-6981 (6.0mg/kg) (9.23 ± 7.60, $p<.05$) and a combination of both Ro-25-6981 and NVP-AAM077 (16.10 ± 9.58, $p<.05$) were able to significantly attenuate KA-induced neurodegeneration relative to the amount seen in controls (28.5 ± 5.92, $p<.05$) (Figure 14). These findings suggest that NR2B-containing NMDARs play a critical role in mediating KA-induced excitotoxicity, and demonstrate Ro-25-6981 to be highly neuroprotective agent against kainate treatment.

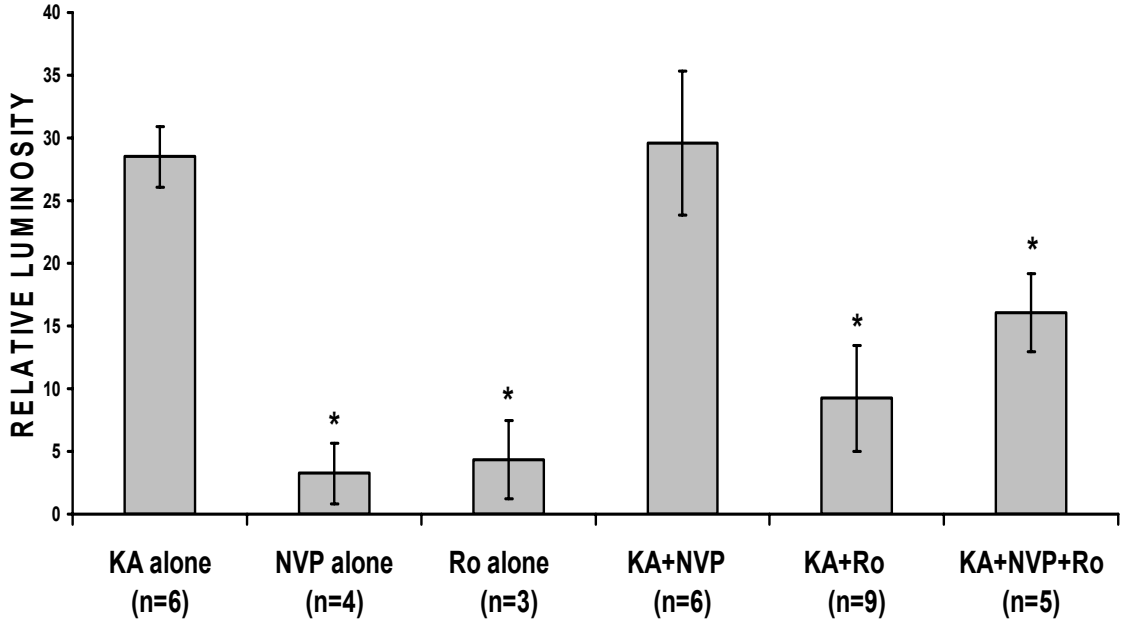

Fig. 13. The neurodegenerative effects of KA administration (18mk/kg i.p.) following different NMDAR subunit antagonist pre-treatments. Pre-treatments were given 1 hour prior to KA injection and FJ staining was performed on 25μm sections prepared 24 hours following the injection of KA.

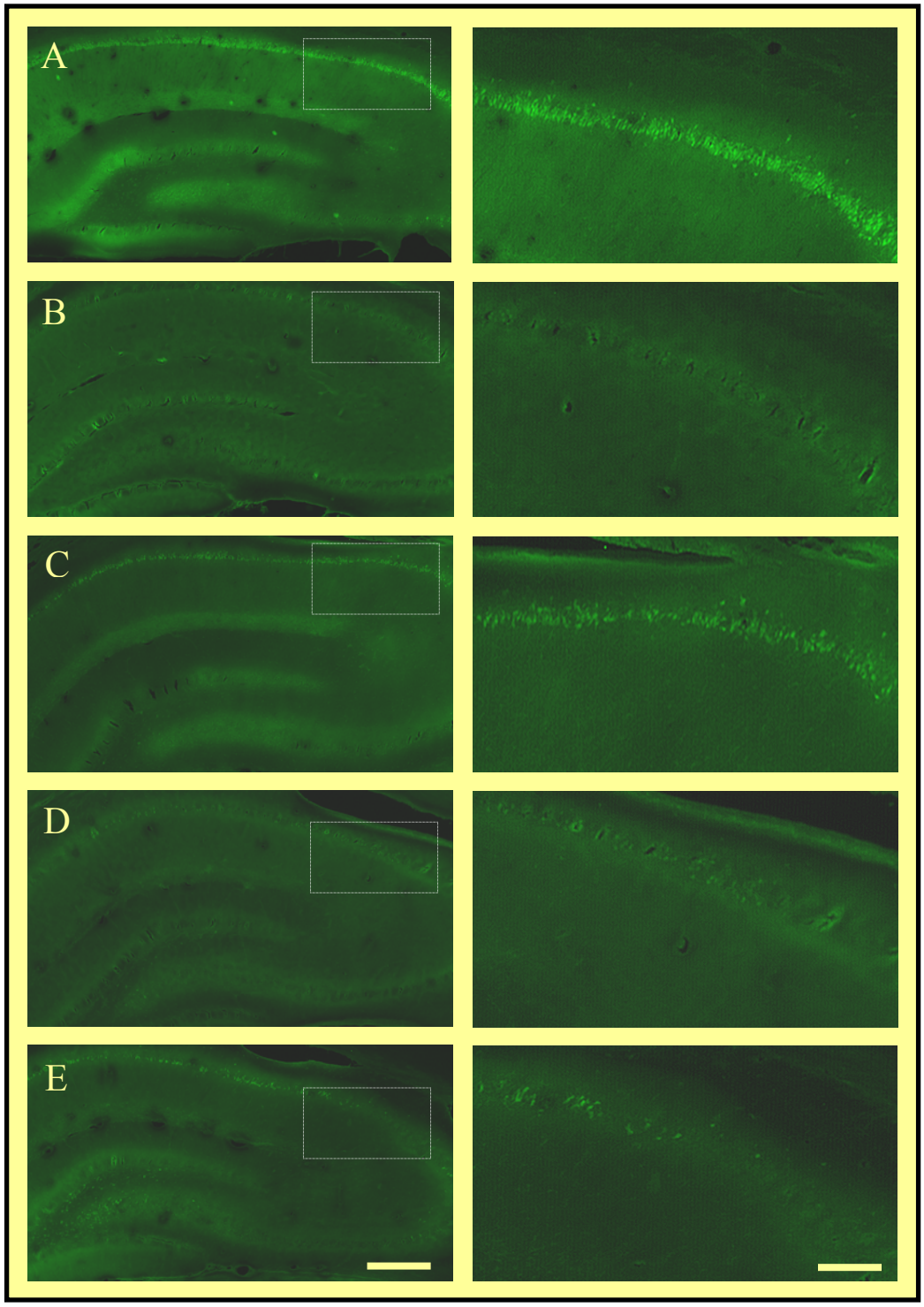

Fig. 14. Pictures of representative FJ-labeled sections of the hippocampus following NMDAR subunit antagonist pre-treatment and the i.p. administration of KA (18mg/kg). The pictures in the right column are blown-up images of the outlined areas from the pictures directly to the left (5X Magnification). **(A)** KA given alone. **(B)** NVP-AAM007 (2.4mg/kg) given alone. **(C)** NVP-AAM007 given 1 hour before KA. **(D)** Ro-25-6981 (6mg/kg) given 1 hour before KA. **(E)** NVP-AAM007 and Ro-25-6981 given 1 hour before KA. Scale bars are 500μm for pictures in the left column and 100μm for pictures in the right column.

3.8 The effect of the induction and expression of LTP on KA-induced excitotoxicity in the CA1

It has been shown *in vitro* that NR2A- and NR2B-containing NMDAR subtypes may dictate the polarity of synaptic plasticity (Liu et al., 2004; Massey et al., 2004), however, there has been little research exploring these findings *in vivo*. In this study, the respective NR2A and NR2B antagonists NVP-AAM077 and Ro-25-6981 were used to investigate their effects on the induction and expression of LTP in the anesthetized rat and the subsequent influence on KA-induced cell death. It was found that pre-treatment with NVP-AAM077 (0.6mg/kg) was sufficient to completely block the induction of LTP, and that a dose of 2.4mg/kg in fact converted the production of LTP into the production of LTD (-28.73± 1.42, $p<.05$) (Figure 15). On the other hand, pre-treatment with Ro-24-6981 (6.0mg/kg) had no significant effect on the induction of LTP (Figure 16). Additionally, the administration of both NVP-AAM077 and Ro-25-6981 together (2.4 and 6.0mg/kg, respectively) prior to HFS resulted in a significantly diminished degree of LTP (15.32 ± 1.13, $p<.05$) relative to controls (44.56 ± 1.50, $p<.05$), and the complete abolishment of NVP-HFS induced LTD that was seen with NVP-AAM077 (2.4mg/kg) pre-treatment alone (Figure 17). Together, these results suggest that the generation of LTP is NR2A dependent while NR2B-containing NMDARs play little or no role in LTP induction in the CA1 region. Furthermore, it would appear that NVP-HFS induced LTD is NR2B dependent as this effect is blocked by Ro-25-6981. 24 hours after pharmacological pre-treatments and electrophysiology, animals were administered KA (18mg/kg, i.p.), and neuronal damage in the CA1 was assessed a further 24 hours later with Fluorojade B. It was found that preventing the induction of LTP using NVP-AAM0077 (1.2mg/kg, i.p.) resulted in the restoration of KA-induced cell death while Ro-25-6981 pre-treatment was found to have no effect on the neuroprotection that LTP-inducing HFS produces relative to controls (Figure 18). When NVP-AAM077 was administered 1 hour after HFS (1.2mg/kg, i.p.)(Figure 19A), the maintenance of LTP was completely unaffected and LTP-associated neuroprotection was preserved (Figure 19B). Because NVP-AAM077 does not block neuroprotection when given after the induction of LTP, this data indicates that LTP mediates neuronal survival in the CA1. Moreover, the neuroprotection demonstrated throughout this research is due to the activation of NR2A-containing NMDARs by LTP protocol, and it is the production of LTP, specifically, that is responsible for the observed neuroprotection.

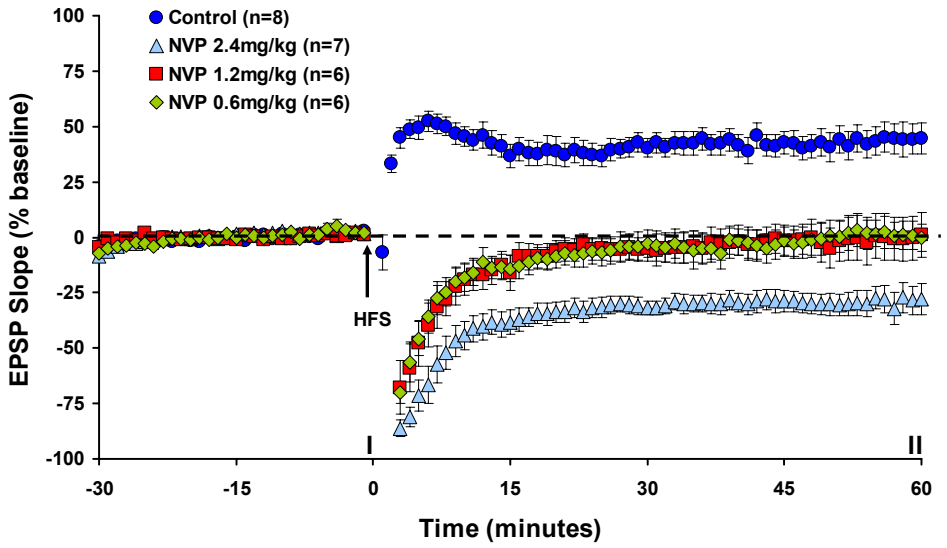

Fig. 15. The effect of different doses of the NR2A antagonist NVP-AAM077 administered IP 1 hour prior to LTP induction in the rat CA1 using 100 Hz tetanus.

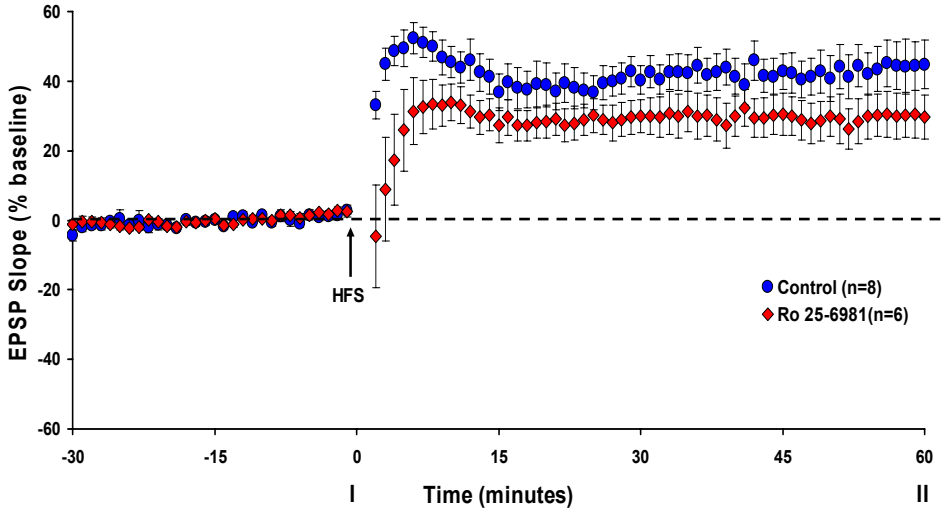

Fig. 16. LTP induction is not significantly altered, relative to controls, when an IP injection (6.0mg/kg) of the NR2B antagonist Ro-6981 is administered 1 hour prior to 100 Hz tetanus.

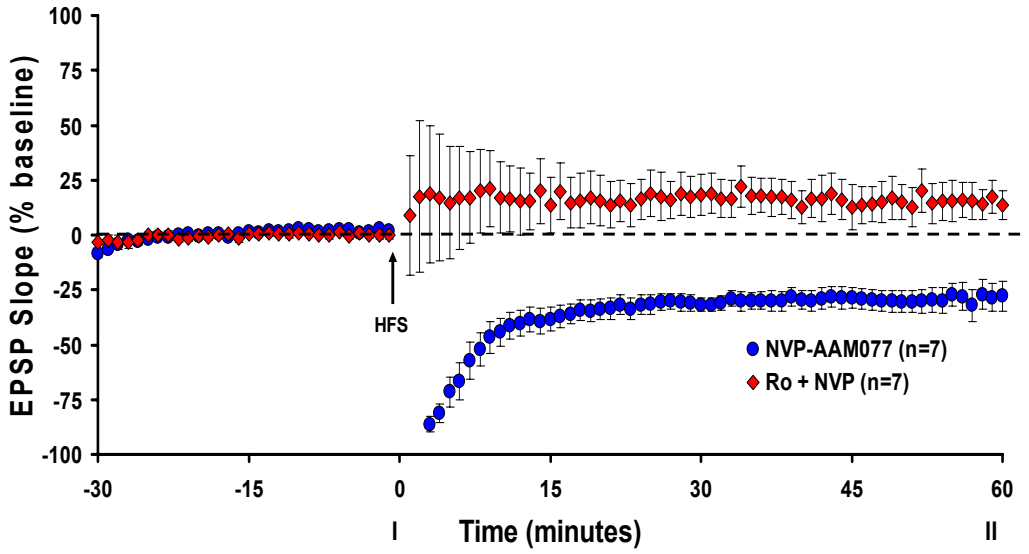

Fig. 17. NVP-HFS induced LTD is prevented when both Ro-6981 (6.0mg/kg) and NVP-AAM077 (2.4mg/kg) are administered IP 1 hour prior to tetanus. The group receiving the 2.4mg/kg dose of NVP-AAM077 has also been shown in Figure 15.

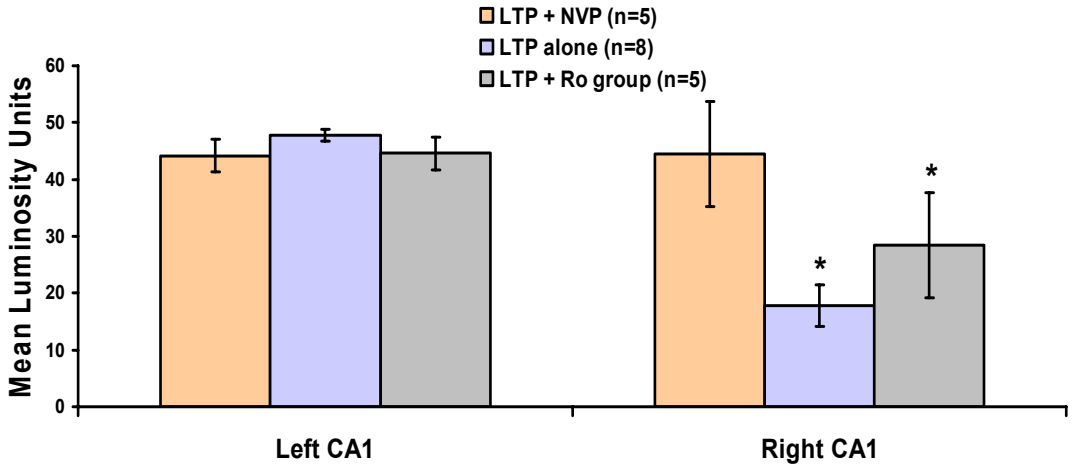

Fig. 18. The blockade of LTP using NVP-AAM077 (1.2mg/kg) results in a loss of neuroprotection against KA whereas pre-treatment with Ro-25-6981 (6.0mg/kg) has no effect on LTP-induced neuroprotection relative to controls when KA is administered 24 hours following electrophysiology experiments. FJ staining is used to assess neuronal damage in 25µm sections 24 hours after KA injection.

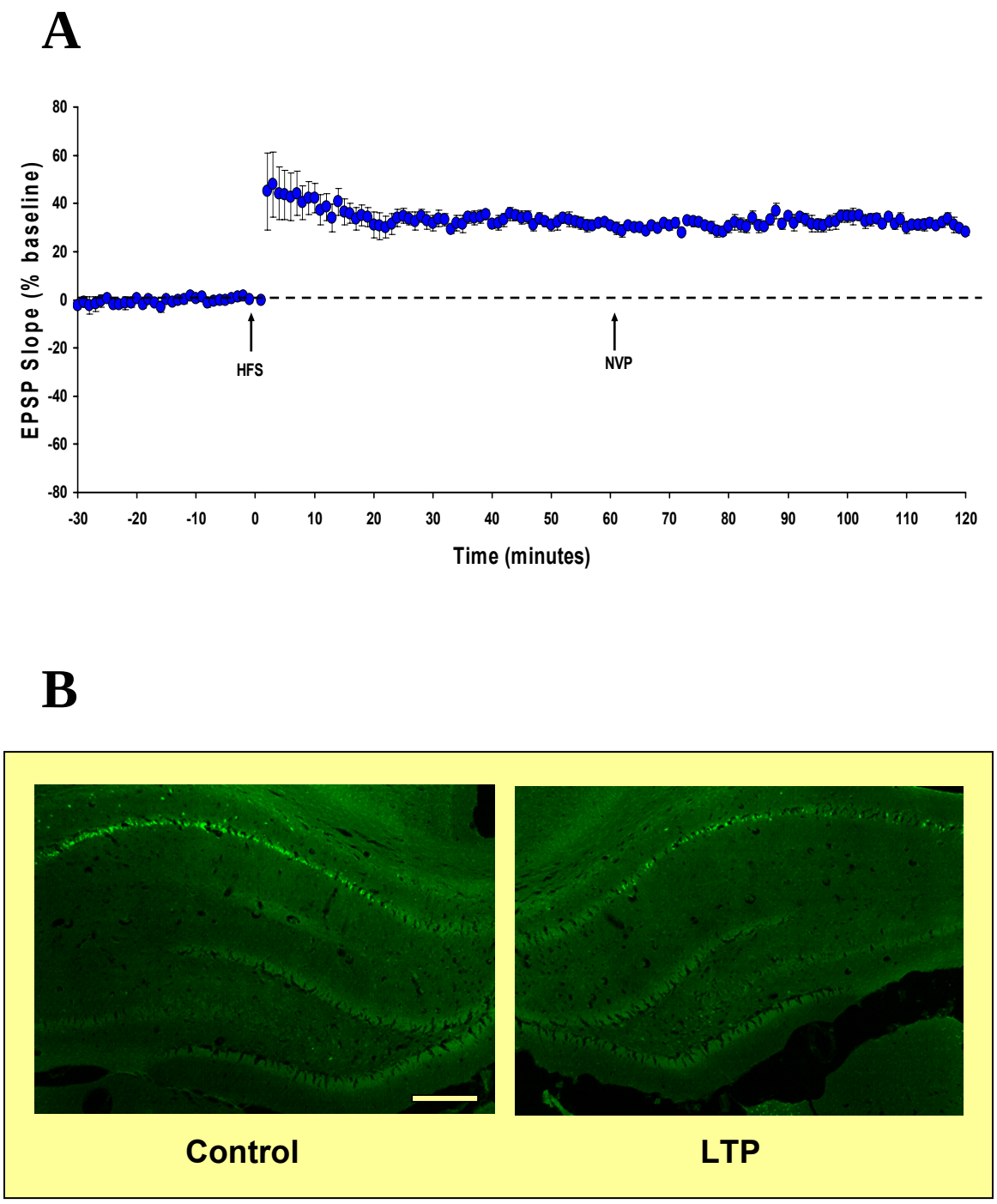

Fig. 19. (A) NVP-AAM077 administered 1 hour after the induction of LTP in the rat CA1 using 100 Hz. tetanus has no effect on the maintenance of LTP (n=5). **(B)** Representative sections (25µm) showing LTP induced neuroprotection is maintained when NVP-AAM077 is administered 1 hour after HFS (Mag. =10X). Scale bar is 400µm.

4. DISCUSSION

This research demonstrates that the mechanism associated with the activation of particular NMDAR populations not only influences the direction of CA1 synaptic plasticity, but that the presence of different NR2 subunits confers distinct pharmacological properties upon these heteromeric NMDARs and couples them to divergent intracellular signalling pathways that ultimately promote either cell survival or cell death. The activation of NMDARs is required for the induction of LTP at hippocampal CA1 synapses, which plays an important role in learning and memory (Collingridge et al., 1992). In particular, a requirement for the activation of NR2A-containing NMDARs in LTP production has been demonstrated by studies of genetically altered mice that lack the NR2A subunit (Sakimura et al, 1995). More recently, research has shown that NR2A-containing NMDARs are essential for the induction of LTP *in vitro* (Liu et al., 2004), and the results of this study support this finding *in vivo* as the NR2A-specific antagonist NVP-AAM077 was found to completely block LTP induction. In fact, the administration of a high dose of the NR2A antagonist (2.4mg/kg) resulted in LTP-producing HFS leading to LTD instead. It has been believed for some time that the degree of NMDAR activation, and hence the level of postsynaptic Ca^{2+} elevation, dictates the direction of NMDAR-dependent synaptic modification and a similar conversion of CA1 LTP into LTD was found using this drug in whole-cell patch clamping (Liu et al., 2004) and has also been reported using a low dose of APV (Nishiyama et al., 2000). The ability to convert the outcome of LTP-producing protocols to LTD after blockade of the NR2A-containing NMDARs indicates that these protocols may be sufficient to activate both NR2A- and NR2B-containing NMDARs and that NR2B-mediated LTD is unmasked after the blockade of NR2A-containing receptors. Mallon et al. (2005) also reported that NMDA-dependent LTP induced by electrical stimulation was prevented by the selective blockade of the NR2A subunit. However, they also reported that Ro25-6981 potentiated the response of NR2A-containing NMDARs to NMDA and concluded that NR2B subunits exert inhibitory restraint over NR2A receptors. In this study, the administration of Ro25-6891 (6.0mg/kg) did not have any significant impact on the production of LTP using HFS. Interestingly though, there appears to be a weak component of LTP that is not NR2A-dependent which becomes evident when both NR2 subunit antagonists are given together. The LTD that was shown to result from HFS in the presence of NVP-AAM077 was abolished and a small but significant LTP was produced. It is possible that this component was indeed dominated by

the activation of NR2B-containing NMDARs that occurs under HFS and this would help to explain why no potentiation is seen in the LTP experiments where NR2A antagonists are used alone. Thus, when HFS is applied, NR2B-type NMDARs are only able to induce LTD when NR2A-type signalling is pharmacologically impaired. In support of this notion, Massey et al. (2004) reported that LTD can be readily induced in adult animals under conditions that enable the activation of extrasynaptic NMDARs. During HFS in the presence of NVP-AAM077, it is possible that glutamate spill over led to the preferential activation of extrasynaptic NR2B-containing receptors because the NR2A-containing receptors were blocked, and that this is why LTD was seen under these conditions. Further studies will be required to establish the physiological conditions that enable NMDAR-dependent LTD to be induced and its role *in vivo*.

Unfortunately, it was not possible to determine whether NR2B-selective antagonists block LTD induction *in vivo* because, despite intensive efforts, no electrical stimulation protocol could be established that produced robust and reliable LTD. It has been reported that NMDAR-dependent LTD is not readily induced in adult animals, for reasons that are not known (Bear and Abraham, 1996; Kemp and Bashir, 2001). However, possibilities that may account for this adversity include the subunit composition and location of NMDARs in adult tissue. For example, NR2B receptors required for LTD are post-synaptically located in the extrasynaptic membrane, raising the possibility that a reason for the difficulty in inducing LTD in adult tissue may be attributable to a more efficient glutamate uptake mechanism. Moreover, during post-natal development, the hippocampal signalling cascades for LTP induction switch (Yasuda et al., 2003) and the NMDA receptor NR2B/NR2A ratio decreases (Sans et al., 2000). By postnatal day 28 (P28), rodents express comparable amounts of these NR2 subunits (Sans et al., 2000), however, the mechanisms by which distinct subpopulations of NMDARs that contain different NR2 subunits determine the polarity of synaptic plasticity remain to be resolved. In addition to the distinct kinetics of Ca^{2+} influx gated through NR2A and NR2B-containing NMDARs (Chen et al., 1999), different NR2 subunits may couple to different post-synaptic signalling pathways and evidence for this has begun to emerge (Ivanov et al, 2006). Indeed, the synaptic plasticity response appears to be directed by the pattern of synaptic activation, which recruits the major NMDAR subtypes to variable extents and therefore triggers distinct signalling cascades which lead to opposing forms of plasticity. These results support the hypothesis that the activation of NR2A-

containing NMDARs is critical for the production of LTP while NR2B-containing NMDARs play a critical role in the induction of LTD in the hippocampal CA1 area.

Well-documented evidence from both *in vitro* and *in vivo* models of stroke strongly supports the critical involvement of NMDA receptor-mediated toxicity in neuronal damage following stroke (Marini et al., 1997). On the other hand, NMDARs also clearly provide a major survival input to CNS neurons, however, NMDAR-activated protective signalling is poorly understood (Hetman & Kharebava, 2006). This study examined the effects of two subunit-specific NMDAR antagonists on KA-induced neuronal death to test the possibility that there may be an opposing action of NR2B- and NR2A-containing NMDARs in mediating cell death and cell survival. Intraperitoneal pre-treatment of the NR2B antagonist Ro25-6981 (6.0 mg/kg) was found to prevent KA-induced neuronal apoptosis while pre-treatment with the NR2A subunit-specific antagonist NVP-AAM077 (2.4 mg/kg) had no measurable effect on KA-induced apoptosis. The efficient blockade of NMDA receptor-dependent cell death by Ro25-6981, but not by NVP-AAM077, strongly suggests that it is the NR2B-containing, but not NR2A-containing, NMDA receptor subpopulation that plays a primary role in triggering cascades that lead to NMDA-induced neuronal apoptosis. Under some pathological conditions, such as stroke and brain trauma, there is usually a transient and rapid increase in extracellular glutamate concentrations, and consequently extrasynaptic receptors, which are predominantly NR2B-containing and not usually activated by synaptically released glutamate during normal synaptic transmission, become activated, resulting in the activation of the NR2B-containing receptor-dependent cell death pathway. This is why blocking these receptors can be neuroprotective under pathological conditions. Under regular physiological conditions, a NR2A-mediated cell survival-promoting effect dominantly suppresses the apoptotic tendency of NR2B, thereby maintaining normal neuronal survival. Thus, it would be reasonable to assert that NR2A antagonists should inhibit this survival signal and promote cell death following the administration of KA, however, increased neuronal death was not seen with NVP-AAM077 pre-treatment most likely because the high dose of KA (18mg/kg) that was given already produced maximal degeneration. An interesting set of experiments that could potentially shed some light on this matter would involve administering NVP-AAM077 with a sub-convulsive dose of KA to unmask this hypothesized increase in neurodegeneration. Nevertheless, these findings support the idea that both NR2A- and NR2B-containing NMDARs are activated by glutamate excitotoxicity and that

they tonically exert opposing influences with respect to promoting cell survival or death. The activation of NR2B-containing NMDA receptors initiates apoptotic signalling cascades and promotes neuronal death, whereas NR2A-containing NMDA receptors stimulate pro-survival signalling and could be potentially harnessed to provide neuroprotective action against NMDA and non-NMDA receptor-dependent neuronal injuries. Ultimately, the net impact of NMDA receptor activation on neuronal survival and death is dictated by the balance between the activation of NR2A and NR2B-containing NMDA receptor subpopulations. Theoretically then, NR2A agonists could protect neurons against brain damage by promoting neuronal survival following an injury, rather than by preventatively blocking the activation of a death signal that is initiated by an insult with NR2B antagonists. Ogita et al. (2003) were able to demonstrate a neuroprotective role of NMDARs using NMDA pre-treatment against kainite-induced excitotoxicity in murine hippocampus *in vivo*; however, limited attempts to replicate this finding in the rat were unsuccessful perhaps due to different dosage requirements between species. It is possible that pre-treatment with an effective dose of NMDA could preferentially activate NR2A-containing NMDARs at the synapse and therefore activate an anti-apoptotic signalling cascade that protects neurons sensitive to KA excitotoxicity, but this is just speculation and more research needs to done in this area.

Altogether, this research supports the hypothesis that NR2A subunits are associated with a cell-survival pathway while NR2B subunits are linked to a cell-death pathway, however, the downstream mechanisms by which NR2A and NR2B-containing receptors exert their opposing roles remain to be fully elucidated. This research does indicate that there is a link between the activation of NR2A-containing receptors using HFS and the phosphorylation of Akt, which plays a critical role in controlling survival and apoptosis. It seems likely that the activation of NR2A-containing NMDARs triggers different signalling events than the activation of their NR2B-containing counterparts, resulting in sub-unit specific outcomes. Unfortunately, all clinical trials to date have failed to demonstrate the effectiveness of NMDAR antagonists in reducing brain injuries (Kemp, 2002). On the other hand, it may be possible that the selective activation of NR2A-containing NMDA receptors can promote neuronal survival and exert a neuroprotective action against both NMDA receptor and non-NMDA receptor mediated neuronal damage. Therefore selective agonists need to be developed and tested to determine if the selective activation of NR2A-containing NMDA receptors constitutes a promising therapy for stroke or other neurodegenerative diseases.

In summary, the activation of the synaptic pool of NR2A-containing NMDARs is implicated in LTP induction, Akt phosphorylation, CREB activation, and reveals a neuroprotective effect, whereas activation of the extrasynaptic NR2B-containing NMDARs has been shown to support LTD, CREB shut-off and induce neuronal death (Hardingham et al, 2002; Massey et al 2004; Lu et al, 2001; Liu et al., 2004). Thus, the preferential activation of NR2A-containing NMDAR's triggers different signalling events from the activation of their NR2B-containing counterparts, and such subunit-specific signalling outcomes determine the direction of synaptic changes as well as cell survival/cell death pathways. In fact, the induction of LTP itself was shown to be neuroprotective against the excitotoxic effects of KA, suggesting that synaptic modification is directly related to neuronal fate. Furthermore, the NR2A subunit appears to underlie this relationship as the inhibition of NR2A-containing NMDARs was found to abolish both LTP induction and the neuroprotection that it confers upon hippocampal CA1 neurons exposed to KA. Conversely, the inhibition of NR2B-containing NMDARs using the selective antagonist Ro25-6981 was found to attenuate the neurodegenerative effects of KA administration while having no effect on the induction of LTP. The electrophysiological and pharmacological approaches utilized in this research have provided some converging evidence that supports the theory that there is a direct link between synaptic plasticity and delayed cell death, and that these two interdependent processes are mechanistically related through a shared codependence upon the specific subunit composition of NMDA-receptors. In the last several years, the promise of targeting brain plasticity in rehabilitation treatment has been actualized, and demonstrated to be a real factor for ameliorating neurological impairments in both laboratory and clinical trials. However, the specific molecular signals to initiate the functional reorganization of synaptic pathways remain to be elucidated (Weingarden & Ring, 2006). This research points to NMDA subunit specific mechanisms which can be manipulated to harness the beneficial effects of synaptic plasticity by activating survival-promoting pathways or inhibiting apoptotic pathways to encourage recovery.

5. CONCLUSION

This research offers a novel approach towards the treatment of neurodegenerative disease from two different angles. First, pharmacologically, a connection has been established between synaptic plasticity and neurodegeneration by demonstrating that drugs specifically targeting NMDAR subunits which have already been shown to exert an influence on the polarity of synaptic plasticity can also be used to shift the balance that exists between neuronal survival and neuronal loss. Second, from a physiological perspective, this research demonstrates that synaptic plasticity itself, a normal cellular process, can be used to deliver beneficial effects to neurons through common intracellular pathways even in the absence of such drugs. By identifying an innate neural mechanism that operates along the same neuroprotective pathways as these drugs, it could be possible to promote cell survival through a combination of these two approaches. Thus, this research is unique in that it offers new insights into the mechanisms underlying neuroprotection, and it suggests adjunctive neuroprotective measures which can substitute or complement the administration of NMDAR blockers in order to counteract excitotoxicity. Although there are no antiapoptotic drugs in clinical use, all of the existing literature supports the inhibition of apoptosis as a promising strategy for treatment of neurodegenerative disease (Yuan et al., 2003). It is quite conceivable that current NMDAR antagonists protect against excitotoxicity while also reducing the pro-survival activity of NMDA. Thus, future research should seek to develop and test drugs that can specifically activate NR2A-containing NMDARs in order to determine their potential efficacy in combating a wide range of neurodegenerative disorders. It is imperative that the switches controlling pro-survival vs. pro-excitotoxic outcome of NMDAR stimulation are fully understood in order to develop drugs that can selectively block the excitotoxicity while, at the same time, enhance the protective NMDAR signalling. Moreover, the exact downstream signalling pathways of NR2A and NR2B-containing NMDARs will need to be elucidated because it will only be possible to develop suitable neuroprotective agents to promote cell survival and/or prevent cell death when these pathways are fully understood. Finally, the neuroprotective properties of HFS induced LTP that this research has demonstrated should be investigated using different animal models of neurodegeneration, such as stroke, to see if this treatment is universally effective across a broad range of conditions.

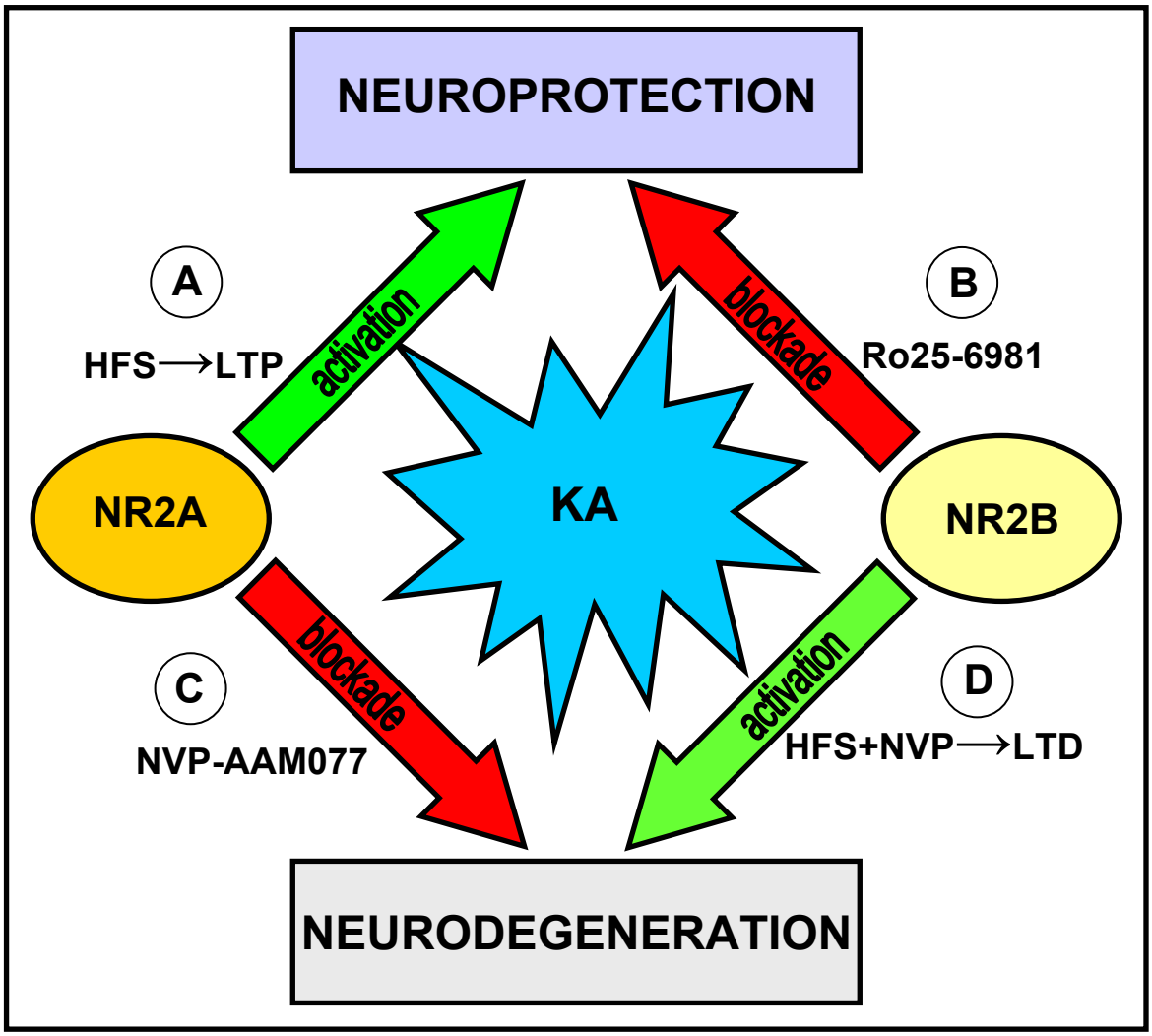

Fig. 20. Summary diagram outlining the opposing roles that NMDAR subtypes play in determining neuronal fate when either blocked or activated in an excitotoxic environment produced via the IP administration of kainic acid (18mg/kg). **(A)** HFS selectively activates NR2A-containing NMDARs and leads to the induction of LTP which is neuroprotective against KA induced excitotoxicity **(B)** Ro25-6981 selectively blocks NR2B-containing NMDARs and this is also neuroprotective against KA induced excitotoxicity **(C)** NVP-AAM077 selectively blocks NR2A-containing NMDARs and this treatment offers no protection against KA induced neurodegeneration **(D)** HFS with NVP-AAM077 pre-treatment selectively activates NR2B-containing NMDARs and leads to the induction of LTD which offers no protection against KA induced neurodegeneration.

6. REFERENCES

Andersen, P., Sundberg, S. H., Sveen, O., and Wigstrom, H. (1977). Specific long-lasting potentiation of synaptic transmission in hipppocampal slices. *Nature*, 266, 736-737.

Albensi, B. C. (2001). Models of brain injury and alterations in synaptic plasticity. Journal of *Neuroscience Research*, 65, 279-283.

Arias, C., Montiel, T. & Tapia, R. (1990). Transmitter release in hippocampal slices from rats with limbic seizures produced by systemic administration of kainic acid. *Neurochem Res.*, 15(6), 641-5.

Arundine M., & Tymianski, M. (2004). Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol Life Sci*., 61(6), 657-68.

Bear, M., F. & Abraham, W. C. (1996) Long-term depression in hippocampus. *Annu Rev Neurosci*., 19, 437-62.

Bliss, T. V. P. & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361, 31-39.

Bliss, T. V. P., Douglas, R. M., Errington, M. L., and Lynch, M. A. (1986). Correlation between long-term potentiation and release of endogenous amino acids from dentate gyrus of anaesthetized rats. Journal of Physiology 377, 391-408.

Bliss, T. V. P. & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol*., 232, 331-356.

Chandler, L. J., Sutton, G., Dorairaj, N., R. & Norwood, D. (2001) N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. *J Biol Chem*., 276(4), 2627-36.

Chen, N., Luo, T. & Raymond, L., A. (1999) Subtype-dependence of NMDA receptor channel open probability. *J Neurosci.*, 19(16), 6844-54.

Collingridge, G. L., Kehl, S. J., and McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *Journal of Physiology*, 334, 33-46.

Collingridge, G., L., Randall, A., D., Davies, C., H. & Alford, S. (1992) The synaptic activation of NMDA receptors and Ca2+ signalling in neurons. *Ciba Found Symp*., 164, 162-71.

Collingridge, G., L. & Bliss., T. V. (1995). Memories of NMDA receptors and LTP. *Trends Neurosci*., 18, 54-56.

Cummings, J. A., Mulkey, R. M., Nicoll, R. A., and Malenka, R. C. (1996). Ca2+ signalling requirements for long-term depression in the hippocampus. *Neuron*, 16: 825-33.

Davis, S. N., Butcher, S. P., and Morris, R. G. M. (1992). The NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5) impairs spatial learning and LTP *in vivo* at intracerebral concentrations comparable to those that block LTP *in vitro*. *Journal of Neuroscience*, 12, 560-573.

Dudek, S. M., and Bear, M. F. (1993). Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. *Journal of Neuroscience* 13, 2910-2918.

Fischer, G., Mutel, V., Trube, G., Malherbe, P., Kew, J. N., Mohacsi et al. (1997) Ro 25-6981, a highly potent and selective blocker of N-methyl-D-aspartate receptors containing the NR2B subunit. characterization in vitro. *J Pharmacol Exp Ther*., 283(3), 1285-92

Fox, C.J., Russel, K.I., Wang, Y.T. & Christie, B.R. (2006) Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus *in vivo*. *Hippocampus*, 16:907-915.

Freir, D. B., Holscher, C. & Herron, C. E. (2000). Blockade of long-term potentiation by β-amyloid peptides in the CA1 region of the rat hippocampus in vivo. *J Neurophysiol*., 85(2), 708-713.

Goldin, M. & Segal, M. (2003) Protein kinase C and ERK involvement in dendritic spine plasticity in cultured rodent hippocampal neurons. *Eur J Neurosci*., 17(12), 2529-39

Hardingham, G. E, Fukunaga, Y. & Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci*., (5), 405-14.

Hardingham, G., E. & Bading, H.(2002) Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. *Biochim Biophys Acta*., 1600(1-2), 148-53.

Hawkins, R. D., Zhuo, M, & Arancio, O. (1994). Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. *J Neurobiol*., 25(6), 652-65.

Hebb, D. O. (1949). The Organization of Behaviour: A Neuropsychological Theory. Wiley, New York.

Hetman, M. & Kharebava, G. (2006) Survival signalling pathways activated by NMDA receptors. *Curr Top Med Chem*., 6(8), 787-99

Heynen, A. J., Abraham, W. C., and Bear, M. F. (1996). Bidirectional modification of CA1 synapses in the adult hippocampus *in vivo*. *Nature*, 381: 163-166.

Hollmann, M., & Heinemann, S. (1994) Cloned glutamate receptors. *Annu Rev Neurosci.*, 17, 31-108.

Humphrey, W. M., Dong, H. X., Csernansky, C. A. & Csernansky, J. G. (2002). Immediate and delayed hippocampal neuronal loss induced by kainic acid during early postnatal development in the rat. *Developmental Brain Research*, 137, 1-12.

Hunsberger, J. G., Bennett, A. H., Selvanayagam, E., Duman, R. S. & Newton, S. S. (2005). Gene profiling the response to kainic acid induced seizures. *Molecular Brain Research*, 141, 95-112.

Ivanov, A., Pellegrino, C., Rama, S., Dumalska, I., Salyha, Y., Ben-Ari, Y., et al. (2006). Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the ERK activity in cultured rat hippocampal neurons. *J Physiol*, Epub.

Kauderer, B. S., and Kandel, E. R. (2000). Capture of a protein synthesis-dependent component of long-term depression. *Proc Nat Acad Sci USA*, 97:13342-13347.

Kauer, J. A., Malenka, R. C., and Nicoll, R. A. (1988). A persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron*, 1, 911-917.

Kelso, S. R., Ganong, A. H., and Brown, T. H. (1986). Hebbian synapses in hippocampus. *Proc Nat Acad Sci USA*, 83, 5326-5330.

Kemp N. & Bashir, Z. I. (2001). Long-term depression: a cascade of induction and expression mechanisms. *Prog Neurobiol.*, 65(4), 339-65.

Kemp, J. A. & McKernan, R. M. (2002). NMDA receptor pathways as drug targets. *Nature Neuroscience*. 5, Suppl:1039-42.

Kim, J. H., Anwyl, R., Suh, Y. H., Djamgoz, M. B. A. & Rowan, M. J. (2001). Use-dependent effects of amyloidogenic fragments of β-amyloid precursor protein on synaptic plasticity in rat hippocampus in vivo. *J Neurosci*., 21(4), 1327-1333.

Kohr, G., Jensen, V., Koester, H. J., Mihaljevic, A., L, Utvik, J. K., Kvello, A, et al. (2003) Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. *J Neurosci*., 23(34), 10791-9.

Korhonen, L., Belluardo, N. & Lindholm, D. (2001) Regulation of X-chromosome-linked inhibitor of apoptosis protein in kainic acid-induced neuronal death in the rat hippocampus. *Mol Cell Neurosci*., 17(2), 364-72.

Korhonen, L., Belluardo, N., Mudo, G. & Lindholm, D. (2003). Increase in Bcl-2 phosphorylation and reduced levels of BH3-only Bcl-2 family proteins in kainic acid-mediated neuronal death in the rat brain. *European Journal of Neuroscience*, 18, 1121-1134.

Laube, B., Hirai, H., Sturgess, M., Betz, H. & Kuhse, J. (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. *Neuron*, 18(3), 493-503.

Leonard, A. S., Bayer, K., U, Merrill, M., A., Lim, I., A., Shea, M., A., Schulman, H. & Hell, J. W. (2002) Regulation of calcium/calmodulin-dependent protein kinase II docking to N-methyl-D-aspartate receptors by calcium/calmodulin and alpha-actinin. *J Biol Chem*., 277(50), 48441-8.

Levy, W. B., and Steward, O. (1979). Synapses as associative memory elements in the hippocampal formation. *Brain Research*, 175, 233-245.

Li, B., Chen, N., Luo, T., Otsu, Y., Murphy, T. H. & Raymond, L. A. (2002) Differential regulation of synaptic and extra-synaptic NMDA receptors. *Nat Neurosci*., (9), 833-4.

Liu, L., Wong, T.P., Pozza, M. F., Lingenhoehl, K., Wang, Y., Sheng, M. et al. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*, 304, 1021-1024.

Liu Y., Wong T.P., Aarts, M., Rooyakkers, A., Liu, L., Lai, T.W, Wu, D.C., Lu, J., Tymianski M., Craig, A.M., Wang, Y.T. (2007). NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J Neurosci.,* 27(11), 2846-57.

Lopez-Picon, F.R., Kukko-Lukjanov, T.K., Holopainen, I.E. (2006). The calpain inhibitor MDL-28170 and the AMPA/KA receptor antagonist CNQX inhibit neurofilament degradation and enhance neuronal survival in kainic acid-treated hippocampal slice cultures. *Eur J Neurosci.*, 23, 2686-94.

Lu, H., C., Gonzalez, E. & Crair, M., C. (2001). Barrel cortex critical period plasticity is independent of changes in NMDA receptor subunit composition. *Neuron*, 32(4), 619-34.

Lu, W., Man, H., Ju, W., Trimble, W. S., MacDonald, J. F., Wang, Y. T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* 29: 243-254.

Lynch, M. A. (2004). Long-term potentiation and memory. *Physiol Rev*, 84, 87-136.

McNaughton, B. L., Douglas, R. M., and Goddard, G.V. (1978). Synaptic enhancement in fasia dentata: cooperativity among coactive afferents. *Brain Research*, 157, 277-293.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature*, 340, 554-557.

Malenka, R. C., Lancaster, B., and Zucker, R. S. (1992). Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron*, 9: 121-128.

Malenka, R. C. (2003). The long-term potential of LTP. *Nat Rev Neurosci,* 4(11), 923-6.

Mallon, A., P., Auberson, Y., P. & Stone, T. W. (2005) Selective subunit antagonists suggest an inhibitory relationship between NR2B and NR2A-subunit containing N-methyl-D-aspartate receptors in hippocampal slices. *Exp Brain Res.*, 62(3), 374-83.

Man, H. Y., Lin, J. W., Ju, W. H., Ahmadian, G., Liu, L., Becker, L. E., Sheng, M., Wang, Y. T. (2001). Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron*, 3:649-62.

Manahan-Vaughn, D. (1997). Group 1 and 2 metabotropic glutamate receptors play differential roles in hippocampal long-term depression and long-term potentiation in freely moving rats. The *Journal of Neuroscience*, 17(9): 3303-3311.

Manilow, R. and Miller, J. P. (1986). Postsynaptic hyperpolarisation during conditioning reversibly blocks induction of long-term potentiation. *Nature*, 320, 529-530.

Mattson, M. P. & Scheff, S. W. (1994) Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. *J Neurotrauma*, 11(1), 3-33

Marini, A. M, Spiga, G. & Mocchetti, I. (1997) Toward the development of strategies to prevent ischemic neuronal injury. in vitro studies. *Ann N Y Acad Sci*., 825, 209-19.

Marini, A., M., Jiang, X., Wu, X., Tian, F., Zhu, D., Okagaki, P. & Lipsky, R. H. (2004) Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: from genes to phenotype. *Restor Neurol Neurosci*., 22(2), 121-30.

Massey, P. V., Johnson, B. E., Moult, P. R., Auberson, Y. P., Brown, M. W., Molnar, E. et al. (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potential and long-term depression. *J Neurosci*, 24(36), 7821-7828.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B. & Seeburg, P., H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*, 12(3), 529-40.

Neuhoff, H., Roeper, J. & Schweizer, M. (1999). Activity-dependent formation of perforated synapses in cultured hippocampal neurons. *Eur J Neurosci*., 11(12), 4241-50.

Nishiyama, M., Hong, K., Mikoshiba, K., Poo, M., M. & Kato, K. (2000) Calcium stores regulate the polarity and input specificity of synaptic modification. *Nature*, 408(6812), 584-8.

Ogita, K., Okuda, H., Yamamoto, Y., Nishiyama, N. & Yoneda, Y. (2003). In vivo neuroprotective role of NMDA receptors against kainite-induced excitotoxicity in murine hippocampal pyramidal neurons. *Journal of Neurochemistry*, 85, 1336-1346.

O'Neil, M. J., Bleakman, D., Zimmerman, D. M. & Nisembaus, E. S. (2004) AMPA receptor potentiators for the treatment of CNS disorders. *Curr Drug Targets CNS Neurol Disord*., 3(3), 181-94.

Poirier, J. L., Capek, R. & De Koninck, Y. (2000). Differential progression of dark neuron and fluoro-jade labelling in the rat hippocampus following pilocarpine-induced status elepticus. *Neuroscience*, 97, 59-68.

Pollard, H., Charriaut-Marlangue, C., Cantagrel, S., Represa, A., Robain, O., Moreau, J. & Ben-Ari, Y. (1994). Kainate-induced apoptotic cell death in hippocampal neurons. *Neuroscience*; 63(1), 7-18.

Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E. et al. (1995) Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. *Nature*, 373(6510), 151-5.

Sans, N., Petralia, R. S., Wang, Y. X., Blahos, J., Hell, J. W. & Wenthold, R. J. (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. J *Neurosci*., 20(3), 1260-71.

Sato, M., Chang, E., Igarashi, T. & Noble, L. J. (2001). Neuronal injury and loss after traumatic brain injury: time course and regional variability. *Brain Research* 917, 45-54.

Staubi, U., and Scafidi, J. (1997). Studies on long-term depression in area CA1 of the anesthetised and freely moving rat. *The Journal of Neuroscience* 17(12): 4820-4828.

Schuman, E. M. (1999). Neurotrophin regulation of synaptic transmission. *Curr Opin Neurobiol*., 9(1), 105-9.

Schmued, L., C. & Hopkins, K. J. (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res*., 874(2), 123-30.

Simonian, N., A., Getz, R., L., Leveque, J. C., Konradi, C.& Coyle, J. T. (1996) Kainic acid induces apoptosis in neurons. *Neuroscience*, 75(4), 1047-55.

Sperk, G. (1994). Kainic acid seizures in the rat. *Prog Neurobiol*., 42(1), 1-32.

Tang, Y. P., Shimizu, E., Dube, G., R., Rampon, C., Kerchner, G., A., Zhuo, M., Liu, G.& Tsien, J. Z. (1999). Genetic enhancement of learning and memory in mice. *Nature*, 401(6748), 63-9.

Tang, K., Zhang, J. T. (2003). (-)Clausenamide improves long-term potentiation impairment and attenuates apoptosis after transient middle cerebral artery occlusion in rats. *Neurol Res*. 25(7), 713-7.

Thiels, E., Kanterewicz, B. I., Knapp, L. T., Barrionuevo, G., and Klann, E. (2000). Protein phosphatase-mediated regulation of protein kinase C during long-term depression in the adult hippocampus *in vivo*. *The Journal of Neuroscience*, 20(19): 7199-7207.

Thomas, G., M., Huganir, R. L. (2004) MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci*., 5(3), 173-83.

Tooyama, I., Bellier, J. P., Park, M., Minnasch, P., Uemura, S., Hisano, T. et al. (2002). Morphologic study of neuronal death, glial activation, and progenitor cell division in the hippocampus of rat models of epilepsy. *Epilepsia*, 43, 39-43.

Weingarden, H. & Ring, H. (2006) Functional electrical stimulation-induced neural changes and recovery after stroke. *Eura Medicophys*., 42(2), 87-90

Weitlauf, C., Honse, Y., Auberson, Y., P, Mishina, M., Lovinger, D.,M. & Winder, D., G. (2005) Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. *J Neurosci*., 25(37), 8386-90.

Yasuda, H., Barth, A. L., Stellwagen, D. & Malenka, R. C. (2003) A developmental switch in the signalling cascades for LTP induction. *Nat Neurosci*., 6(1), 15-6.

Yang, S. N., Tang, Y. G. & Zucker, R. S. (1999). Selective induction of LTP and LTD by postsynaptic $[Ca2+]_i$ elevation. *J Neurophysiol*., 81(2), 781-7.

Ye, X., Carp, R., I., Schmued, L., C. & Scallet, A., C. (2001) Fluoro-Jade and silver methods: application to the neuropathology of scrapie, a transmissible spongiform encephalopathy. *Brain Res Brain Res Protoc*., 8(2), 104-12.

Yoshioka, A., Hardy, M., Younkin, D.P., Grinspan, J.B., Stern, J.L. & Pleasure, D. (1995) α-Amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. *Neurochemistry*, 64, 2442–2448.

Yuan, J., Lipinski, M. & Degterev, A. (2003). Diversity in the mechanisms of neuronal cell death. *Neuron*, 40, 401-413.

Zalutsky & Nicoll (1990) Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science*, 248(4963), 1619-24.

Zhang, L., Hsu, J. C., Takagi, N., Gurd, J. W., Wallace, M., C. & Eubanks, J. H. (1997). Transient global ischemia alters NMDA receptor expression in rat hippocampus: correlation with decreased immunoreactive protein levels of the NR2A/2B subunits, and an altered NMDA receptor functionality. J Neurochem., 69(5), 1983-94.