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Non-Ionotropic Activation of the NMDAR, Leading to ERK 1/2 Phosphorylation

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Melissa Slocumb

Dr. Robert Renden, Thesis Advisor

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MELISSA SLOCUMB

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Robert Renden, Ph. D., Thesis Advisor

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Tamara Valentine, Ph. D., Director, **Honors Program**

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Abstract

N-methyl-D-aspartate receptors (NMDARs) are important to neuron function. NMDARs are transmembrane ligand-gated and voltage-gated ion channels that pass sodium, potassium, and calcium (MacDermott et al., 1986). They are composed of a tetramer of proteins in the postsynaptic cell membrane of neurons. The NMDAR is unique in the sense that it requires two agonists to stimulate its activation: the excitatory transmitter glutamate, and the co-agonist glycine. It is also unique in voltage-dependent regulation via a magnesium block in the ion channel. When the neuron is depolarized, this block is removed and ions can pass freely through the channel (Nowak et al., 1984). These three properties of passing calcium (MacDermott et al., 1986), being ligand-gated by glutamate, and being voltage-gated with a magnesium plug (Nowak et al., 1984), make the NMDAR important for regulating activity-dependent postsynaptic plasticity, a mechanism believed to underlie learning and memory (Nicoll, 2003). Ionotropic activation of NMDARs by ligands has been implicated in extracellular signal-regulated kinase (ERK) signaling (Martel et al., 2009). ERK is a protein that promotes synaptic plasticity by regulating the membrane trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which is closely linked to learning and memory formation (Sweatt, 2004).

It is unknown whether NMDARs have a non−ionotropic capacity. My hypothesis is that the single NMDAR agonist glycine is capable of regulating ERK activity in the absence of ion channel activity. NMDAR activation and coupling to intracellular signaling cascades were probed using a pharmacological and molecular biology approach. N-methyl-d-aspartate (NMDA) was used to stimulate the receptor at the glutamate binding site, while the ionotropic pore was pharmacologically and physically

blocked. Cultured mouse neurons and transfected Human Embryonic Kidney (HEK) 293 cell cultures were used to determine a subunit-specific role of the NMDAR. ERK1 and ERK2 phosphorylated protein and total ERK protein were measured using Western blot standard procedures (Sambrook and Maniatis, 1989). This research is significant because learning how to regulate NMDAR signaling cascades independent of ionotropic activity with a single ligand could lead to the development of treatments that could promote neuron survival and plasticity in patients with ischemia or neuronal insult.

The results presented here must be considered inconclusive since there are several issues with the experimental protocols, only discovered late in the production of this work. While the results cannot be reliably used for any definitive conclusions, they are useful in troubleshooting and in refining these procedures. The understanding that these experiments have brought can be used to create new experiments that will produce results that can be reliably assessed, and these new results may be used to address the hypothesis.

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Introduction

Ion channels in the cell membrane are crucial to cellular function. The lipid bilayer of cell membranes blocks the passive diffusion of ions. Ions can only cross the cell membrane via an ion pump or an aqueous pore. Ion pumps actively regulate the concentration of specific ions inside and outside of the cell so that a relative negative charge within the cell is achieved (Bear et al., 2007). This activity maintains an electrochemical gradient across the cell membrane, which creates potential energy that the cell utilizes for rapid transmembrane electrical signaling. The difference in voltage across the cell is called the membrane potential, which is about -70 mV in neurons, relative to extracellular media. These voltages are maintained in a steady state and are referred to as the neuron's resting potential. When ion channels are activated, an aqueous pore is formed across the cell membrane, and ions passively diffuse across the cell membrane down their individual electrochemical gradients, rapidly changing the relative voltage of the cell. These local electrical changes can be propagated within the neuron, facilitating intracellular communications (Hodgkin and Huxley, 1952).

Ion channels can be voltage-gated or ligand-gated. Voltage-gated ion channels are regulated by the electrical charge across the cell membrane and open in a voltage-dependent manner, and may be selective for specific ions. Ligand-gated ion channels are regulated by the hydrostatic binding of substances to specific chemical moieties of the channel structure. Ligand binding results in a reversible, allosteric change in the physical shape of the receptor protein in the cell membrane. This change in shape is what causes the ion channel pore to open or close (Campbell, 2009). N-methyl-D-aspartate receptors (NMDARs) are one type of ion channel. NMDARs are sodium, potassium, and calcium

ion permeable channels on the postsynaptic membrane of neurons (MacDermott et al., 1986), and they exhibit characteristics of both voltage-gated and ligand-gated ion channels. NMDARs require the binding of both of the ligands glutamate and glycine at separate sites, and cell depolarization to activate the receptor and to open its ion channel (Nowak et al., 1984; Bear et al., 2007).

The result of ion channel activity can signal the activation of intracellular cascades of enzyme activity, modulating protein function. For example, protein kinase activation by calcium ions leads to the phosphorylation of a specific target protein, transferring a phosphate group from adenosine triphosphate (ATP) to a specific amino acid residue on the target protein. Gaining a phosphate group can either activate or deactivate that protein due to its resulting change in molecular shape, which in turn can regulate other intracellular activity. Other enzymes, acting as phosphatases, reset the protein's activity by removing the phosphate group from the target protein. In this way, networks of protein kinase intracellular signaling cascades regulate large-scale changes in cellular activity due to specific stimuli, such as patterns of calcium ion influx into the cell (Campbell, 2009).

The synchronized activity of ion channels can also generate massive, rapid electrical signaling in neurons. When ion flux causes the membrane potential to depolarize to a threshold of about -55 mV, the neuron potential rapidly depolarizes due to the activation of voltage-sensitive sodium channels, which permit an explosive flow of positive sodium ions across the cell membrane into the cell. This influx of sodium ions results in the inside of the cell rapidly becoming positive and depolarizing, peaking at about +40 mV. After a delay of around 1msec, voltage-sensitive potassium channels are

activated, and potassium rushes out of the cell, driving the membrane potential towards hyperpolarizing potentials. Simultaneously, sodium channels inactivate, and the cell hyperpolarizes to around -80mV. ATP-dependent sodium and potassium pumps reset the chemical balance of these ions and restore the cell to its resting potential to -70mV (Hodgkin and Huxley, 1952). This process is called an action potential, and it diffuses rapidly throughout the neuron, which can be up to a meter in length. The frequency and pattern of action potentials between neurons is how information is encoded throughout the central and peripheral nervous systems (Bear et al., 2007; Campbell, 2009). Myelination of neuronal projections speeds the transmission of electrically encoded information between different regions of the brain by acting as an insulator for the electrical signals (Hodgkin, 1976). The synchronized activity of ion channels plays a pivotal role in the generation of and perpetuation of these action potentials in neurons.

The depolarizing effects of an action potential propagate through the entire neuron until the electric current reaches a specialized intercellular contact between two neurons, termed a synaptic bouton. The voltage change at the presynaptic site causes an influx of calcium, which signals to synaptic vesicles in the bouton to fuse with the presynaptic membrane. Synaptic vesicles are small membrane-enclosed pockets that contain neurotransmitters. Synaptic vesicles release their contents into the synaptic cleft when they fuse with the presynaptic membrane (Sudhof, 2004). These neurotransmitters travel across the synaptic cleft to ligand-gated receptors on the postsynaptic cell where this chemical signal is converted back into an electrical signal that can be propagated through the postsynaptic neuron (Bear et al., 2007; Alberts et al., 2009). This process repeats itself over and over again through many neurons, effectively regenerating an

electrical signal in every cell that receives transmitter. This process is used for all functions of the brain such as input, information storage, processing, and output. It also creates a long-distance signaling mechanism through the nervous system (Bear et al., 2007; Campbell, 2009).

NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are ion channels on the postsynaptic membrane of neurons. Glutamate is an excitatory neurotransmitter that is released from the presynaptic neuron during an action potential, and it binds to both NMDARs and AMPARs. AMPARs are ion channels composed of a tetramer of proteins that pass sodium and potassium ions upon activation by glutamate binding. They are also capable of passing small amounts of calcium. AMPARs contribute to the depolarization of the postsynaptic neuron when they allow the passage of these ions across the membrane. NMDARs also pass sodium and potassium ions, but they pass significantly greater amounts of calcium than AMPARs (MacDermott et al., 1986). NMDARs are different from AMPARs in that they require both glutamate and glycine binding, and cell membrane depolarization, to become active. In this way, NMDARs become activated when AMPARs contribute to the depolarization of the postsynaptic neuron (Bear et al., 2007). Active NMDARs allow calcium into the cell, and this calcium influx triggers an intracellular signaling pathway that involves the phosphorylation of extracellular-signal regulated kinase (ERK; Martel et al., 2009), leading to increased AMPAR trafficking to the cell membrane (Li and Keifer, 2009). Measuring the phosphorylation of ERK1 (molecular weight of 44kDa) and ERK2 (molecular weight of 42kDa) can be used to measure the level of NMDAR activation and the degree of AMPAR regulation in neurons.

There is a close relationship between the functions of these two ion channels, and their activities are linked to changes in synaptic strength like long-term potentiation (LTP) and long-term depression (LTD). Increased synaptic activity in glutamatergic neurons leads to the increased function of glutamate receptors like AMPAR (Kauer et al., 1988; Perkel and Nicoll, 1993). This increased AMPAR function increases NMDAR activation, and the resulting calcium influx in turn increases the expression of AMPARs on the postsynaptic membrane (Li and Keifer, 2009). Increased expression and trafficking of AMPA receptors in and out of the cell membrane are associated with LTP (Groc & Choquet, 2006); defined as an increase in synaptic strength, lasting for at least an hour (Soderling & Derkach, 2000). In neurons, LTP can be induced by repeated correlated presynaptic firing and postsynaptic depolarization, or due to a burst of high-frequency synaptic activity. Synaptic LTP is associated with behavioral changes in learning and memory, and the downregulation of LTP in animal models correlates well with loss of memory formation (Nicoll, 2003). Thus, NMDAR activity can be linked to AMPA activity and changes in synaptic strength and LTP (Kauer et al., 1988). The opposite of LTP is long-term depression (LTD), which is a decrease in synaptic strength lasting for at least an hour, and can be thought of as a synaptic correlate to memory extinction. This synaptic depression can be induced by the uncorrelated firing of neurons or by sustained low-frequency action potential stimulation. Decreased NMDAR activity and intracellular calcium concentration initiate a signaling cascade for the removal of AMPA receptors from the postsynaptic membrane, decreasing synaptic strength (Bear and Malenka, 1994; Bear et al., 2007). Activation of NMDARs and AMPARs initiate both LTP and LTD.

This study focuses on NMDARs and how they regulate intracellular signaling cascades. Preliminary unpublished data from Dr. Wan's lab have demonstrated that glycine alone, the co-agonist of the NMDAR, increases AMPAR receptor function via a non-ionotropic signaling capacity of the NMDAR. Non-ionotropic activation of the NMDAR to regulate AMPAR function has been shown to be mediated by an ERK-dependent signaling mechanism. This intracellular mechanism is independent of glycine receptor activation in mature hippocampal neurons. Dr. Wan's lab also has functional evidence that glycine alone can activate a non-channel activity of the NMDAR to confer neuroprotection in an excitotoxicity model. Non-ionotropic activation of the NMDAR to confer neuroprection in the neuronal injury model was shown to be dependent on a protein kinase B (AKT) signaling cascade. These findings suggest that ligand binding, but not ion flux through the NMDAR, can regulate physiological activity in neurons.

The work presented here explores these preliminary findings, and attempts to validate them using standard protein detection techniques in appropriately controlled experiments. The hypothesis for this thesis is that the single agonist glycine can activate the NMDAR's intracellular signaling cascades, regulating ERK1/2 phosphorylation independent of the NMDAR's ionotropic activities. The hypothesis was addressed using a pharmacological approach, treating mouse and Human Embryonic Kidney (HEK) 293 cells in vitro and assaying protein expression with the Western blot technique. A change in the expression levels of phosphorylated ERK proteins would indicate that specific agonists can regulate NMDAR activity independent of its ionotropic properties. Learning how to regulate NMDAR signaling cascades independent of ionotropic activity with a

single ligand could lead to the development of treatments that could promote neuron survival and plasticity in patients with ischemia or neuronal insult.

Literature Review

N-methyl-d-aspartate receptors (NMDARs) are ion channels on the postsynaptic membrane of neurons. They are composed of a tetramer of protein subunits. An obligate pair of GluN1 protein subunits associates with GluN2A-D and/or GluN3A-B protein subunits to form functional NMDARs. The most common isoforms of the NMDAR expressed in mammalian telencephalon (cortex and hippocampus) are composed of the GluN2A and GluN2B subunits (Dingledine et al., 1999). The NMDAR is unique in the sense that it requires the simultaneous binding of two separate ligands, or agonists, to stimulate its activation. The agonist glycine binds to the GluN1 subunit, and the coagonist glutamate binds to the GluN2 subunit, regulating channel gating (Laube et al., 1997). The NMDA receptor also displays characteristics of voltage-dependent activation, requiring cell membrane depolarization to remove a magnesium ion residing in the channel pore before ions can pass through the channel. Magnesium is present physiologically at a 2mM concentration, and it has an electrostatic attraction to the NMDA pore protein domain to create a channel plug, much like the stopper in the drain of a kitchen sink. Cell membrane depolarization creates a strong enough electrical change to repel the magnesium ion out of the channel pore. Cell membrane repolarization drops the magnesium plug back into position by electrostatic attraction, blocking the NMDA receptor ion channel again. After glutamate and glycine agonist binding, NMDAR

activation is primarily dependent on the removal of this magnesium plug in its ion pore (Nowak et al., 1984).

NMDARs have been implicated in the regulation of synaptic plasticity and neuronal survival and death signaling. Greater synaptic activity leads to more NMDAR activity and a higher concentration of intracellular calcium (MacDermott et al., 1986), which leads to increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) expression and long-term potentiation (LTP; Sweatt, 2004). Lower synaptic activity and calcium concentrations have the opposite effect. Thus, the calcium concentration within the cell may determine the degree of NMDAR subunit-specific activation, leading to either LTP or long-term depression (LTD) promotion (Liu et al., 2007). The pathological activation of NMDARs can also cause a toxic increase in intracellular calcium, which can result in apoptotic cell death. Influencing LTP and LTD are how NMDARs regulate synaptic plasticity, and NMDARs play a role in neuronal survival and death signaling through their regulation of calcium influx.

NMDARs have been directly implicated in the mechanisms underlying LTP and LTD (Bear and Malenka, 1994). A transient increase in the frequency of action potentials, if sufficiently high and/or sustained, makes synaptic connections between two neurons stronger by causing an increase in the number of receptors on the receiving neuron. This strengthening of neuronal pathways within the brain is induced through the exocytosis and endocytosis of cell membrane ion channels (Groc & Choquet, 2006) and, when these changes are sustained for at least an hour, is called long-term potentiation (LTP; Soderling & Derkach, 2000). When the activity between neurons is not well correlated, their synapses tend to be subjected to long-term depression (LTD), which is

the weakening of synaptic connections through the elimination of receptors on the postsynaptic membrane. Extreme instances of LTD can lead to neuronal death, especially during early development in neuronal pruning. Eliminating weak pathways leaves room for stronger pathways to develop. The global regulation of changes in LTP and LTD throughout the nervous system is called metaplasticity, or the plasticity of activity-induced synaptic plasticity, and acts as a baseline reset for synapses on a longer time scale (Abraham and Bear, 1996). Action potentials, cell membrane depolarization, and NMDAR activity are all closely linked, and this is why NMDARs are directly implicated in the mechanisms underlying LTP and LTD (Bear and Malenka, 1994).

The GluN2A subunit of the NMDAR is associated with cell survival signaling, and the GluN2B subunit is associated with cell death signaling (Liu et al., 2007; Chen et al., 2008). NMDA receptors composed of GluN2A subunits are associated with the promotion of cell survival and LTP through their upregulation of ERK phosphorylation, leading to greater AMPA receptor expression (Liu et al., 2004). Activation of NMDARs has been implicated in extracellular-signal-regulated-kinase (ERK) signaling (Martel et al., 2009). Calcium ion influx through GluN2A-containing NMDA receptors activates a signaling pathway involving calcium/calmodulin-dependent protein kinase II (CaMKII), Ras, and ERK1/2 that traffics AMPA receptors into the postsynaptic membrane (Zhu et al., 2002). Phosphorylated ERK promotes synaptic plasticity through the regulation of intracellular signaling cascades, regulating the AMPA receptor proteins to be trafficked to the postsynaptic membrane (Hardingham, Arnold, & Bading, 2001). NMDA receptors composed of GluN2B subunits are associated with the inhibition of cell survival, resulting in LTD due to the downregulation of AMPA receptor expression (Liu et al.,

2004). Calcium ion influx through GluN2B-containing receptors activates a signaling pathway involving Rap and ERK6 that traffics AMPA receptors out of the postsynaptic membrane via endocytosis (Zhu et al., 2002). Therefore, the GluN2A subunit is associated with cell survival, and the GluN2B subunit is associated with cell death (Liu et al., 2007; Chen et al., 2008; Lujan et al., 2012). Figure 1 visually outlines the known intracellular signaling pathways that lead to LTP and LTD from the NDMAR.

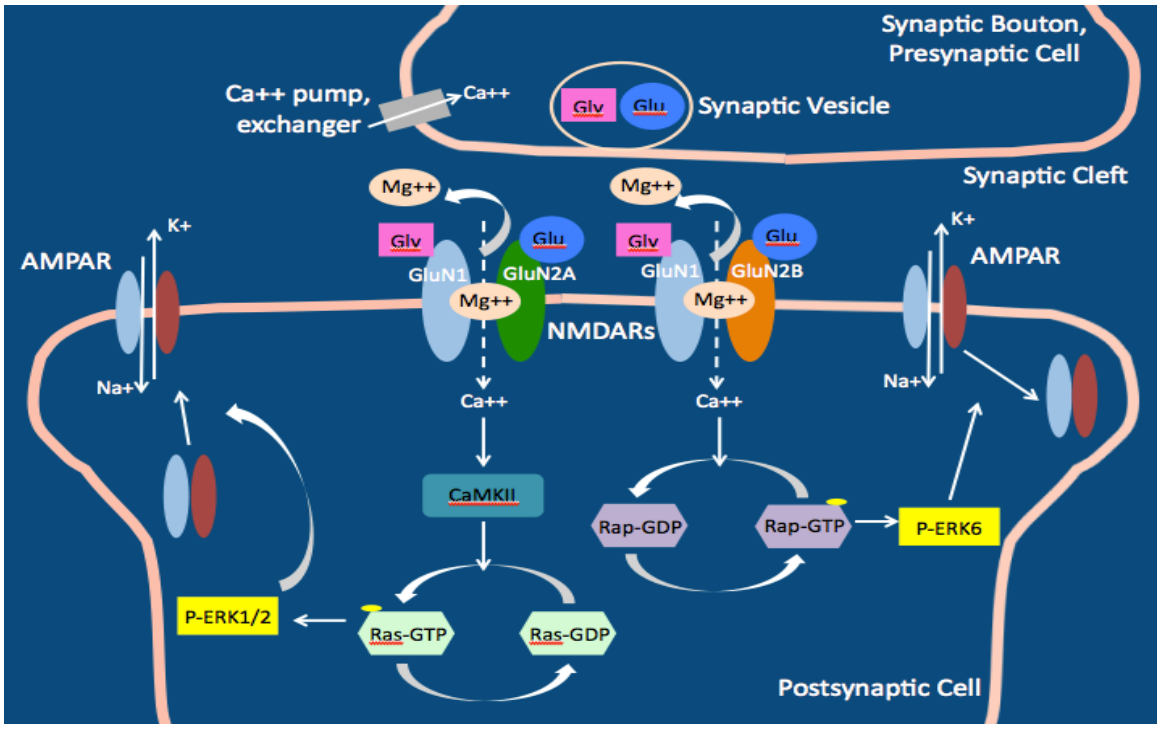

Figure 1: Visual abstract of the intracellular signaling pathways leading to LTD and LTP through the NMDAR. Calcium influx to the presynaptic bouton triggers the release of synaptic vesicles containing glycine (Gly) and glutamate (Glu), which cross the synaptic cleft and bind to NMDARs on the postsynaptic membrane. Postsynaptic cell depolarization releases the magnesium plug in the NMDAR, allowing calcium into the cell. Activation of NMDARs containing the GluN2A subunit leads to the activation of CaMKII, which phosphorylates Ras, which phosphorylates ERK1 and ERK2. ERK1 and ERK2 traffic AMPARs into the synaptic membrane, which increases LTP. Activation of NMDARs containing the GluN2B subunit leads to the phosphorylation of Rap and the phosphorylation of ERK6. ERK6 traffics AMPARs out of the synaptic membrane and increases LTD.

NMDARs can also contribute to a detrimental process of cell death called glutamatergic excitotoxicity by allowing too much calcium into the neuron. Apoptosis is the controlled destruction of a cell, ensuring that the digestive lysosomal enzymes in the cell's cytoplasm do not leak into the extracellular space to digest and damage neighboring, healthy cells. Neuronal injury and the sudden deprivation of blood flow or oxygen, as is the case with neuronal insult or ischemia, can result in the unprogrammed death of neurons, or necrosis, which has very detrimental results. When neurons experience a lack of blood flow and/or oxygen, their metabolic systems cannot function, and the neuron cannot produce the ATP needed to maintain its ion pumps and resting potential. The resulting break down of the electrochemical gradient causes chronic depolarization, allowing calcium to leak into the cell. NMDA receptors play a key role in this excitotoxicity process because they are a major route for calcium passage into the cell (MacDermott et al., 1986). Unregulated calcium influx triggers the uncontrolled release of glutamate, as if repetitive action potentials were occurring. A feedforward cycle is propagated throughout the tissue, since the release of glutamate further depolarizes neighboring cells, increasing the intracellular concentrations of calcium, and triggering the release of still more glutamate. The pathological increase of extracellular glutamate, combined with the calcium-induced activation of cellular digestive enzymes within the cell, quickly results in neuronal death. This process is called glutamatergic excitotoxicity (Bear et al., 2007).

NMDA receptor activation during excitotoxicity and neuronal pruning has been shown to have differing effects on neuronal survival, depending on the subunit composition of the receptor. NMDARs containing the GluN2A protein subunit seem to

protect the cell from damage and promote cell survival, whereas NMDARs containing the GluN2B subunit seem to promote cell death (Liu et al., 2007; Lujan et al., 2012). The localization of specific NMDAR subunits on the cell membrane supports this idea. During neural development, GluN2B subunits are expressed on the cell membrane at the synapse more than GluN2A subunits, promoting cell death during neuronal pruning. Neuronal pruning is the refinement of neuronal pathways through the elimination of weak synaptic connections. After neural maturation, the GluN2B subunits relocate from the synapse to the edges of the synapse, leaving the GluN2A subunits more prominently activated by synaptic activity, and promoting cell survival (Groc and Choquet, 2006). Thus the GluN2A subunit protects the cell from damage and promotes cell survival, whereas the GluN2B subunit promotes cell death (Liu et al., 2007; Lujan et al., 2012).

The ionotropic roles of NMDARs are well established (Dingledine et al., 1999), but it is not known whether NMDARs possess non-ionotropic activity. NMDARs activated ionotropically have been shown to regulate ERK phosphorylation and AMPAR expression on the postsynaptic membrane (Krapivinsky et al., 2003; Thomas and Huganir, 2004; Kim et al., 2005; Martel et al., 2009), but it is unknown whether any non-ionotropic activity of NMDARs is involved in the regulation of this process. This study focuses on NMDARs and how they regulate the ERK intracellular signaling cascade. Preliminary unpublished data from Dr. Wan's lab have demonstrated that glycine alone, the co-agonist of the NMDAR, increases AMPAR receptor function via a non-ionotropic signaling capacity of the NMDAR. Non-ionotropic activation of the NMDAR to regulate AMPAR function has been shown to be mediated by an ERK-dependent signaling mechanism. This intracellular mechanism is independent of glycine receptor activation in

mature hippocampal neurons. Dr. Wan's lab also has functional evidence that glycine alone can activate a non-channel activity of the NMDAR to confer neuroprotection in an excitotoxicity model. Non-ionotropic activation of the NMDAR to confer neuroprection in the neuronal injury model was shown to be dependent on a protein kinase B (AKT) signaling cascade. These findings suggest that ligand binding, but not ion flux through the NMDAR, can regulate physiological activity in neurons. This study aims to determine if NMDARs have non-ionotropic properties that can be activated with the single agonist glycine, which confers synaptic plasticity and cell survival.

Methodology

Cell Culture

Embryonic mouse neurons from cortex and hippocampus, and cultured Human Embryonic Kidney (HEK) 293 cells, were used for this study. Neuronal cells were used to look at intracellular kinase regulation due to NMDAR activation, and regulation as a result of pharmacological ion channel blockade. All animal experiments were approved and carried out in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Reno. A primary in vitro cell culture of prenatal hippocampus murine neurons was made from 16-day-old mouse embryos. Pregnant C57BL/6 mice were anesthetized by inhalation with isoflurane (Abbot Laboratories, North Chicago, IL). Hippocampi from the embryonic mice were isolated in ice-cold calcium- and magnesium-free Hank's balanced salts solution (HBSS; Life Technologies, Grand Island, NY) so that no calcium or magnesium ions could interact with the cells. This precaution was important since both calcium and magnesium directly

interact with NMDARs. The cells were dissociated into culture dishes, and the neurons were allowed to mature for 9 to 11 days in vitro prior to treatment and protein collection, using standard culture protocols (Kaech and Banker, 2006).

HEK293 cells were used to determine the subunit-specific functions of the NMDAR. HEK293 cells are a standard tool for molecular transfection and expression of ectopic genes. These cells were transfected with specific NMDAR subunits using standard procedures (Lipofectamine 2000, Invitrogen) since HEK293 cells do not express NMDARs naturally. The expression of specific subunits was manipulated so that the role of specific NMDAR functional tetramer subunits could be examined, as is represented in Figure 2. HEK293 cells do endogenously express both ERK1/2 (Dong et al., 2011) and CaMKII (Tsui et al., 2005), which are the intracellular signaling mechanisms manipulated downstream of NDMAR activation. It was necessary to have these kinases expressed in HEK293 cells in order to determine if NMDAR activation was occurring in the transfected HEK293 cells. The GluN1 598M mutant was used because it has a point mutation that substitutes asparagine in position 598 of the protein to glutamine (Q) or arginine (R). This amino acid change causes the NMDA receptor's ion channel pore to be malformed, creating a channel block that prevents the voltage-independent passage of divalent ions like calcium through the channel (Single et al., 2000). This physical blockade was a different technique used to prevent calcium from passing through the NMDAR ion channel and to prevent its ionotropic activity. Levels of GluN1, GluN2A, and GluN2B subunit expression were assayed independently to ensure their successful transfection. A measure of phosphorylated ERK1/2 protein demonstrated

the successful expression of functional NMDAR ion channels on the HEK293 cell membrane.

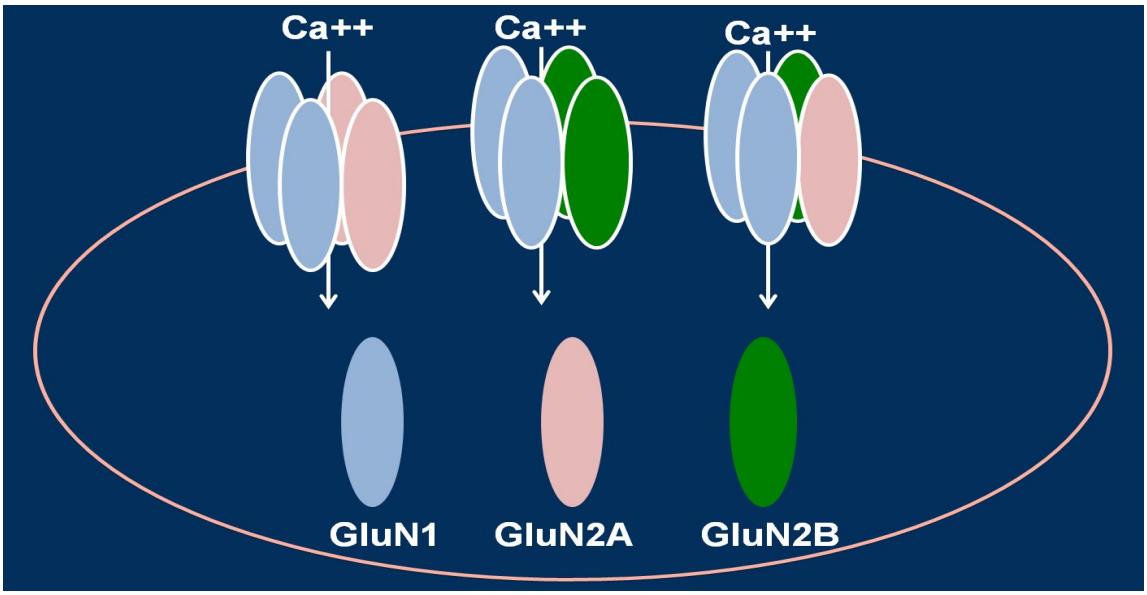

Figure 2: NMDAR subunit transfection in HEK293 cells. Subunits expressed in the cytoplasm do not create functional NMDA receptors. Tetramers of subunits comprising a pair of GluN1 subunits that are expressed on the cell membrane yield functional NMDA receptors that can pass calcium.

Western Blotting

Cell samples were treated, collected, and prepared for analysis by Western blot, all on the same day (see Results). Samples were run through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate all of the proteins expressed in the cells by molecular weight. Afterwards, protein in the gels from SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and assayed with an immunoblot technique to measure phosphorylated ERK1/2 and total ERK1/2 proteins, as well as the presence of NMDAR subunits in transfected HEK293 cells. The Western blot assay was performed using standard procedures (Sambrook and Maniatis, 1989).

The PVDF membranes were first incubated with primary antibody against phosphorylated ERK1 and ERK2 (p-ERK1/2) and, for HEK293 cells, GluN1 (Cell Signaling Technology, Beverly, MA). The p-ERK1/2 and GluN1 primary antibodies were raised in a rabbit host. Staining of these primary antibodies was reported with horseradish peroxidase-conjugated secondary antibody, and the protein bands were imaged using chemiluminescent SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). An EC3 Imaging System (UVP, LLC, Upland, CA) was used to obtain blot images directly from the PVDF membrane. The membrane was then stripped and re-probed with primary antibody against total ERK1 and ERK2 protein (t-ERK1/2) and, for HEK293 cells, GluN2A (Cell Signaling Technology, Beverly, MA) and GluN2B (Millipore Corporation, Billerica, MA) subunits. The t-ERK1/2 primary antibody was raised in a rabbit host, the GluN2A primary antibody was raised in a goat host, and the GluN2B primary antibody was raised in a mouse host. The GluN1 and GluN2B primary antibodies were used in a 1:500 ratio in 5% fat free milk in trizma buffer saline (TBS) solution. The p-ERK1/2 primary antibody was used in a 1:1500 ratio, the t-ERK1/2 primary antibody was used in a 1:1000 ratio, and the GluN2A primary antibody was used in a 1:400 ratio. All secondary antibodies were used in ratios as recommended by their manufacturers.

Quantification of the Western blot image data was performed using ImageJ software, measuring the integrated optical density of the bands in each trial. A darker band in the Western blot indicates a greater density of expressed protein in the sample, which corresponds to a higher integrated optical density in analysis. The level of

phosphorylated ERK relative to total ERK is reported as a ratio of p-ERK/t-ERK, and this ratio is normalized across trials against a control.

Materials

The N-methyl-D-aspartate (NMDA), the Strychnine, and the glycine used for these experiments were from Sigma-Aldrich (St. Louis, MO). MK-801, Ifenprodil, and L689 were from Tocris Biosciences (Ellisville, MO).

Statistics

The ratio of the integrated optical densities of phosphorylated ERK to total ERK proteins was taken for every trial, and the mean of all trials was graphed. A minimum of three replicates showing the same result was considered sufficient to draw conclusions, as is consistent with accepted practice in the field. An ANOVA was used to examine the statistical significance of the differences between groups of data using GraphPad Prism Version 6.0d. Significance was placed at $p < 0.05$.

Results

Treatments

Changes in ERK1/2 intracellular signaling due to NMDAR activation were probed using pharmacological and physiological approaches. The possibility for calcium to pass through the ion channel was eliminated by treating the cells in a calcium free extracellular solution (ECS) containing ethylene glycol tetraacetic acid (EGTA) (Research Organics, Cleveland, OH), which binds to divalent cations such as calcium and magnesium, making these ions unavailable for interaction with the NMDAR (Auld, 1995). Further, the ion channels were physically blocked using MK-801 (10μM), which

permanently blocked the ion channel pore of the NMDAR, to prevent ionotropic activation. In experimental pre-treatment protocols, cells treated with MK-801 were stimulated with glycine and NMDA to remove this magnesium plug so that the plug could be replaced with MK-801, creating a receptor that was pharmacologically blocked and no longer ionotropically functional. Cell cultures were stimulated with N-methyl-D-aspartate (NMDA; 50μM), a synthetic agonist replacement for glutamate that is specific to the NMDA receptor ligand-binding site for glutamate. A synthetic agonist was used because it specifically targets the activation site of interest on the GluN2 subunit of the NMDAR. Strychnine (10μM) was used as a glycine receptor antagonist, blocking peripheral glycine receptor activation on the cell membrane. L689 (5μM) is a drug that blocks the primary glycine-binding site on the GluN1 subunit of the NMDAR. Ifenprodil (10μM) is a drug that blocks the glycine-binding site on the GluN2 subunit, with a higher affinity for the GluN2B subunit of the NMDAR. Refer to Figure 3 for a schematic of the NMDAR and its agonist and antagonist binding sites.

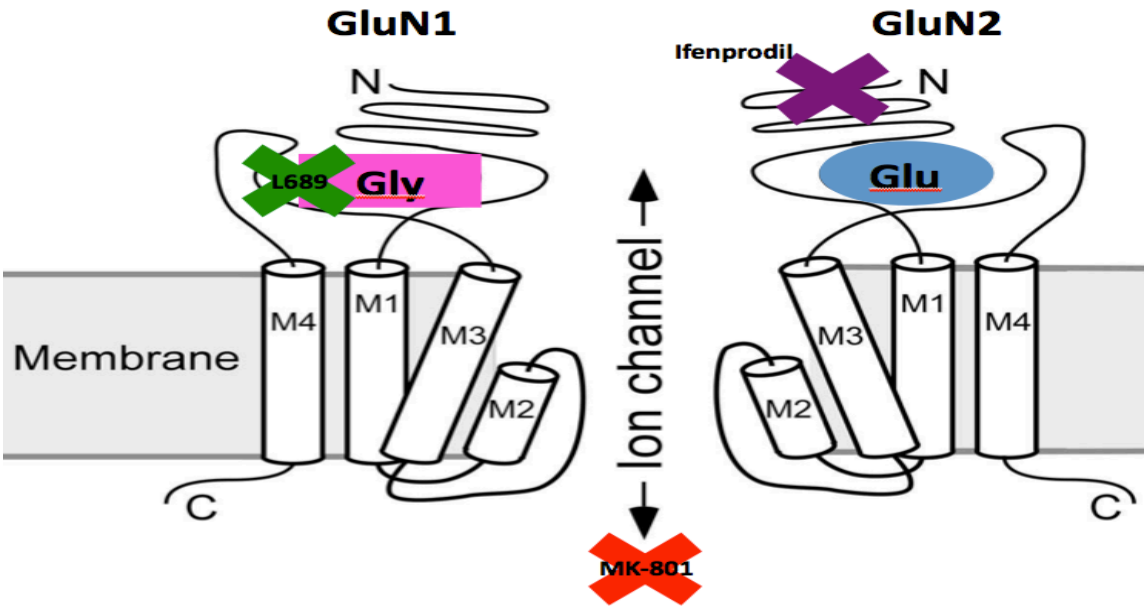

Figure 3: NMDAR subunit structures, ligand binding sites, and drug target areas. Glycine binds to the GluN1 subunit and glutamate binds to the GluN2 subunit. Ifenprodil (10μM) blocks the peripheral glycine-binding site on GluN2, with a higher affinity for GluN2B. L689 (5μM) blocks the primary glycine-binding site on GluN1. MK-801 (10μM) permanently pharmacologically blocks the ion channel pore after NMDAR activation removes the magnesium plug in the ion channel (adapted from Siegler, Retchless et al., 2012).

Neuron Pharmacological Experiment: Proof of Concept

The hypothesis for this experiment was that NMDAR activation could occur non-ionotropically, resulting in the phosphorylation of ERK1/2. This treatment compared neurons stimulated with NMDA (50μM) in ionotropic conditions versus metabotropic conditions. Experimental conditions consisted of both normal extracellular solution (ECS) and calcium-free ECS, with each condition consisting of both MK-801 (10μM) blocked channel treatments and MK-801 free treatments. All treatments contained strychnine (10μM). Phosphorylated ERK1/2 was measured against total ERK1/2 protein. Schwarzschild et al. (1999) found that the presence of extracellular calcium and

glutamate was necessary to stimulate the phosphorylation of ERK1/2 through the NMDA receptor. They also found, at minimum, a two-fold increase in ERK1/2 phosphorylation levels in striatal cells over conditions not containing extracellular calcium or glutamate. A two-fold increase in phosphorylated ERK1/2 was therefore expected in the normal ECS, MK-801 free, NMDA-stimulated condition over the non-stimulated condition for this experiment. A 0.5-fold increase was expected in non-ionotropic NMDA-stimulated conditions (i.e. in the presence of MK-801 or calcium-free ECS) over non-stimulated conditions consistent with preliminary data in Dr. Wan's lab.

Figure 4 shows the control results for neurons not stimulated with NMDA. There was no significant difference between the baseline ERK1/2 phosphorylation levels for ionotropic channel activation of the NMDAR versus metabotropic, non-channel activation. Figure 5 shows the normalized ERK1/2 phosphorylation levels after stimulating NMDARs with NMDA. There were no significant differences in phosphorylation between treatment and control groups, and there were no significant differences between ionotropic and metabotropic activities.

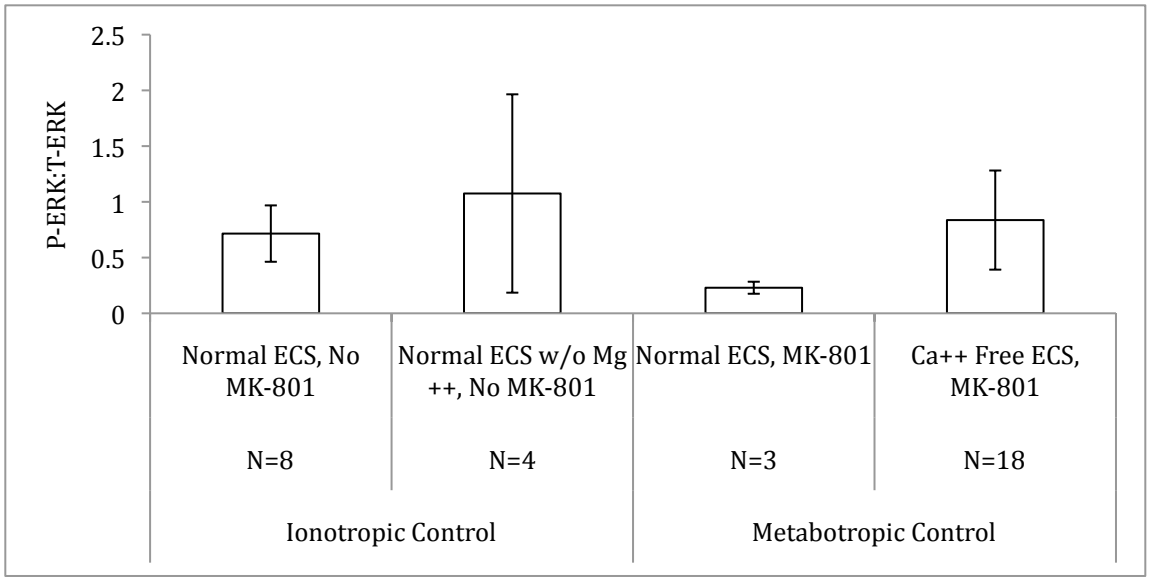

Figure 4: Neuronal NMDAR ERK1/2 phosphorylation levels for ionotropic and metabotropic controls. Samples were not stimulated with NMDA, and ion channels were blocked with MK-801 (10μM) in metabotropic controls only. NMDAR ionotropic and metabotropic activities were measured by assaying ERK1/2 phosphorylation against total ERK1/2 protein using Western blot. When ionotropic activity was inhibited, there was less ERK phosphorylation. However, these differences were not significant.

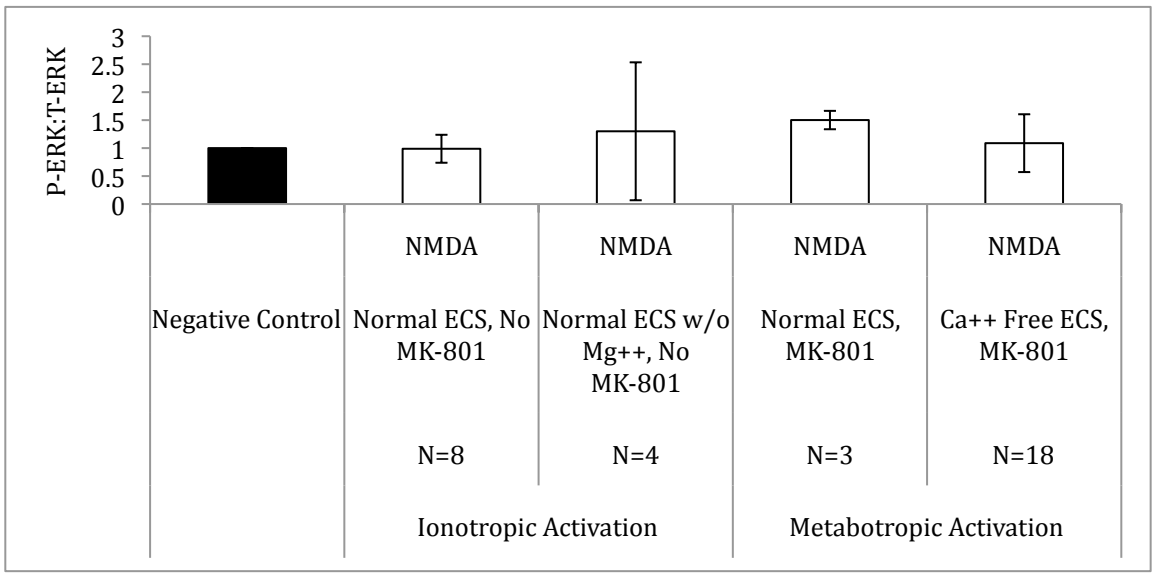

Figure 5: Neuronal NMDAR ERK1/2 phosphorylation levels after stimulation with NMDA. Ion channels were blocked with MK-801 (10μM). NMDAR ionotropic and metabotropic activities were measured by assaying ERK1/2 phosphorylation against total ERK protein after treatment with NMDA (50μM) using Western blot. No significant differences were found between treatment with NMDA and controls, nor between ionotropic and metabotropic activities. Results are normalized to a negative control with no NMDA stimulation.

Neuron Pharmacological Experiment: Blocking Ion Channel Pore and NMDAR Glycine Receptors, Testing Overall Response

The hypothesis for this experiment was that NMDAR activation could occur non-ionotropically via a secondary glycine-binding site on the GluN2 subunit, resulting in the phosphorylation of ERK1/2. This experiment was designed to try to describe where glycine is binding in order to activate the NMDAR, if the primary receptor site on the GluN1 subunit was not responsible for ERK1/2 phosphorylation. Conditions for this experiment consisted of calcium-free ECS with MK-801 (10μM) blocked channel treatments and strychnine (10μM). Experimental conditions included L689 (5μM) to block the primary glycine-binding site on the GluN1 subunit, Ifenprodil (10μM) to block the hypothesized secondary glycine-binding site on the GluN2 subunit, and then both L689 (5μM) and Ifenprodil (10μM). Phosphorylated ERK1/2 was measured against total ERK1/2 protein in all conditions. No significant increase in ERK1/2 phosphorylation levels was expected in the control condition or in the Ifenprodil conditions. A 0.5-fold increase was expected in the L689, NMDA (50μM) condition, based on Dr. Wan's preliminary data showing metabotropic activation. Figure 6 shows that ERK1/2 phosphorylation levels through NMDAR metabotropic activities are not significantly affected by antagonizing glycine binding with L689 and/or Ifenprodil.

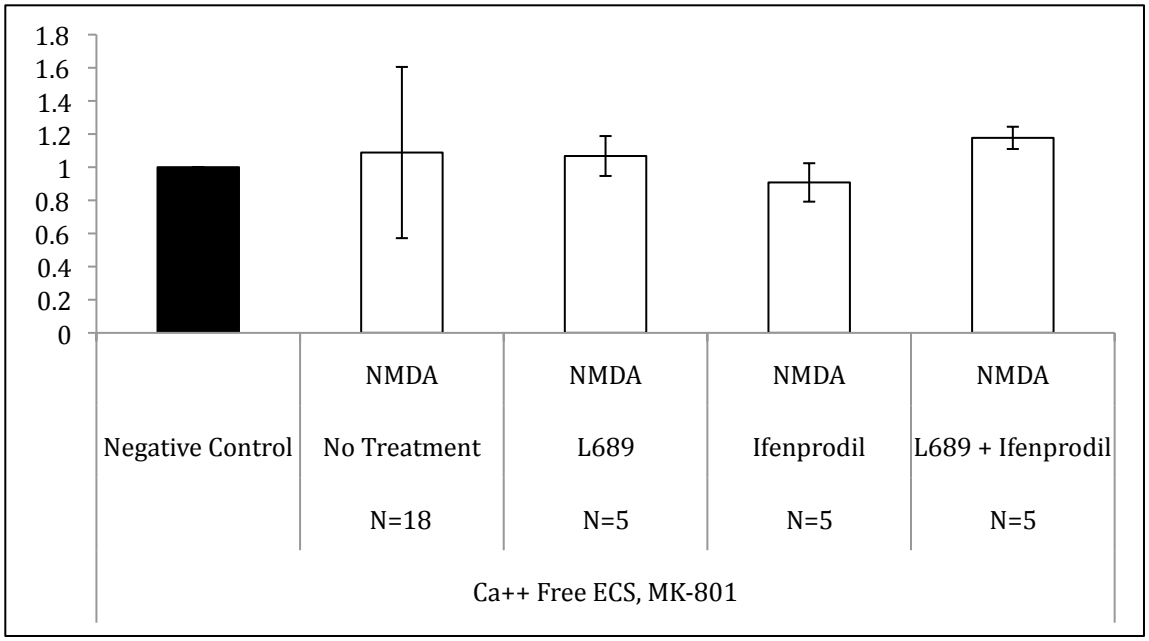

Figure 6: Neuronal NMDAR ERK1/2 phosphorylation levels after treatment with L689 and Ifenprodil and stimulation with NMDA. Calcium-free ECS was used, and ion channels were blocked with MK-801 (10μM). L689 (5μM) and Ifenprodil (10μM) were used to block glycine binding. NMDAR metabotropic activities were measured by assaying ERK1/2 phosphorylation after treatment with NMDA (50μM) with Western blot. No significant differences were found between treatment with NMDA and controls, and no significant difference was found between drug treatments. Results are normalized to a negative control with no NMDA stimulation.

HEK293 Control Experiment

This experiment was designed to show that the NMDAR subunits were being successfully transfected into the HEK293 cells, that ERK1/2 phosphorylation was not the result of a single NMDAR subunit acting by itself, and that ERK1/2 phosphorylation is not a result of NMDA-stimulated cellular activity outside of the NMDAR. A negative result was hypothesized, showing no significant ERK1/2 phosphorylation above the negative control without NMDA stimulation. Four transfection conditions were evaluated in calcium-free ECS with MK-801 (10μM) and strychnine (10μM). The four conditions were naïve HEK293 cells with no transfection, GluN1 transfection only, GluN2A

transfection only, and GluN2B transfection only. Phosphorylated ERK1/2 was measured against total ERK1/2 protein, and the presence of the GluN1, GluN2A, and GluN2B subunits was assayed. Figure 7 shows that the subunits were transfected successfully, though the faint presence of bands in HEK293 cells not transfected for the GluN2A and GluN2B subunits suggest that HEK293 cells may have some endogenous NMDAR proteins. It is also possible that some nonspecific antibody binding occurred giving a false result. Figure 8 shows that the HEK293 cells stimulated with NMDA (50μM) did not have a significant increase in ERK phosphorylation when compared to the HEK293 cells not stimulated with NMDA, demonstrating that a single NMDAR subunit is not sufficient to phosphorylate ERK1/2.

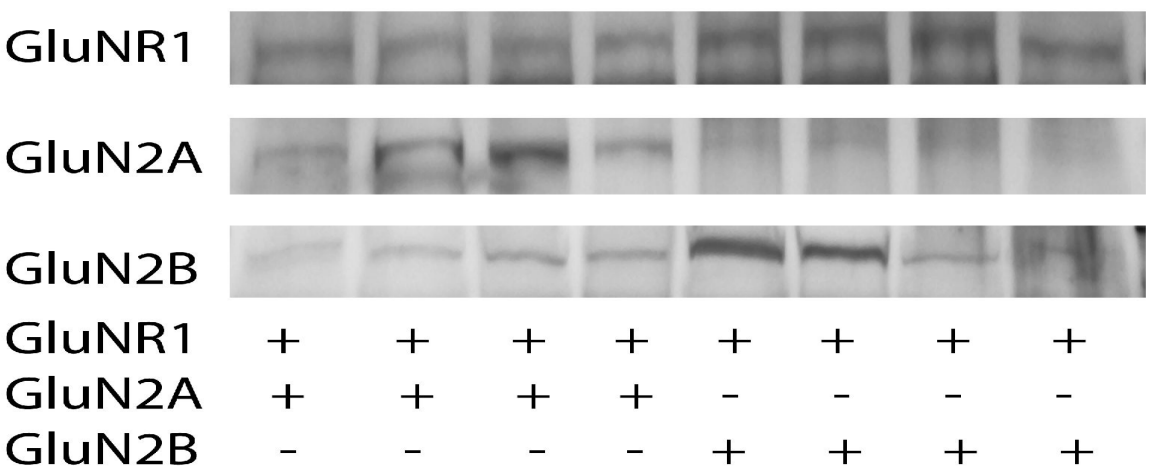

Figure 7: HEK293 transfection efficiency for NMDAR subunits. The GluN1, GluN2A, and GluN2B subunits were successfully transfected and expressed in every trial. The GluN1 row is representative of both the WT and 598M GluN1 subunit types. There appears to be either some nonspecific antibody binding or some endogenous NMDAR subunit protein present even in non-transfected HEK293 conditions.

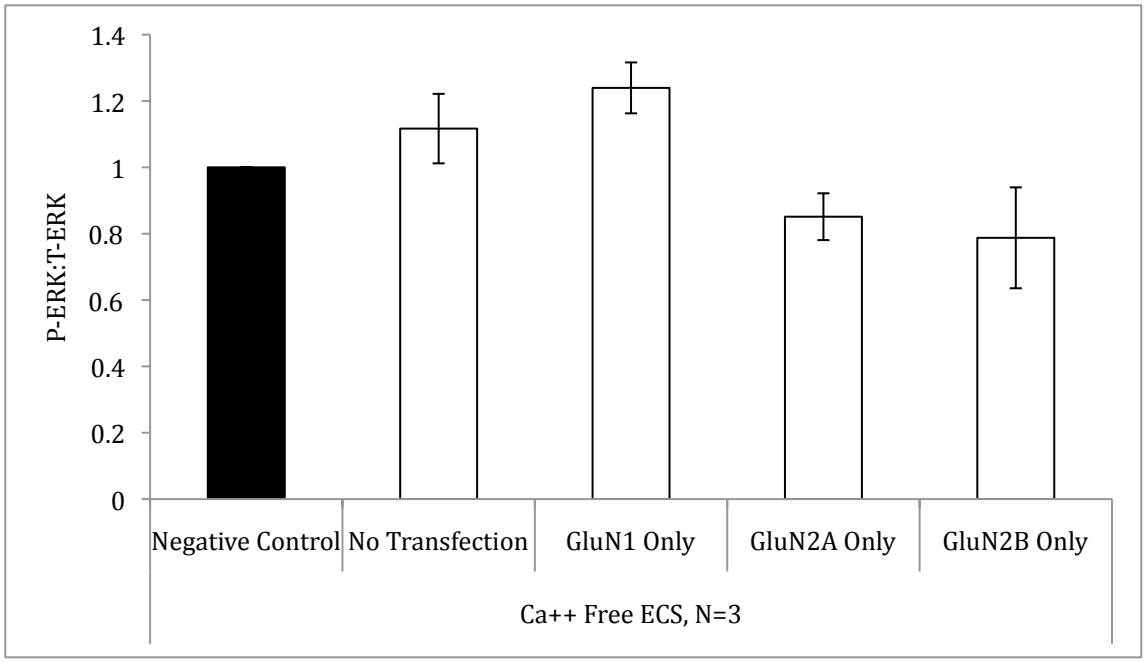

Figure 8: ERK1/2 phosphorylation is not a result of a single NMDAR subunit stimulated with NMDA. HEK293 cells were transfected with no NMDAR subunits, GluN1 subunit only, GluN2A subunit only, or GluN2B subunit only. ERK1/2 phosphorylation levels were measured against total ERK protein. No significant differences were found between cells stimulated with NMDA (50μM) and non-stimulated cells. Results are normalized to a negative control with no NMDA stimulation.

HEK293 Pharmacological Experiment: Blocking Ion Channel Pore and NMDAR Glycine Receptors, Testing Subunit-Specific Response

The hypothesis for this experiment was that NMDAR activation could occur non-ionotropically via a secondary glycine-binding site, resulting in the phosphorylation of ERK1/2. HEK293 cells were transfected with a combination of GluN1 plus GluN2A subunits, or of GluN1 plus GluN2B subunits. Conditions for this experiment consisted of calcium-free ECS with strychnine (10μM) and MK-801(10μM) blocked channel treatments. Experimental conditions included L689 (5μM) to block the primary glycine-binding site on the GluN1 subunit, or Ifenprodil (10μM) to block the hypothesized secondary glycine-binding site on the GluN2 subunit. Phosphorylated ERK1/2 was

measured against total ERK1/2 protein in all conditions. No significant increase in ERK1/2 phosphorylation levels was expected in the control condition and in the Ifenprodil conditions. A 0.5-fold increase was expected in the L689, NMDA (50μM) condition, based on Dr. Wan's preliminary data. Figure 9 shows that NMDARs containing GluN2A or GluN2B subunits do not have activities significantly affected by antagonizing glycine binding with L689 or Ifenprodil. No significant differences were found between treatment with NMDA and controls, and no significant differences were found between cells expressing receptors composed of the GluN2A or GluN2B subunits.

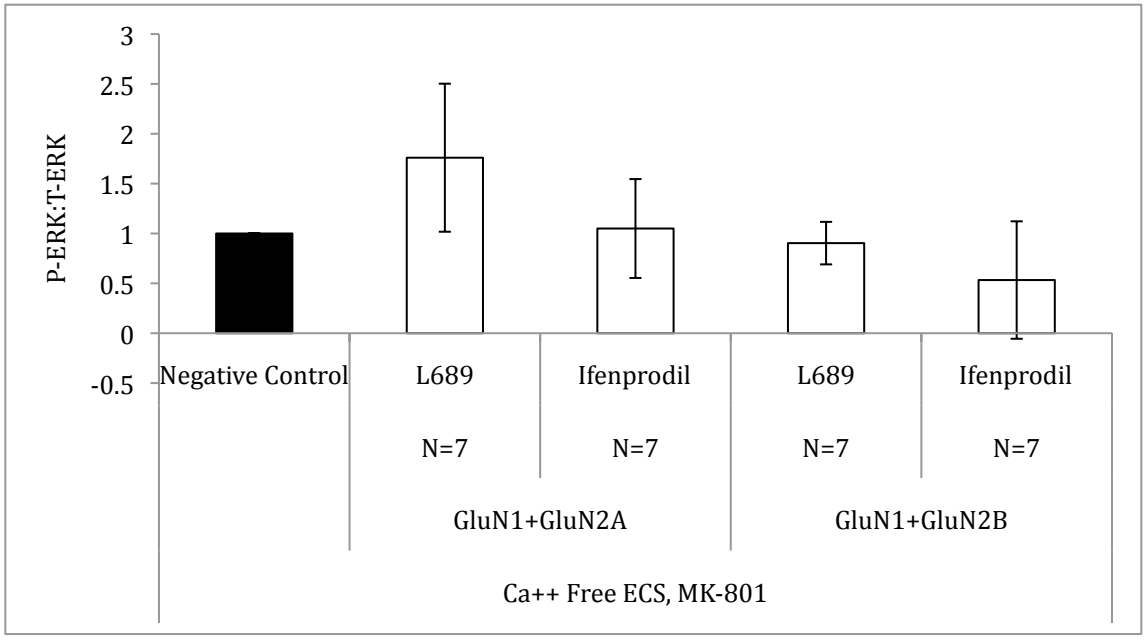

Figure 9: ERK1/2 phosphorylation levels after GluN2A and GluN2B NMDAR subunit treatment with L689 and Ifenprodil in HEK293 cells. HEK293 cells were transfected with GluN1 plus GluN2A or GluN2B NMDAR subunits. Calcium was made unavailable for passage through the NMDAR ion channel with calcium-free ECS, and ion channels were blocked with MK-801 (10μM). L689 (5μM) and Ifenprodil (10μM) were used to block glycine binding. NMDAR metabotropic activities were measured by assaying ERK1/2 phosphorylation after treatment with NMDA (50μM) using Western blot. No significant differences were found between treatment with NMDA and controls. No significant difference was found between drug treatments. No significant differences were found between cells expressing receptors composed of the GluN2A or GluN2B subunits. Results are normalized to a negative control with no NMDA stimulation.

HEK293 Physiological Experiment: Molecular Block of Ion Channel Pore, Testing Subunit-Specific Response

This experiment was designed to determine if there were any NMDAR subunit-specific correlations for non-ionotropic ERK1/2 phosphorylation. HEK293 cells were transfected with a combination of GluN1 mutant 598M plus GluN2A subunits; Wild Type (WT) GluN1 plus GluN2A subunits; GluN1 mutant 598M plus GluN2B subunits; or WT GluN1 plus GluN2B subunits. The GluN1 598M mutant has a point mutation that substitutes asparagine in position 598 of the protein to glutamine (Q) or arginine (R). This amino acid change causes the receptor's ion channel pore to be malformed, creating a channel block that prevents the voltage-independent passage of divalent ions like calcium through the channel (Single et al., 2000). Using the 598M mutant physiologically prevents ionotropic activation of the NMDAR, similar to how MK-801 pharmacologically blocks ionotropic activation of the NMDAR by permanently replacing the magnesium plug in the ion channel. Conditions for this experiment consisted of calcium-free ECS and strychnine (10μM), further creating a non-ionotropic, metabotropic activation environment for the NMDA receptors. Metabotropic, subunit-specific activation of the NMDAR was being tested.

Phosphorylated ERK1/2 was measured against total ERK1/2 protein in all conditions. A greater increase in ERK1/2 phosphorylation was expected in the NMDA (50μM) stimulated conditions than in the non-stimulated conditions. Figure 10 shows that, after metabotropic activation, there were no significant differences in ERK1/2 phosphorylation levels of NMDARs containing GluN2A versus GluN2B subunits. Physiologically blocking the NDMAR ion channel pore via expression of the 598M

GluN1 subunit did not yield a significant difference in ERK1/2 phosphorylation levels when compared to Wild Type (WT) GluN1 metabotropic activation.

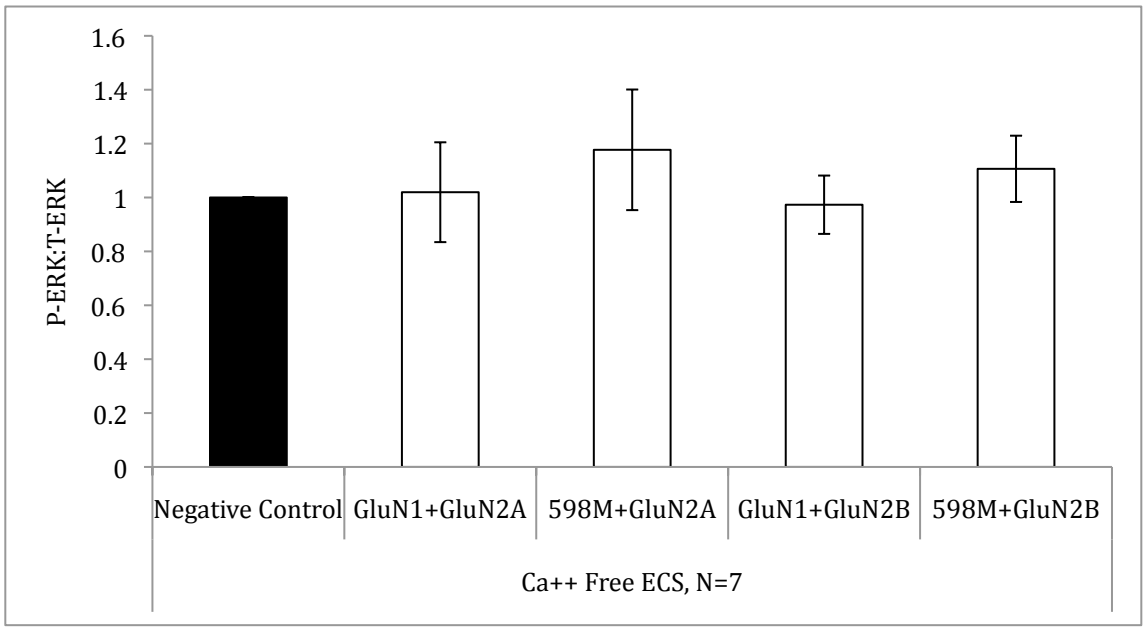

Figure 10: ERK1/2 phosphorylation levels of 598M and WT GluN1 subunit NMDARs after NMDA stimulation in HEK293 cells. HEK293 cells were transfected with Wild Type (WT) or 598M mutant GluN1 NMDAR subunits plus GluN2A or GluN2B subunits. The 598M subunits physically blocked the NMDAR ion channel pore. Calcium was made unavailable for passage through the ion channel with calcium-free ECS. NMDAR metabotropic activities were measured by assaying ERK1/2 phosphorylation after treatment with NMDA (50μM) using Western blot. No significant differences were found between treatment with NMDA and controls, and no significant differences were found between WT and 598M transfected HEK293 cells, with either GluN2A or GluN2B subunits. Results are normalized to a negative control with no NMDA stimulation.

Protein Concentration Dilution Curve

This experiment was conducted after analyses of the previous experiments showed insignificant results. The purpose of this experiment was to show that I am capable of completing the Western blot successfully, thus demonstrating that the results in my previous experiments were indeed accurate and not the result of poor technique. A cortical brain tissue sample was prepared from an adult mouse and diluted to create a

concentration curve. Gel wells were filled with brain tissue samples containing total protein concentrations of 0.5 - 40 µg/µl, and the Western blot was conducted to assay for total ERK1/2 protein. Two samples from the same culture were run, and Figure 11 shows the Western blot results of the second run showing a clear dilution curve. The results for the 30µg/µl and 40µg/µl samples in the first Western blot were faulty and were therefore not included. Analysis of the bands in ImageJ yielded the pixel integrated densities of each sample, and these values were plotted along both a linear scale and a logarithmic scale, as can be seen in Figure 12. The top graph plots the results along a linear scale, and a logarithmic function fits the mean results with an equation of $y=1.6561\ln(x) + 2.0973$ and $R^2=0.9819$. The bottom graph plots the results along a logarithmic scale, and a linear function fits the mean results with an equation of $y=1.6561(x) + 2.0973$ and $R^2=0.9819$. These results indicate data with a strong fit to the regression lines, as is indicated by how the R^2 value is so close to 1. Such a strong fit is indicative of a reliable result for the standard curve.

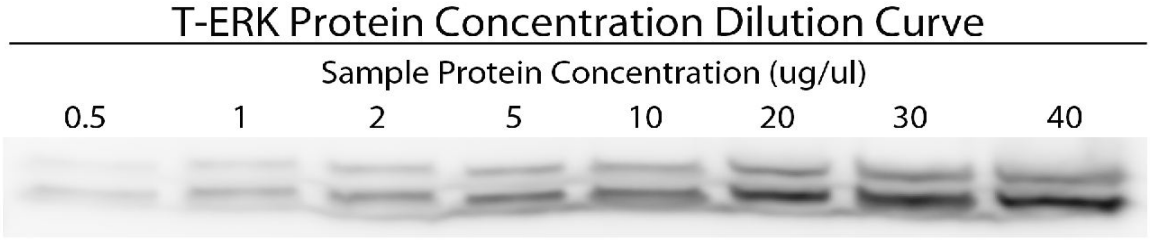

Figure 11: Western blot of total ERK1/2 protein present in a protein concentration dilution curve. A mouse cortical brain tissue sample was diluted to create a concentration curve. This is a successful, clean Western blot result showing the steady increase in t-ERK1/2 concentration as the overall protein concentration of the samples increases.

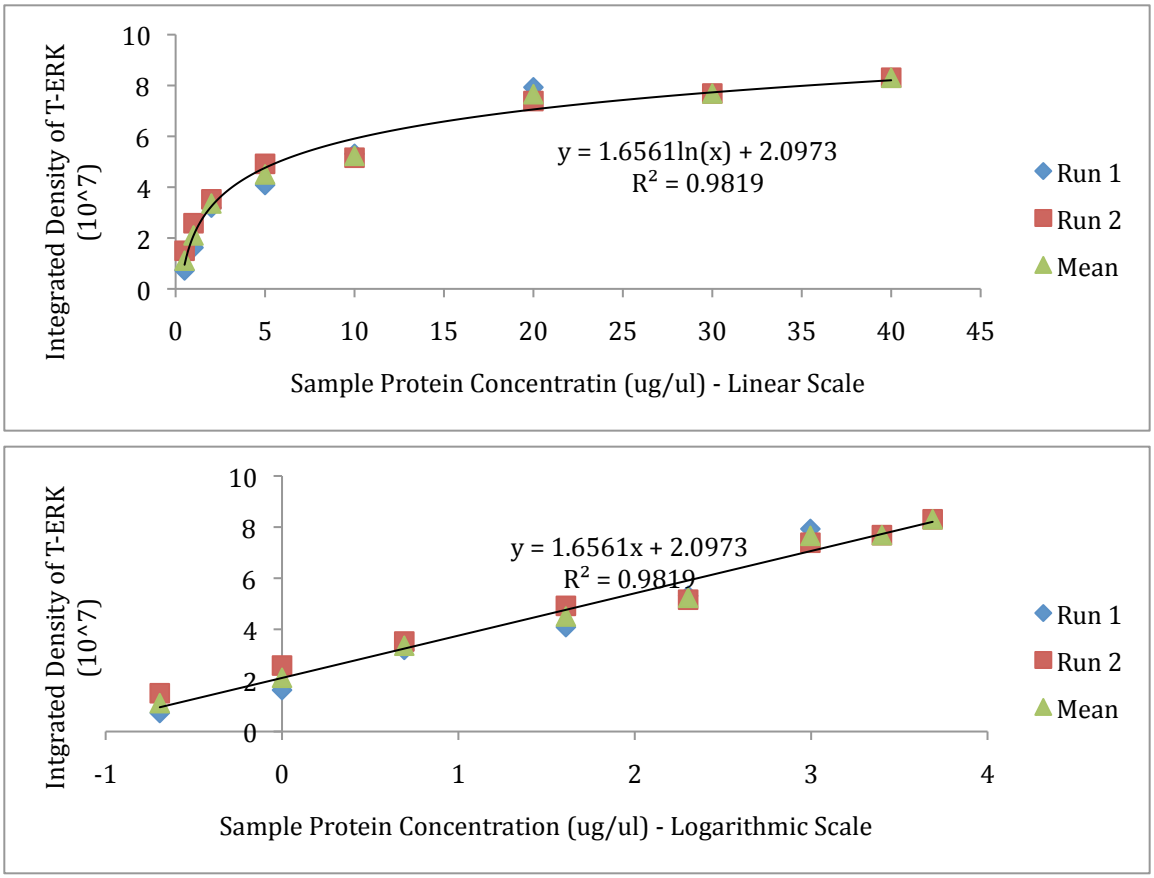

Figure 12: Integrated density of t-ERK1/2 with a best-fit function for the protein concentration dilution curve. The integrated densities of two Western blots containing dilutions of a mouse cortical brain tissue sample create a dilution curve. The results for the 30µg/µl and 40µg/µl samples in the first Western blot were faulty and therefore not included. The top graph plots the results along a linear scale, and a logarithmic function results along their mean with an equation of y=1.6561ln(x) + 2.0973 and R^2=0.9819. The bottom graph plots the results along a logarithmic scale, and a linear function results along their mean with an equation of y=1.6561(x) + 2.0973 and R^2=0.9819.

Discussion

The neuron pharmacological proof-of-concept experiment was meant to be a positive control experiment providing a baseline of ERK1/2 phosphorylation levels in both physiological ionotropic conditions and non-ionotropic conditions. Though the differences in phosphorylation levels across conditions were not significant, an expected pattern of phosphorylation was generally observed, as is depicted in

Figure 4. In the ionotropic controls, there was greater ERK1/2 phosphorylation in the magnesium-free ECS condition than in the normal ECS condition. This makes sense since a shortage of free magnesium available to plug the NMDAR ion pore would result in ion channels that are free to pass calcium, independent of cell depolarization. The magnesium-free condition represents NMDAR activation levels, and thus ERK1/2 phosphorylation levels, higher than what is likely to happen in normal, physiological conditions. The metabotropic conditions were expected to have less phosphorylation than the ionotropic conditions, and this is seen in the normal ECS, MK-801 (10μM) condition, where MK-801 is blocking the NMDAR ion pore and preventing calcium passage. The calcium-free ECS, MK-801 condition should have had lower phosphorylation levels still, since there was no calcium available to pass through the ion channel, which was blocked by MK-801, anyway. However, this condition appears to have phosphorylation levels comparable to the normal, ionotropic condition. This was unexpected, and it can perhaps be explained by how large the error bar is, suggesting that there were issues with reproducibility.

These same conditions were then stimulated with NMDA (50μM), presented in Figure 5. The ionotropic conditions did not show the expected two-fold increase in ERK1/2 phosphorylation over the non-stimulated condition (Schwarzschild et al., 1999). This was a troubling finding, and it is perhaps due to the small sample size and the large degree of error showing difficulty in reproducibility. However, the metabotropic conditions did show the expected minimum 0.5-fold increase that was seen in the preliminary data from Dr. Wan's lab. This was encouraging since it

showed that metabotropic activation of the NMDAR is possible and predictably measureable.

The neuron pharmacological experiment that used L689 (5μM) and Ifenprodil (10μM) was meant to determine if glycine binding played a role in the metabotropic phosphorylation of ERK1/2 in the NDMAR (Figure 6). Since there were no significant differences in ERK1/2 phosphorylation across all experimental conditions, this would suggest that glycine binding might not effect metabotropic activation of the NMDA receptor. However, since there are so many issues with the experimental protocols, I cannot confidently make this conclusion. I do not feel that I was able to reliably detect changes in phosphorylation levels, even if they had been present. The results for this experiment are inconclusive, and the experiment should be repeated with improved procedures.

The HEK293 control experiment was meant to demonstrate that there was successful HEK293 transfection of NMDAR subunits and that valid NMDAR subunit experiments could be conducted in HEK293 cells. It was also meant to show that ERK1/2 phosphorylation is not the result of a single NMDAR subunit acting by itself, and that ERK1/2 phosphorylation is not a result of NMDA-stimulated cellular activity outside of the NMDAR. Figure 7 shows the Western blot results of NMDAR subunit assays, proving that the HEK293 transfections were successful. There appears to either be endogenous GluN2A and GluN2B subunit protein in the HEK293 cells, or nonspecific binding of the antibodies in the Western blot procedure giving a false positive result. The ERK1/2 phosphorylation results in Figure 8 show there is no significant difference in phosphorylation levels between subunits acting by themselves. There does, however,

appear to be slightly higher phosphorylation levels in the GluN1-only transfection condition. This observation combined with the Western blot results suggests that there are endogenous NMDAR subunit proteins being expressed in HEK293 cells. Although, if there are GluN2A and GluN2B NMDAR subunits being endogenously expressed in HEK293 cells, they are not being expressed at a high enough level to significantly impact the results of the experiments being conducted in HEK293 cells.

The HEK293 pharmacological experiment involving Ifenprodil (10μM) and L689 (5μM) was meant to determine if there are any subunit-specific functions of the NMDAR in ERK1/2 phosphorylation. HEK293 cells were transfected with combinations of either GluN1 plus GluN2A subunits, or GluN1 plus GluN2B subunits, and then treated in calcium-free ECS and MK-801 (10μM) to create a metabotropic condition. L689 and Ifenprodil are glycine-binding inhibitors for the GluN1 and GuN2 subunits, respectively, with Ifenprodil having a slightly higher affinity for the GluN2B subunit. The results of this experiment are depicted in Figure 9. Despite the lack of significant difference in results between the experimental conditions, it appears that Ifenprodil had a greater inhibitory effect on ERK1/2 phosphorylation than L689. It also looks like GluN2B subunits had less phosphorylation than GluN2A subunits overall. This makes sense since Ifenprodil has a higher affinity for the GluN2B subunit, inhibiting its activity more than the GluN2A subunit. The only 0.5-fold increase in ERK1/2 phosphorylation levels observed was in the GluN1 plus GluN2A, Ifenprodil experimental condition. This suggests that metabotropic activation of the NMDAR is only possible in the receptors composed of the GluN2A subunit, and that glycine binding to the GluN2A subunit is necessary for this activation, since this glycine-binding site was not being inhibited.

The HEK293 physiological experiment involved physically blocking the NMDAR ion channel pore with the GluN1 598M subunit mutant. This experiment was meant to determine if there were any subunit-specific of the NMDAR in ERK1/2 phosphorylation. Physically blocking the ion channel and treating the cells in calcium-free ECS created a metabotropic environment for the HEK293 cells transfected with combinations of GluN1 598M plus GluN2A, or GluN1 598M plus GluN2B NMDAR subunits. The results of this experiment are seen in Figure 10. While there are no significant differences in ERK1/2 phosphorylation levels between any of the experimental conditions, there does appear to be greater phosphorylation in the GluN1 598M mutant NMDA receptors than in the WT receptors.

The HEK293 cell transfection experiments may be considered valid because the HEK293 cells are an appropriate model for NMDAR experiments. HEK293 cells must be transfected in order to express NMDAR subunits, and they do endogenously express ERK1/2 (Dong et al., 2011) and slight levels of CaMKII (Tsui et al., 2005). Since ERK1/2 and CaMKII are the intracellular signaling mechanisms manipulated downstream of NDMAR activation, it is necessary to have these kinases expressed in HEK293 cells in order to determine if NMDAR activation is occurring in the transfected HEK293 cells. The measure of phosphorylated ERK1/2 protein demonstrates the successful expression of functional NMDAR ion channels on the HEK293 cell membrane. Since these mechanisms are present, all of the HEK293 cell experiments with the NMDA receptor can be considered valid.

Because no significant results were found in the proceeding experiments, a protein dilution curve was created in order to demonstrate that the Western blot technique

was being conducted correctly. This experiment was demonstrates that I can prepare experimental samples, accurately assess and dilute those samples' overall protein levels, and complete the Western blot properly to yield clear, analyzable results. This experiment was conducted as a necessary troubleshoot to eliminate the possibility that the preceding insignificant results were due to failed technique. Adult mouse cortical brain tissue samples were prepared, and total ERK1/2 protein was assayed. Two samples were run, and Figure 11 shows the Western blot results of the second run showing a clear dilution curve. The results for the 30µg/µl and 40µg/µl samples in the first Western blot were faulty and were therefore not included. This suggests that reproducibility becomes more difficult at these higher protein concentrations. The integrated densities of each sample were plotted along both a linear scale and a logarithmic scale, as can be seen in Figure 12. The top graph plots the results along a linear scale, and is fit by a logarithmic function, reflecting expected signal response from the antibody staining technique. The bottom graph plots the results along a logarithmic scale, and is fit by a linear function with an equation of $y=1.6561(x) + 2.0973$ and $R^2=0.9819$. The regression lines for both graphs have a very high fit to the data with $R^2=0.9819$, indicating that there is little variance between runs and a high degree of reproducibility. The clarity of the results and their reproducibility show that I am proficient at the Western blot technique and that the results are not insignificant due to failed technique.

When looking at the protein concentration dilution curve plotted in Figure 12, it becomes apparent that using a sample concentration of 40µg/µl for these experiments may not have been ideal. The samples with a concentration of 30µg/µl and 40µg/µl were less reproducible, and they fall on the end of the logarithmic regression line where there

is a diminishing slope. This means that a two-fold increase in phosphorylated ERK1/2 protein concentration at these sample concentrations would be less apparent and more difficult to detect. However, at sample concentrations between 10µg/µl and 20µg/µl, changes in protein concentrations along the logarithmic regression line are more drastic. A two-fold increase in protein concentration is easy to detect along this part of the standard curve because it correlates with the largest difference in integrated density. Since a two-fold increase in phosphorylated ERK1/2 levels between NMDA-stimulated and non-stimulated conditions is expected in the experiments (Schwarzchild et al., 1999), I would recommend using a protein concentration between 10µg/µl and 20µg/µl for the experimental samples. This concentration range would be ideal and sufficient to see the expected results in a neuron positive control, and it would give an adequate and measurable range of potential protein expression to each experimental condition.

In addition, I recommend that a p-ERK1/2 primary antibody dilution curve should be run on samples using this new recommended sample concentration range of 10µg/µl to 20µg/µl. Running a dilution curve using the new sample concentrations will help to find the ideal concentration of p-ERK1/2 primary antibody that should be used in future experiments. Having an antibody concentration high enough to detect the presence of the desired protein, yet not too high so as to saturate the image, is important because saturating the image will convolute the analysis conducted with ImageJ. The artificially high pixel integrated density values produced in ImageJ will make it seem that there are higher concentrations of protein present than there truly are. Using the proper concentration will ensure that the images are not saturated and that the pixel integrated

density values produced more accurately reflect the actual p-ERK1/2 protein concentrations in each sample.

NMDAR metabotropic activation leading to ERK1/2 phosphorylation was not shown at significant levels in these experiments. However, there are enough issues with the experimental protocol that these results may be considered inconclusive. These results cannot be reliably used for any definitive conclusions, but they are useful in troubleshooting and in refining the procedures. The understanding that these experiments have brought can be used to create new experiments with altered protocols that will produce results that can be reliably assessed. These new results may then be used to address the hypothesis.

Creating a reproducible positive control with anticipated results that are consistent with previous knowledge is important to establish before moving forward with experiments. This was not done adequately for these experiments. The neuron positive controls seen in Figures 4 and 5 show favorable patterns of ERK1/2 expression, but they do not show the differential levels of ERK1/2 expression that are consistent with previous knowledge. The minimum two-fold increase in phosphorylated ERK1/2 between non-stimulated and NMDA-stimulated conditions, as was seen by Schwarzchild et al. in 1999, is not observed in this positive control. This baseline needs to be better established in future experiments. The experimental protocol needs to be troubleshot and altered sufficiently to create these expected results before any experimental conditions can be rerun. Without these necessary alterations, any results seen in future experimental conditions cannot be trusted since they do not have an adequate positive control to be compared against.

For the HEK293 experiments, I would suggest including experimental conditions that are conducted in normal ECS. This would create an ionotropic condition that the metabotropic conditions can be compared against, looking for that 0.5-fold increase in phosphorylated ERK1/2 protein that was previously observed in Dr. Wan's lab.

Even if further experimentation does yield reliable results that conclusively refute the hypothesis, this would not suggest that the NDMAR could not be metabotropically activated. It is still true that metabotropic activation of the NDMAR could lead to the activation of another intracellular signaling cascade that does not include the phosphorylation of ERK1/2. Future Western blot studies could investigate this idea, measuring the phosphorylation of other proteins in NMDAR intracellular signaling cascades under metabotropic conditions. For example, Nabavi et al. (2013) found metabotropic activation of the NMDA receptor by measuring phosphorylated p38 MAP kinase against total p38 using the Western blot. They concluded that ligand binding to the NMDA receptor under basal calcium conditions was sufficient to induce LTD, and that ion flux through the ion pore was not necessary.

Additionally, experiments involving other techniques measuring things that the Western blot cannot measure could show that NMDAR metabotropic activation is occurring. For example, immunohistochemistry staining of AMPA receptors could show visual changes in AMPAR expression levels under different NMDAR activation conditions. Also, patch clamp experiments measuring local changes in the cell membrane potential at the postsynaptic membrane could be used to demonstrate that NMDAR activity is occurring in metabotropic conditions. For example, Nabavi et al. (2013) were able to show NMDAR metabotropic activity and LTD after brief high-frequency

stimulation using the patch clamp technique. Similarly, Kessels et al. (2013) used the patch clamp technique to take field potential recordings of neurons, measuring AMPAR currents and inferring changes in LTD as a result of metabotropic NMDAR activity.

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