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College of Liberal Arts

Allosteric Modulation of mGlu4 Metabotropic Glutamate Receptors is Protective Against NMDA Induced Toxicity in Primary Neuronal Cell Culture

A Thesis in Neuroscience

By

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Submitted in Partial Fulfillment
Of the Requirements
For the Degree of
Bachelor of Arts
With Specialized Honors in Neuroscience

April 2017

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Abstract

For many of the key mental processes that occur in the brain daily, the brain relies on the regulation of glutamate, the primary excitatory neurotransmitter. As the primary excitatory neurotransmitter in the brain, about 50% of cortical synapses are dedicated to glutamate signaling (Danysz and Parsons 2012). Due to its high prevalence, dysregulation of the glutamate system can contribute to the development of several neurodegenerative diseases, including Alzheimer's Disease. While glutamate blockers are prescribed as potential treatment for Alzheimer's Disease, this treatment has had only minimal impact on patient cognition and no impact on patient progression. One area of research focuses on allosteric modulation of metabotropic glutamate receptors, specifically mGlu4, where prior research establishes activation of this receptor to be protective *in vitro* (Bruno et al., 2000). This research uses two compounds, RD82 and RD87, both of which are allosteric modulators of mGlu4, synthesized by Dr. Ronald Doll's group at Drew University, to assess their protective abilities against induced toxicity in primary neuronal cell cultures. In this research, we used MTS assay, immunocytochemistry, and intracellular calcium assays to assess the protective abilities of RD82 and RD87 in cultures exposed to glutamate and NMDA induced toxicity. Using the combined results of MTS assay and immunocytochemistry, we found that RD82 is protective against induced toxicity, while a pilot study of RD87 did not demonstrate protective abilities. Following confirmation of activity, RD82 was used for a pilot study to address the hypothesis that RD82's mechanism of action could involve mediating calcium levels.

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1. Introduction

1.1 The Importance of Glutamate

For the occurrence of many key mental processes, the brain relies on the careful regulation of several neurotransmitters, including the primary excitatory neurotransmitter glutamate. Precise release of specific neurotransmitters at specific specialized sites on neurons called synapses allows the brain to perceive and respond to stimuli, as well as heavily involved in processes essential to learning and memory. For example, regulating the amount of glutamate in Aplysia leads that organism to respond either in a habituate or sensitized fashion to being poked (Kandel et al.). With about 50% of the synapses of the cortex being dedicated to glutamate signaling (Purves et al., 2012), dysregulation of the glutamate system and subsequent changes in the resting levels of glutamate in the brain can produce a wide range of consequences, with symptoms being simply subtle changes in mood but ranging to completely forgetting one's own identity. With this in mind, it is not surprising that a neurotransmitter that is involved so intimately in the functioning of the brain, that its dysregulation is involved in several neurodegenerative diseases and conditions. For a diverse group of diseases of the brain, both acute and chronic dysregulation of the glutamate system are found to contribute to the disease state. These diseases include acute diseases of the central nervous system, such as cerebral ischemia, traumatic brain injuries, and status epilepticus, as well as chronic neurodegenerative diseases, such as amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease (Lewerenz and Maher 2015). Although these diseases are extremely diverse in nature, dysregulation of the glutamate system including over activity is suspected to be involved in the pathology and progression of many of these diseases. This has produced a large interest in the

research of the glutamate system in attempts to better comprehend the nature of these diseases, as well as create therapies and treatments more successfully.

1.2 Glutamate in the Healthy Brain

Within the central nervous system, there are several vital neurotransmitters, each with responsibilities crucial to the very nature of central nervous system. These neurotransmitters are classified based on the types of effect that they typically produce, such as excitation or inhibition of neurons. Without the function of both excitatory and inhibitory neurotransmitters, neuronal signaling would cease to continue successfully. Of the excitatory neurotransmitters, glutamate plays the largest role in neuronal excitation and central nervous system functioning, as it is active at about 70% of excitatory synapses within the central nervous system are glutamatergic (Danysz and Parsons 2012), and these synapses appear very widespread within the brain. As glutamate is a nonessential amino acid that is unable to cross the blood-brain barrier, it is synthesized locally within neurons, most commonly from the precursor glutamine (Purves et al., 2012). Presynaptic synthesis proceeds vesicular packaging of glutamate by glutamate transporters, prior to glutamate release. Upon release into the synapse between cells, glutamate will bind its receptors on the postsynaptic cell, typically producing an excitatory response. Although glutamate is known predominantly for its role in cellular excitation, the specific effect produced by glutamate released is mediated by the receptor subtypes present on the postsynaptic cell, which are classified into two main classes, ionotropic and metabotropic.

1.2.1 Ionotropic Glutamate Receptors

Ionotropic glutamate receptors are ligand-gated ion channels that mediate synaptic transmission upon glutamate release (Mayer 2005). As ligand-gated ion channels, these receptors function by converting the electrical signal of an action potential to the chemical

release of neurotransmitter (Yashiro and Philpot 2008). These receptors are further classified into two broad categories of receptors, which are NDMA receptors and non-NMDA receptors, all of which are located postsynaptically within neurons. Differences between these two broad categories are established by differences in the structural components of the ion channels, specifically in the assembly of receptor subunits present, and by the functional components, such as functional activation of the receptors. The non-NMDA glutamate receptors, which are further broken down into two categories, AMPA and Kainate receptors, function similarly to other ligand-gated ion channels seen in the central nervous system (Kandel et al., 2012). Upon binding of glutamate, these channels open, allowing influx and efflux of sodium (Na+) and potassium (K+), which drives the depolarization of the cell, ultimately resulting in neuronal excitation. The pore size of the entry to these channels is relatively small, preventing larger cations, such as calcium (Ca+2) from entering the cell. In contrast, NMDA receptors, which contain 2 GluN1 subunits and 2 GluN2A-D subunits, have a much larger pore size, allowing larger cations such as Ca+2 to enter the cell, creating a more drastic depolarization of the cell. Unlike non-NMDA receptors, NMDA receptors have a magnesium (Mg+2) block within the inner pore, which prevents ion flow necessary for depolarization from occurring. The affinity of the Mg+2 block within the pore is determined by the charge of the cell. While the cell is at rest, Mg+2 remains within the pore, preventing ion flow. As stimulation occurs, and the cell becomes less negative, magnesium is expelled from the cell, and the flow of ions through the channel can occur. In addition to glutamate binding and Mg+2 expulsion, the activation of these receptors requires the extracellular binding of glycine. For these channels, there is a large component of timing, where simultaneous occurrence of glutamate binding, glycine binding, and cell depolarization is significant enough to expel Mg+2, and allow rush of Ca+2 into the cell.

As ligand-gated ion channels, all ionotropic glutamate receptors are relatively fast acting, allowing for quick cellular excitation (Mayer 2005). That being said, due to the timing component of the NMDA receptors, non-NMDA receptors are considerably faster acting, and are deemed responsible for the majority of the more quickly occurring neuronal transmission (Zhou and Danbolt 2014). In contrast, NMDA receptors are more typically activated during neuronal processes that require synaptic plasticity, and are known to be heavily involved in long-term potentiation (LTP) and long-term depression (LTD) (Yashiro and Philpot 2008). These processes, which are vital to synaptic plasticity, are Ca+2 dependent processes that occur only by the activation of NMDA glutamate receptors, allowing Ca+2 to act as a second messenger and make changes as the level of the synapse. During the process of long-term potentiation, Ca+2 activity mediates several vital cellular processes that allow for synaptic modifications, such as changes in rates of translation, vesicular transport, receptor location and quantity, upregulation of neurotransmitter production, and posttranslational modifications that increase ionic conductance (Kandel). While long-term potentiation is not produced solely by changes in these processes, the combination of any of these modifications results in stronger synapses. These modifications only occur while intracellular Ca+2 levels, which are are kept at relatively low levels in a resting cell, are physically increased, so repetition needs to occur for these changes to become more permanent.

1.2.2 Metabotropic Glutamate Receptors

In contrast to ionotropic glutamate receptors which are fast acting ligand-gated ion channels, metabotropic glutamate receptors function drastically differently. Metabotropic glutamate receptors (mGluRs), are composed of eight subtypes, mGluR1- mGluR8, which are categorized into three subgroups (I-III), based on shared characteristics, such as amino acid

sequences, pharmacological profiles, and intracellular signal transduction pathway coupling (Bruno et al., 2000). Activation of these receptors modulates postsynaptic ion channel activity indirectly, often by inhibition of postsynaptic Na+ and Ca+2 channels (Purves et al., 2012). These postsynaptic effects occur slowly compared to ionotropic glutamate receptors, and can lead to either the excitation or inhibition of postsynaptic cells. Although specifics regarding mGluR structure are limited, it is known that these receptors contain similar binding-domains to those seen in ionotropic glutamate receptors, and contain similar structures to other metabotropic receptors, as they are composed of seven helical transmembrane spanning domains. These seven transmembrane spanning domains connect the extracellular binding-domain to the intracellular component of the receptor, which activate various g-proteins, leading to the activation of intracellular cascades. As activators of g-proteins, these receptors produce much slower but longer lasting cellular responses, that can produce changes in gene transcription, activation of phosphatases and kinases, and numerous other intracellular responses. Group 1 mGluRs, which include mGluR1 and mGluR5, are coupled to phospholipase C through G_q and G_{11} proteins, while Group 2 mGluRs, including mGluR2 and mGluR3, and group 3 mGluRs, including mGluR4 and mGluRs 6-8, are coupled to inhibit adenylyl cyclase by G_i and G_o proteins (Revett et al., 2013). Evidence supports that for mGluRs coupled to both phospholipase C and adenylyl cyclase that almost every step involved in the signaling pathways is modulated by Ca+2 involvement. Intracellular and extracellular Ca+2 involvement both can mediate mGluR activity, as depletion of extracellular Ca+2 has proven to inhibit the postsynaptic effects of mGluRs. Distribution of mGluRs throughout the brain varies by subtype, with mGluRs 1, 3, 5, and 7 found distributed throughout the brain, and mGluRs 2 and 4 localized to specific regions of the brain. The specific cellular location also varies by subtype, with Group 1 mGluRs found

primarily in the somatodendritic domains of neurons, Group 2 mGluRs found in the somatodendritic compartments and axonal domains of neurons, and Group 3 mGluRs located primarily at the active zones of presynaptic axon terminals.

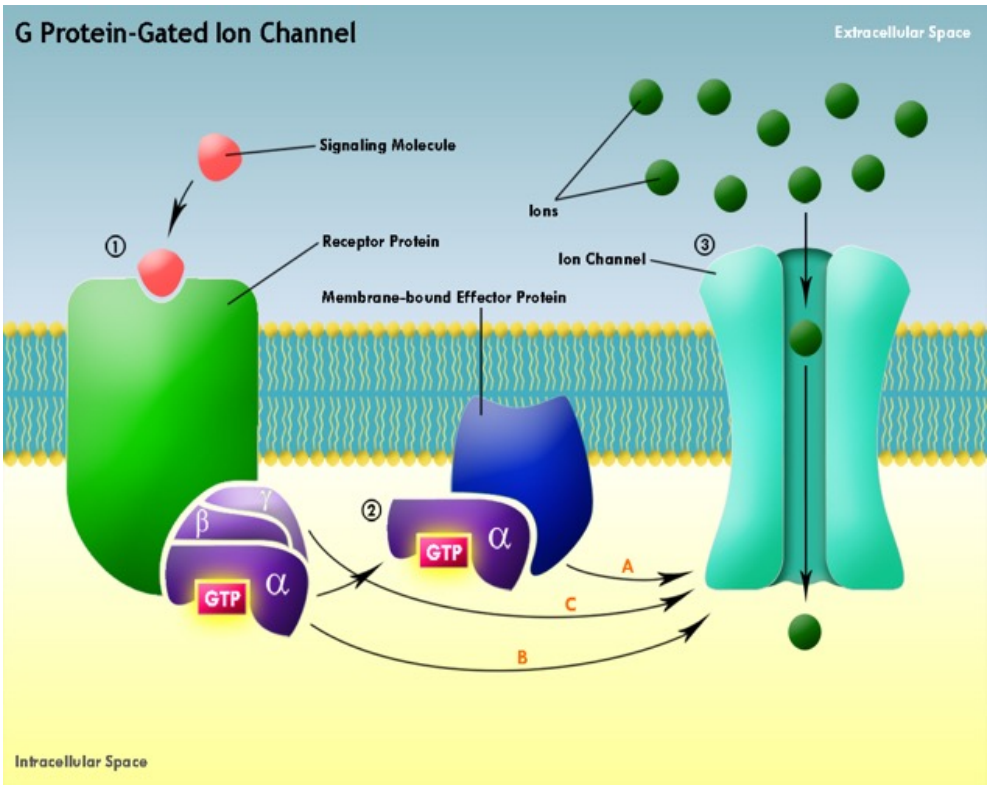


Figure 1. Metabotropic and Ionotropic Receptors

(https://en.wikipedia.org/wiki/G_proteingated_ion_channel)

1.3 Glutamate Toxicity

Although it is well understood that glutamate is vital to neuronal signaling and central nervous system functioning, there is evidence that dysregulation or over stimulation of the glutamate system has an extremely detrimental effect on neurons. In a healthy functioning brain, glutamate clearance is tightly regulated, to limit the intensity and duration of its activity upon release, by both glutamate transporters and glutamine synthetase (Danysz and Parsons 2012). While a cell is undergoing normal resting conditions, glutamate concentration levels are typically

in the low micromolar range. At the peak of synaptic transmission glutamate concentration levels can transiently reach upwards of low millimolar, but this however lasts for merely a few milliseconds before returning back to the considerably lower resting levels of glutamate. The extent of extracellular glutamate levels is tightly regulated by glutamate transporters, which are responsible for the re-uptake and clearance of extracellular glutamate levels. Excess glutamate taken back into the cell is metabolized by glutamine synthetase back to glutamine, the main precursor of glutamate production, which functions as a transmitter recycling mechanism. Glutamate transporters, which are highly expressed in glial cells, are in part responsible for glutamate clearance, which limits the extracellular presence of glutamate, and subsequently mediates the activity of the glutamate system (Zhou and Danbolt 2014). When this function is compromised, excess glutamate levels remain in the extracellular fluid of the brain, producing unfavorable concentrations of glutamate to neuronal survival. Unfavorably high resting glutamate levels in the brain increases the excitability of the neurons to the extent that they essentially excite themselves to death in a process known as excitotoxicity. More specifically, glutamate excitotoxicity is the result of mild but chronic activation of NMDA receptors, ultimately compromising cell viability, resulting in neurodegeneration (Danysz and Parsons 2012). The term excitotoxicity itself was first coined in 1986, describing the ability of glutamate and other structurally similar amino acids to kills nerve cells (Lewerenz and Maher 2015). Evidence from several scientific studies support the hypothesis that this process underlies the cell death seen in several acute and chronic diseases of the central nervous system.

Excitoxicity is the result of irregular, often excessive activation of NMDA ionotropic glutamate receptors (iGluRs) which typically results in the loss of cell bodies and dendrites, both of which are postsynaptic structures. Chronic activation is in a way self activating, where excess

levels of extrasynpatic glutamate influences the excitability of NMDA iGluRs. These excess levels keep the cells at a resting potential that is much closer to the threshold for firing an action potential, so that even the slightest stimulation is sufficient to remove the internal Mg+2 block, allowing for a substantial influx of Ca+2 (Danysz and Parsons 2012). Increased sensitivity results, keeping cells irregularly active, producing over activity within the cells. Over activity produces an abnormal Ca+2 influx, and ultimately produces irregularities in synaptic function, toxicity at the level of the synapse, and ultimately cell death due to the inability to perform normal cellular transmission. In the next sections, several neurological diseases will be reviewed in which evidence of glutamate toxicity plays a role in either the initiation of the progression of the diseases.

1.3.1 Traumatic Brain Injury (TBI)

Traumatic brain injury (TBI), which can be the result of external physical insults or internal disruptions such as strokes, can produce both short-term and long-term chronic disabilities that when severe can even shorten one's life (Bramlett and Dietrich 2015). Some of the well-known side effects that can be experienced due to TBI are seizures, sleep disorders, and psychiatric issues. In addition to these well-known side effects, another severe consequence of TBI is an increased risk of developing a neurodegenerative disease following the injury. Of interest specifically for this research is that even mild TBIs can produce progressive neurodegeneration in the brain resulting in sever atrophy, as well as increase the likelihood of developing neurodegenerative diseases. To understand the extent of atrophy that occurs with TBI, studies have been conducted using staining techniques to compare the brain atrophy that occurs in control animals to brain atrophy that occurs in TBI animals. These techniques have shown significant atrophy in TBI animals, in addition to expansion of ventricles, compared to the

control animals, indicating that there is a mechanism for neurodegeneration occurring (Bramlett and Dietrich 2015). Other studies have specifically addressed the involvement of the glutamate system, one of which induces TBI in male mice using controlled cortical impact (CCI) (Cantu et al., 2015). In this study, using FRET-based glutamate biosensors to map glutamate signaling, it was found that glutamate signaling was increased following CCI induced injury. In addition, GABAergic interneuron activity decreased, suggesting that both glutamate and GABA activity are impacted in TBI. For this reason, excitotoxicity of the glutamate system is a targeted area of treatment following TBI, as studies have indicated that neurons and oligodendrocytes are more vulnerable to glutamate receptor activation.

1.3.2 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressive neurodegenerative disease characterized by the loss of motor neurons in the spinal cord and brainstem. ALS is broken down into two subtypes, familial and sporadic. Familial cases, which make up about 10% of diagnosed cases are the result of a rare gene mutations on genes including SOD1, C9ORF72 and TDP-43 (Bonifacino et al., 2016). In contrast, the cause of sporadic cases is unknown. Currently, the disease is fatal, as there is no known cure for either the familial or sporadic cases. However, the glutamate system is believed to be involved in the loss of motor neurons, with several glutamate processes being of suspect. In contrast to some of the other neurodegenerative diseases of which the glutamate system is suspected to be involved, ALS is thought to primarily involve AMPA iGluRs, rather than NMDA iGluRs, as AMPA iGluRs are highly expressed in motor neurons (Lewerenz and Maher 2015). A study conducted using spinal cord slices from SOD1 transgenic mice reported synergism between the SOD1 mutation and cell loss by AMPA receptors (Hong et al., 2012). For this experiment, cultures were stimulated with a glutamate uptake inhibitor to

compare cell death in the ventral horn. Wild type cultures stimulated with the inhibitor demonstrated very minute cell death, while SOD1 cultures that were not stimulated with the inhibitor showed more significant cell death than the wild type that were stimulated. In addition, SOD1 cultures that were stimulated with the inhibitor showed significant cell death when stimulated with the inhibitor, resulting in survival of only about 20% of cells in the ventral horn, compared to the wild type, indicating that the SOD1 mutation is synergistic to cell death by glutamate induced toxicity. Due to the suspected involvement of the glutamate system, glutamate blockers are one of the diagnosed therapies used to attempt to slow down disease progression and reduce discomfort (ALS Association 2016).

1.3.3 Alzheimer's Disease

Alzheimer's Disease, which is one of the leading causes of death among older adults in the United States, is a disease that is characterized by chronic and irreversible neurodegeneration (Danysz and Parsons 2012). While Alzheimer's Disease is amongst many diseases of the brain characterized by neuronal loss, and shares some of its pathologies with other neurodegenerative diseases, Alzheimer's Disease has its own complex combination of pathologies that make the disease unique. The disease is characterized by multiple stages during which cognitive and functional abilities decline. The first is known as the pre-clinical or pre-dementia stage, where symptoms are so minor that they are often considered to just be a result of aging or stress, such as minor issues with executive functioning and semantic memory (Dominguez et al., 2011). This stage is often relatively long. For some cases, it can take upwards of eight years before a patient meets the clinical criteria for being recognized as having Alzheimer's Disease. Following the pre-clinical stage of Alzheimer's Disease is the stage referred to as mild cognitive impairment, where an individual will demonstrate clinically diagnosable cognitive impairment, however this

impairment is not to the extent of that is seen with full blown dementia. Finally, full blown dementia is diagnosed, and in many cases Alzheimer's Disease is the assumed diagnosis, however it cannot be definitively diagnosed without a post-mortem autopsy. For a definitive diagnosis, an autopsy would need to indicate the presence of extracellular amyloid-beta plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and the accompanied neurodegeneration (Mastunaga et al., 2015). These specific physical markers accompanied by cognitive decline severe enough to produce dementia distinguish the disease apart from other dementias and neurodegenerative diseases.

While it is well understood that there are specific hallmarks of the disease that distinguish it from other neurodegenerative diseases and dementias, the order at which these biological hallmarks are occurring and the causes for them is unknown. There are known genetic predispositions to the disease, with different gene alleles or mutations producing a definitive diagnosis of early onset Alzheimer's, or increasing the likelihood of diagnosis with Alzheimer's later on in life compared to others. Different longitudinal studies have been conducted in attempts to identify the order that which these specific hallmarks or biomarkers occur during the disease progression (Bertens et al., 2015). A longitudinal study conducted using participants chosen using the Alzheimer's Disease Neuroimaging Initiative were broken into groups based their state of cognitive abilities, including cognitively normal, mildly cognitively impaired, and demented, and were grouped based on their based amyloid beta 1-42 (AB1-42) levels. During the duration of the study the biomarkers known to be involved in the disease were measured, including cerebrospinal fluid levels of AB1-42 and tau, as well as cognitive assessment. During the study, the results indicated that the AB pathology occurs prior to the tau pathology, with tau levels changing more dramatically later on in the study. While this evidence would suggest that

the disease progression does occur in an orderly fashion, there is still not concrete evidence that one hallmark occurs first or is the cause of another. However, it is well understood that during the disease progression there is substantial neuronal loss, suggesting the importance of developing therapies that prevent cell death. A primary area of drug development for Alzheimer's disease, as well as other neurodegenerative diseases has focused on limiting the extent of neuronal loss occurring during the disease progression, with a category of the drug development focusing on the glutamate system.

As mentioned previously, glutamate is the major excitatory neurotransmitter in the central nervous system, and is essential to several vital neuronal processes that occur constantly, including synaptic transmission, synaptic plasticity essential to learning and memory, and neuronal differentiation. In Alzheimer's Disease, the areas of the brain containing a high density of glutamatergic neurons are also the areas of the brain where early damage occurs, including layers of the cortex and hippocampal neurons (Revett et al., 2013). Because areas of the brain that contain a high density of glutamatergic neurons are influenced during the disease progression, various stages of the glutamate cycle are interrupted or altered, subsequently resulting in increased resting concentrations of glutamate in the brain. Experimental evidence suggests that the brain's ability to quickly remove excess glutamate release is inhibited in AD brains by a decrease in glutamate transporters expressed in glia cells (Zumkehr et al., 2015). The consequence of excess glutamate in the AD brain may be even more troubling than in most conditions because there is evidence that AB1-42 oligomers can directly increase Ca+2 current through ionotropic glutamate receptors and therefore increase the risk of excitotoxicity (Alberdi et al., 2010).

One of the most common clinical symptoms of Alzheimer's Disease is the progressive dementia and memory loss. The proper functioning of the glutamate system is essential to the formation of new memories through a process known as long-term potentiation (LTP) (Revett et al., 2013). This process, which occurs with high-frequency stimulation and the increased release of glutamate, is dependent on the functioning of both iGluRs and mGluRs. AMPA receptors are activated during the initial immediate glutamate release, while NMDA receptor activation follows continuous and synchronous stimulation, subsequently permitting release of Ca+2 onto the postsynaptic cells, further triggering internal cascades that increase protein transcription, strengthening synapses. The opposite of this process is known as long-term depression, which can be caused by down-regulating the initial glutamate receptors activated in LTP, preventing the activation of NMDA receptors. It has been shown that these processes are influenced in Alzheimer's Disease, with the occurrence of LTP decreasing and the occurrence of LTD increasing (Revett et al., 2013). One of the reasons that these processes are suspected to be influenced during Alzheimer's Disease is due to interactions between amyloid beta and the glutamate system. Many studies have been conducted to assess the relationship between amyloid beta and the glutamate system and it can further be argued that amyloid beta can impact glutamate receptors (Alberdi et al., 2010) and glutamate transport (Zumkehr et al., 2015). For example, studies attempting to identify a mechanism by which amyloid beta influences the glutamate system have focused on spine density in regions of the brain associated with LTP (Knafo et al. 2009), while other studies have used methods such as the patch clamp in CA1 neurons of the hippocampus to show that amyloid beta induced long-term depression is dependent upon glutamate (Hsieh et al., 2006).

With prior evidence suggesting that amyloid beta can modulate the release of peptides involved in learning and memory in brain regions relevant to Alzheimer's Disease, a dual-method approach using both *in vivo* and *in vitro* model systems further demonstrated that the presence of amyloid beta inhibits the release of neuropeptides including glutamate, aspartate, and GABA (Mura et al., 2012). In this research, release of neurotransmitter was stimulated *in vivo* and *in vitro* by using nicotine to stimulate cholinergic nicotinic receptors. Using both model systems, the addition of nicotine increased the release of several transmitters, including glutamate. When amyloid beta treatment was added, the increase of transmitter due to addition of nicotine decreased significantly. These results suggest that amyloid beta modulates the release of neurotransmitter, and this evidence has been seen with glutamate.

1.4 Glutamate Blockers as a Potential Therapy for Alzheimer's Disease

With the understanding that over excitation of the glutamate system can produce severely detrimental effects in the central nervous system, mediating the activity of glutamate has grown to be highly researched in attempts to develop therapies for neurodegenerative disease. For diseases such as Alzheimer's disease, the glutamate system and excitoxicity is a commonly studied cause of the disease (Dominguez et al., 2011). The initial focus for glutamate toxicity therapies was to pharmacologically block the activity of glutamate at the synapse, therefore reducing the glutamate activity occurring, and subsequently prevent excitoxicity. This type of drug is well known as one of the only treatment options for those diagnosed with Alzheimer's disease, but also is a commonly known option for treatment of other diseases including ALS (ALS Association 2016).

Memantine, which is a derivative of amantadine, an anti-viral agent traditionally used to treat Parkinson's disease, is more recently used to treat Alzheimer's Disease (Dominguez et al., 2011), and further is established as the only glutamate receptor ligand that is approved by the Federal Drug Administration to treat Alzheimer's (Johnson et al., 2015). Memantine was first created to counteract high blood sugar levels but did not do so successfully. Instead, it was discovered that the drug acted on the central nervous system, suggesting the potential of its use to treat neurodegenerative disorders. Initially it was believed that the mechanism of action for the drug was by both direct and indirect dopamine, serotonin, and noradrenaline activity. However, it was later discovered that the drug instead was an antagonist of NMDA glutamate receptors (NMDARs), functioning to block NMDA induced currents in the central nervous system. Recent research suggests that memantine mainly inhibits extrasynaptic NMDARs, in contrast to acting on synaptic NMDARs (Johnson et al., 2105). However, the ability of memantine to inhibit synaptic NMDARs can increase with increased stimulation of synaptic NMDARs. Stimulation of synaptic NMDARs is thought to activate cell survival pathways while stimulation of extrasynaptic NMDARs is thought to activate cell death pathways. However, there is not clear agreement on these specific hypothesized roles.

1.4.1 Searching for Evidence for Memantine

Memantine has shown to protect against the toxic effects of amyloid-beta (AB) as well as decrease the extent of cognitive impairment in transgenic mice with high levels of AB, however the direct mechanism of action is not well understood (Alley et al., 2015). With this understanding, George Alley and colleagues conducted a series of experiments to attempt to identify a mechanism by which memantine prevents AB toxicity as well as improves cognitive function in animals, and by doing so provided significant evidence that memantine does in fact

treat AB related pathology in Alzheimer's experimental models. One of the models they employed utilized embryonic rat cortical neurons plated at a density of $1.5x10^6$ cells that were grown in culture for 7 days prior to memantine treatment. Memantine concentrations for stimulation included 4uM, 9uM, and 18uM, and stimulation occurred for 48 hours. MTS assay and calceim AM labeling indicated that memantine treatment of cortical neurons did not produce a significant change in cell viability, as compared to cells treated with a vehicle control. In addition, lactate dehydrogenase release and ethidium homodimer labeling both indicated that memantine did not seems to induce toxicity in culture either across all concentrations, as compared to the vehicle control. Following assessment of the viability of the neurons as well as the extent of memantine induced toxicity, soluble AB1-40 and AB1-42 levels were quantified from the cultures using ELISA. ELISA indicated no statistically significant difference in the soluble AB1-40 levels across all three memantine concentrations compared to the vehicle control, however there was a trend for decrease in the 18uM. In contrast, soluble AB1-42 levels statistically significantly decreased in the 4uM and 18uM memantine conditions as compared to the vehicle control ($p<0.01$). This suggests that memantine treatment increased the ratio of AB1-40 to AB1-42 in culture.

Another experimental approach used by Alley and colleagues demonstrated similar results to those seen in the previously described *in vitro* experiment (Alley et al., 2015). A double transgenic male mouse model with a double mutation to overexpress human APP and human PS-1 was used to assess the effect of oral memantine on AB burden in the brain. The transgenic mouse line was chosen because the double mutation creates a rapid accumulation of AB plaques in the cortex and hippocampus of rats around 3 months old. Once the rats reached 15 weeks old they were assigned to either the experimental (memantine) or placebo group. The

mice assigned to the experimental group were given 20 mg/kg/day of memantine orally via there drinking water for 8 days, with the daily memantine intake controlled by adjusting the concentration present in the drinking water provided based on their daily consumption of drinking water. Blood samples of memantine levels were recorded prior to perfusion of the mice and dissection of their brains. A second experimental group of mice was given 2.5 mg/kg of memantine intraperitoneally daily for 8 days, with a control group receiving saline injections, with blood memantine levels being measured and perfusion occurring following completing treatment. Several different AB measurements were conducted by ELISA including soluble AB1-40, soluble AB1-42, the ratio of soluble AB1-40/AB1-42, and insoluble AB1-42. No statistically significant changes in the soluble AB1-40 levels were measured in the intraperitoneal or oral memantine treatment groups compared to the control. In contrast, statistically significantly lower soluble AB1-42 levels were measured in the oral memantine treatment group compared to the control and injection groups. In addition, in the oral memantine group the ratio of soluble AB1-40/ AB1-42 increased. Finally, no differences were seen in the insoluble AB1-42 levels across all groups, which may have been due to the short duration of the experiment. Together, the results from both the *in vitro* and *in vivo* studies conducted by Alley and colleagues support that there is an interaction between AB1-42 levels and memantine, indicating memantine as a potential treatment for humans.

While *in vivo* and *in vitro* evidence both suggest that memantine is a viable option for the treatment of Alzheimer's Disease, clinical evidence suggests otherwise. A meta-analysis assessing clinical trial evidence of efficacy of memantine on individuals with mild Alzheimer's Disease conducted by Lon Schneider and colleagues in 2011 demonstrated that there is a lack of evidence supporting the use of memantine as treatment for this group of individuals (Schneider

et al., 2011). Within this study it was reported that in 2006, almost 20% of the United States population of individuals diagnosed with mild Alzheimer's disease were being treated with this medication, and that almost 40% of United States neurologists claimed to prescribe memantine for patients with MCI. While these statistics are relatively older, this drug is still prescribed today, either independently from or combined with cholinesterase inhibitors for mild AD or MCI. However, the meta-analysis conducted including clinical trials comparing the effects of memantine to a placebo group on patients with mild AD from manufacturer sponsored meta-analyses, registries, presentations, and publications were confirmed minimal efficacy of the drug in treating those with mild AD. With a total of 12 trials used, no evidence was found supporting the efficacy of memantine on treating patients with mild AD in any of the trials independently or when statistically combining the data from the trials. As evidence supports that the drug does not successfully improve those with mild AD or MCI, which is the point in the disease progression that the disease would most likely be diagnosed and treatment would be encouraged, it is evident that another treatment option is necessary.

1.5 Allosteric Modulation of mGluRs

As glutamate excitotoxicity is believed to underlie several neurodegenerative diseases, development of drugs to prevent or treat the damage caused by irregular glutamate activity is of great interest. For the glutamate system specifically, drug development has shifted to focus on targeting the mGluRs, which are g-protein coupled receptors. In the past, drug development has faced the challenge of producing highly selective compounds for g-protein coupled receptors in general (GPCRs) (Sheffler et al., 2013). Traditionally, drug developments for GPCRs have targeted the orthosteric-binding site on the receptor, which is the binding site for the natural endogenous ligand of the receptor. Upon binding to the orthosteric site, the drug will then act to

either mimic the natural ligand of the receptor, or compete with the natural ligand by blocking it from binding. Because selective activation of GPCRs at the orthosteric site is a challenge to drug development, a different approach has become increasingly popular, which involves activation of an alternate binding site, known as an allosteric binding site, on the receptor instead. Positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) are being developed to either enhance or inhibit the natural activity of a GPCR, by binding to an allosteric location, which is distinctly different from the natural ligand binding-site. Drug development for modulation of GCPRS has shifted from orthosteric to allosteric as there are several known advantages or favorable characteristic of allosteric modulators compared to orthosteric modulators (Williams et al., 2009). The first is that many allosteric modulators are only active when the natural ligand is also present, producing more controlled activity of the drug. Another advantage is that allosteric modulation produces the potential for more selectivity of action of the drugs due to the availability of sequence divergence of allosteric binding sites.

1.5.1 Group III mGluRs

While glutamate is the major excitatory neurotransmitter in the brain, the glutamate system is able to mediate its own action through the modulatory mechanisms of the mGluRs, which are expressed throughout the CNS in its entirety, as mentioned previously. These receptors are found both presynaptically and postsynaptically, and are vital to several different CNS processes, based on the specific subtype. While Group I mGluRs exhibit predominantly excitatory downstream effects, Groups II and III exhibit predominantly inhibitory downstream affects, as they are negatively coupled to adenylyl cyclase.

Group III mGluRs, with the exception of mGluR6 which is expressed primarily postsynpatically on ON-center bipolar cells in the retina, are located primarily presynpatically on

neurons, and are widespread throughout the central nervous system (Sheffler et al., 2013). Excluding mGluR6, the Group III mGluRs are believed to mediate glutamate activity, specifically by inhibiting glutamatergic synaptic potentials by inhibiting glutamate release and voltage-gated Ca+2 entry (Bruno et al., 2000). Thus far, Group III mGluRs are the least investigated group of mGluRs, due to challenges faced when attempting to develop compounds selective for each receptor subtype within the group (Sheffler et al., 2013). With that being said, it is well known that the receptor subtypes within this class are extremely diverse. For example, mGlu4, which is the receptor subtype of interest regarding the glutamate system is highly expressed in the the cerebellum, hippocampus, olfactory bulb, and peripherally in pancreatic islet cells. Studies have demonstrated that knocking mGlu4 knockout mice have impaired synaptic plasticity demonstrated by difficulty learning complex motor tasks, indicative of their high expression in the cerebellum. mGlu7 receptors are also involved in learning in memory, as mGlu7 knockout mice demonstrate learning and memory deficits, however these receptors have a much lower affinity for glutamate and are only activated by very high levels of the transmitter. In contrast to mGlu4 and mGlu7, mGlu8 is expressed widely throughout the brain, and mGlu8 knockout mice demonstrate increased weight gain and anxiety, but do not demonstrate learning and memory deficits. Finally, mGlu6 receptors are only expressed in the retina, and are also not at all linked to learning and memory at this point.

As mentioned previously, the receptor subtypes within Group III are considerably diverse, but with overlapping activity between a few of the subtypes (Sheffler et al., 2012). As Group III mGluRs have several subtypes within the class itself, research has been conducted attempting to establish which subtype within the class is protective against excitoxicity. One study conducted specifically assessed the neuroprotective abilities of mGluR4 in cell culture by

comparing mGlu4 knockout (-/-) mice in mixed cell cultures containing neurons and astrocytes to heterozygous (+/-) and wild type (+/+) mice cultures (Bruno et al., 2000). Evidence prior to this study demonstrated that agonists of Group III mGluRs including L-AP-4, L-SOP, and (R,S)-PPG, protected cortical neuronal cultures against several toxic threats in culture, including substantial exposure to amyloid beta for a prolonged period of time and irregular activity of ionotropic glutamate receptors. In order to test specifically which receptor subtype was allowing or enhancing the protective abilities of these Group III agonists, cell cultures were prepared from wild type mice, mice heterozygous for mGlu4, and mice lacking mGlu4. The mixed cultures were prepared from fetal mice, and were stimulated with NMDA about 13-14 days after plating. Prior to exposing the cultures to NMDA, the cultures with different genetic backgrounds were treated with different group III agonists. The percent NMDA induced neuronal death was measured and quantified, and results indicated that the (-/-) cultures showed statistically significantly higher cell death compared to the (+/+) and (+/-) conditions, for all three group III agonists. For all three agonists, the knockout cultures showed almost double the amount of neuronal death compared to the other two culture conditions. These results suggest that mGlu4 is the group III subtype that plays a protective role against toxic threats involving the glutamate system in cell culture, as cultures lacking mGlu4 showed significantly higher levels of cell death compared to wild type and heterozygous cultures for the receptor.

1.5.2 RD82

Initially, allosteric modulation of mGlu4 was intended to treat Parkinson's Disease, which is a neurodegenerative disease that is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (Bennouar et al., 2013). Degeneration of dopaminergic neurons produces dopamine depletion in the brain, which

ultimately disrupts the synaptic balance of excitatory and inhibitory neurotransmitters in the basal ganglia network, which then produces the motor symptoms that accompany the disease. Initial treatments for Parkinson's primarily focused on the dopamine system by administering dopamine agonists and the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA). Although treating Parkinson's Disease patients with L-DOPA has proven to successfully reduce the disease symptoms, long-term treatment of this kind often produces dyskinesia, which is the involuntary movement of muscles, often most severe with the drug is most significantly reducing Parkinson's disease symptoms. Because of the long-term negative side effects of treating the dopamine system directly, research for the disease has shifted towards treatments that instead indirectly influence the dopamine system by shifting the ratio of excitatory and inhibitory neurotransmitters in the central nervous system. One of the newer focuses in Parkinson's Disease research is the allosteric modulation of mGlu4 metabotropic glutamate receptors. Modulators of this receptor subtype were developed with the intention of acting synergistically with L-DOPA, a drug that increases the amount of dopamine in the CNS, with the intention of decreasing the amount of L-DOPA needed for treatment.

In contrast, allosteric modulators can also be used to treat neurodegenerative diseases by directly influencing the activity of the glutamate system and preventing excitotoxicity. At Drew University, Dr. Ronald Doll and his group have worked to identify lead structures that activate mGlu4, and then synthesize analogous structures to optimize drug characteristics, including potency, selectivity, and drug like properties. The initial compound synthesized by Lundbeck Research as an activator of the mGluR4 receptor (Bennoaur et al., 2013) was resynthesized by the Doll group and is the primary compound used for this research. However, 5 analogues were synthesized in collaboration with Dr. Dario Doller of Lundbeck Research, including RD83,

RD84, RD85, RD86, and RD87 (Figure 2). The data in this experiment includes use of the compounds RD82 and RD87, which differ in chemical structure, molecular weight, and polarity.

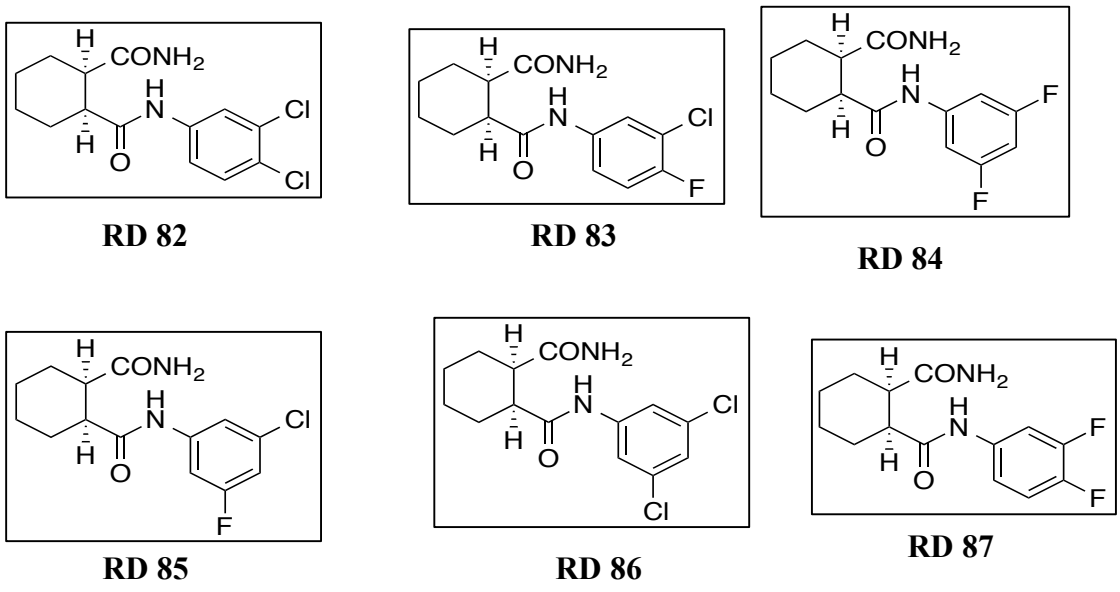


Figure 2. Chemical Structures of RD Compounds

1.6 Experiments and Hypotheses

This experiment aims to further explore allosteric modulation of mGlu4 as a potential protective mechanism against NMDA and glutamate induced toxicity using primary neuronal cell cultures from embryonic rats. The first hypothesis explored in this research was that NMDA and glutamate could be used to induce excitoxicity and subsequent neurodegeneration in a primary neuronal cell culture model system. During this process, various concentrations of glutamate and NMDA were tested to find the optimal concentration to induce excitotoxicity in culture without entirely obliterating the cells. To test this hypothesis, for the majority of these

experiments, NMDA was used at concentrations between 50uM and 100uM, as this range successfully induced neurodegenerative without completely destroying the cultures. To discover the optimal concentration of NMDA to use to induce excitotoxicity, immunocytochemistry and MTS assays were used to quantify the extent of cell death occurring during stimulation. The second hypothesis being tested was that RD82 and RD87 would have concentrations at which they could be applied to cultures without producing neuronal toxicity. The third hypothesis that we tested was that RD82 and RD87would function as PAMs activating mGlu4 and would protect against the neurodegeneration following NMDA and glutamate induced excitotoxicity. To test this hypothesis, initial usage of RD82 and RD87 included assessment of the general toxicity of these compounds independently to embryonic rat cortical neurons by applying varying concentrations of these compounds to the cells, and comparing the toxicity of each. The fourth hypothesis that we explored was that differences in these compounds, including elements such as structure and polarity, would lead RD82 and RD87 to produce different levels of protection against induced toxicity. We hypothesized that these differences in the compounds could potentially impact the compounds' ability to modulate mGlu4, impacting the protective abilities of RD82 and RD87 against neurodegeneration. If either RD82 or RD877 demonstrated protection from glutamate or NMDA induced toxicity, a pilot study would be conducted to examine whether intracellular calcium levels were being altered due to mGlu4 modulation. Finally, we hypothesized that we would be able to detect differences in intracellular calcium levels due to exposure to stressors and RD82.

2. Materials and Methods

2.1 Preparation and Care of Primary Neuronal Cell Cultures

Primary neuronal cell culture preparation began with sacrifice of a pregnant rat at approximately E18. Following sacrificed, which was performed using carbon dioxide, the pregnant rat was dissected for removal of the embryos. Embryos were decapitated to dissect the brain and obtain both hemispheres for culture. During dissection the embryonic head was placed in Hank's Balanced Salt Solution (HBSS) obtained from Gibco by Life Technologies, for tissue preservation. The skull, midbrain, cerebellum, and meninges were all removed and discarded, maintaining only the left and right hemispheres. The hemispheres were separated, dissociated, pipetted into 3 mL of trypsin in a 15 mL conical tube, and placed in the warm water bath for five minutes. Following trypsin, the cells were washed in HBSS twice for three minutes. The cells were pipetted into a 3 mL conical tube of plating media containing NeurobasalQ Medium, Fetal Bovine Serum (FBS), and Primocin antibiotics, and were dissociated entirely using a glass pipette flamed for sterilization. The cells were counted to calculate the appropriate volume of plating media and volume of cells needed to plate $1x10^5$ cells/mL. To plate $1x10^6$ cells/well in culture, 100 uL of the prepared cell solution was pipetted per well, and cultures were placed into the incubator at 37°C for 1-4 hours for initial incubation. Following initial incubation, all plating media was aspirated off of the cultures and replaced with growth media containing NeurobasalQ Medium, B-27 Supplement, and Primocin. For most experiments, cultures were maintained for 12-14 days, with culture maintenance occurring every 2-3 days. Maintenance included aspirating off half of the growth media from each well to exchange for fresh media in order to prevent contamination and maintain nourishment of the cells.

2.1.1 Plate Preparation

Plates for culture were prepared prior to dissection. In a sterile culture room, Poly-L-Lycine Hydrobromide obtained from Sigma was applied to each well of the plates to sit for at

least 12 hours. All Poly-L-Lycine was collected was reuse. The plates were washed with HBSS or autoclaved water three times, with each wash resting for 15 minutes before being aspirated off. Plating media was added and the plates were placed in the incubator. For MTS assay, the inner 60 wells of a 96 well plated were prepped for culture. For Intracellular Calcium assay, the inner 60 wells of a 96 well black plate were prepped for culture. For Immunocytochemistry, a 12 well plated was prepared for culture.

2.2 Stimulation of Cells

2.2.1 Preparation of reagents

RD82 stock was prepared using compound provided by the Doll lab. Most stock was prepared at a concentration of 30mM by dilution in Dimethyl Sulfoxide (DMSO). Initial stock preparation included measuring RD82 at about 0.0035 grams, and calculating the appropriate volume of DMSO needed to achieve 30mM, which was about 370uL. The compound was diluted in DMSO, filtered, and divided into aliquots to be maintained in the freezer for future experiments. The process was later modified to prevent degradation of the compound in DMSO. Doll's lab instead provided 0.0005g aliquots of RD82 compound which were diluted in 530uL of DMSO on the day that they were used for experimentation. Any leftover compound diluted in DMSO was discarded. RD87 stock was prepared using the same initial technique as RD82 with compound provided by the Doll lab. The compound was diluted in DMSO to create a stock at 30mM, filtered, and stored in aliquots for future use.

N-Methyl-D-aspartate (NMDA) stock was prepared by diluting NMDA compound in autoclaved water. Most stock was prepared to be 50mM in concentration by measuring about 0.008g of NMDA to dilute in about 1.07mL of autoclaved water. The solution was filtered for sterilization and stored.

Glutamate was prepared by dissolving glutamic acid (molecular weight=147.13) in 100mL of growth media. 73.35mg of glutamic acid was dissolved in about 90mL of growth media while stirring over heat. NaOH was added to assist in dissolving. Once the glutamic acid was dissolved in solution, the pH sensor was used to determine the pH, and more NaOH was added until the pH was about 7.2-7.4. The remaining 10mL of growth media was added. The glutamate solution was stored in the refrigerator until use.

2.2.2 Serial Dilutions

As stock solutions were created at concentrations significantly higher than used in experimentation, serial dilutions were used to test initial concentration curves of both RD82 and NMDA. RD82 serial dilutions were prepared by first diluting the 30mM stock to 30uM solution, as this was the highest concentration used to stimulate cultures. All other concentrations were further diluted from the 30uM solution. NMDA dilutions were done similarly by first diluting the 50mM stock to 200uM, and preparing all lower concentrations from the 200uM solution. To prepare the serial dilutions of both RD82 and NMDA, stock solutions were diluted in growth media, and then further diluted by adding more substantial volumes of growth media to create the desired concentrations.

2.2.3 Concentration Curves

Initial experimentation with RD82 included only two concentrations (1uM and 30uM) with the intention of simply testing if there was any effect of adding the compound compared to the control. Following positive results, a range of concentrations was tested, ranging from 1uM to 30uM. Other concentrations included 3uM, 5uM, 10uM, 15uM, 20uM and 25uM in order to test for the optimal concentration. Solutions were prepared as described previously by serially diluting RD82 stock in growth media.

An NMDA concentration curve was performed to identify the optimal concentration of NMDA to apply to the cells in culture to induce toxicity and neurodegeneration. Solutions were prepared as described previously by diluting NMDA stock in growth media.

2.2.4 Removal of Media and Addition of Reagents

Cells were grown in culture for about 12-14 days prior to stimulation. Upon stimulation, all media was removed from the cultures to apply the prepared solutions containing RD82 and NMDA. For control wells, all media was removed and replaced with fresh growth media and the cultures were returned to the incubator. For the most part, for the MTS assay and immunocytochemistry, reagents were left on the cultures to stimulate for about 24 hours. In addition, cultures were stimulated for only 30 minutes prior to running assays to test how quickly the compound was effective. For 30 minute cultures, the solutions were added to the cells for 30 minutes and then was exchanged for fresh growth media for about 24 hours. For the intracellular calcium assay the assay was conducted immediately after applying the reagents to the cultures.

2.3 Immunocytochemistry

Immunocytochemistry was used to assess neuron stability after stimulation. Primary and secondary antibodies were used to stain for tubulin, with lighter staining indicating less stable tubulin in the cells. Instable tubulin would indicate neuronal damage due to the induced toxicity in culture. Cultures were grown in 12-well plates. Once cultures were maturely differentiated they were stimulated with glutamate and RD82 for 24 hours. Following stimulation, all media was removed from the cells and the cultures were immediately fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. All paraformaldehyde was removed from the cells and the cells were washed with PBS three times to remove any excess paraformaldehyde. The cells were incubated with 0.5% triton-100 in PBS for ten minutes at room temperature, the triton

was removed, and the cells were washed with PBS. The primary antibody anti-acetylated tubulin was applied and the cells incubated for one hour on a shaking platform. The primary antibody was collected and the cells were washed with PBS three times to remove excess antibody. The secondary antibody was applied to incubate for one hour on a shaking platform. The antibody was collected, the cells were washed with PBS three times, and PBS was left on the cells for imaging by fluorescent microscope using the program NIS elements. Plates were imaged and the average and minimum light intensity values were collected. Minimum values were subtracted from the average values to normalize. Normalized values were averaged for each condition and imported into excel for statistical analysis.

2.4 MTS Assay

Cultures were plated in clear 96 well plates at $1x10^5$ cells per well and maintained for two weeks prior to stimulation with NMDA and RD82. After stimulation, a solution was prepared with 20uL of MTS dye per 100uL of growth media. All media was removed from the cultures and immediately replaced with the MTS dye solution. The cells were returned to the incubator for 2 hours with the dye solution. The microplate reader was used to conduct the MTS Assay using SoftMax Pro 6.2.1 software. The wavelength was set to read at 490, reading both absorbance and endpoint. Data was exported and converted to excel for statistical analysis. All experimental conditions were normalized to control conditions at 100% cell survival.

2.5 Intracellular Calcium Assay

An intracellular calcium assay was conducted to measure changes in calcium gradients following stimulation with RD82 and NMDA. Cells were grown in culture for two weeks prior to stimulation and conduction of the calcium assay. The calcium dye solution was prepared using a Molecular Probes kit by Life Technologies, which is designed to detect small chemical changes

in intracellular calcium levels using fluorescent imaging. The solution was composed of 50uL of Fluo-4 AM 1000X in DMSO, 500uL of Powerload Concentrate 100X, 5mL of Neuro Background Suppressor 10X, and 77mg of Probenecid. The solution was added to the cultures after all growth media was removed. The cultures were returned to the incubator for 30 minutes. All media containing the fluorescent solution and 100 uL of solutions containing RD82 and NMDA were then added to the cultures and the assay was immediately run to measure changes in calcium levels. Immediately after the first calcium assay, 10uL of 50mM glutamate was added per well and the calcium assay was run a second time to assess changes in calcium levels due to the added glutamate. The data was exported and converted to excel for statistical analysis.

3. Results

3.1 Glutamate and RD82's Effect on Microtubules

Primary neurons were harvested from E18 rats and grown in culture for one week prior to stimulation with 12.5mM glutamate and RD82 (Figure 3). Concentrations of RD82 included 1uM and 30uM. Staining for acetylated tubulin using immunocytochemistry was conducted as described previously, and relative light intensity averages were calculated for each condition by averaging controlled values (mean-min). Statistical analysis using a 2x3 two-way ANOVA on relative light intensity by presence of glutamate and dose of RD82 confirms statistical significance of glutamate ($F_{1,114} = 10.08$, $p = 0.002$), as average light intensity was statistically significantly lower with glutamate (M=62.36, SD=27.14), than without glutamate (M = 84.25, SD = 46.17). In addition, there was no statistically significant effect of RD82 by two-way ANOVA, indicating that RD82 was not toxic to 1 week old primary neuronal cultures. However, comparison of means indicates a trend towards an effect of RD82, as both 1uM and 30uM tRD82 treatment groups increased the average light intensity of cells stimulated with glutamate by 25%

and 23% respectively. Finally, there was no statistically significant interaction between glutamate and RD82 ($F_{2,114} = 0.32$, $p = 0.725$).

3.2 RD82's Impact on NMDA Induced Toxicity

Primary neurons were harvested from E18 rats and grown in culture for 12-14 days as described previously. Cultures were stimulated with 5 total treatment groups, including a control condition consisting of growth media, a 50uM NMDA negative control condition, and 50uM NMDA paired with 10uM, 20uM, and 30uM RD82 (Figure 4A). An MTS assay was conducted to assess cell survival following stimulation with 50uM NMDA, and differences across RD82 treatment groups. According to the results of a one-way ANOVA, there is a statistically significant difference between at least two of the treatment group sin the MTS assay, on average ($F_{4,25} = 5.69$, $p < 0.002$). In addition, according to the results of the post-hoc LSD test, the control condition and the 50uM NMDA condition were statistically significantly lower than RD82 treatment groups, on average, with no other statistically significant differences detected. These results suggested that the RD82 treatment groups significantly increased average percent cell survival above that of the control and 50uM NMDA treatment groups, indicating protective abilities of RD82 against NMDA induced toxicity.

In addition, we conducted a 30-minute exposure of cultures to 50uM NMDA and 30uM RD82 to assess the extent of toxicity that could be induced in a relatively short duration of time, as well as assess the speed of action of RD82 as a treatment (Figure 4B). An MTS assay was conducted as described. Results of a one-way ANOVA on percent cell survival by MTS by RD82 treatment after only 30 minutes, there was no statistically significant effect ($F_{2,15} = 2.96$, $p = 0.08$), although there was a trend. Post-hoc LSD testing of the trend indicated that cells

treated with RD82 had a higher average percent cell survival than control cells and cells treated with 50uM NMDA.

Culture preparation for an MTS assay was performed as described previously. Following 12-14 days of growth in culture, primary neurons were stimulated with 100uM NMDA and a concentration curve of RD82 including 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM, and 30uM (Figure 5). MTS Assays were conducted and data was exported and compiled, normalizing all treatment groups to the control condition. Prior to conduction of these assays a NMDA concentration curve was conducted and 100uM NMDA indicating 100uM NMDA to be the ideal concentration to consistently induce toxicity in culture. By comparison of means, the compiled results indicate 100uM NMDA stimulation effectively induced toxicity as the percent cell survival decreased 49% in the NMDA condition compared to the control condition. In addition, statistical analysis by one-way ANOVA confirmed statistically significant differences across groups ($F_{9,11} = 3.21$, p=0.002). Further, MTS data was transformed by subtracting the average percent value for the NMDA negative control condition from each treatment condition to compare cell survival of each treatment group to the NMDA condition in order to evaluate the percentage of cells saved by each treatment group. Transformed data indicated the 1uM, 10uM, 25uM and 30uM conditions most effectively increased cell survival from 100uM NMDA induced toxicity, with treatments increasing cell survival by 25%, 21%, 24% and 28% respectively, compared to the 100uM NMDA condition.

3.3 RD87's Impact on NMDA Induced Toxicity

A parallel MTS Assay was conducted as described previously (Figure 5), using 100uM NMDA as a stressor, as well as a concentration curve of RD87 (Figure 6). Concentrations of RD87 included 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM and 30uM. Statistical analysis by

one-way ANOVA determined a statistically significant effect of all treatment groups differing statistically significantly from the control group ($F_{9,50} = 5.11,\ p < 0.0005$). Post-hoc LSD indicated that all treatments had a statistically significant lower average percent cell survival than the control condition. Although inferential statistics did not indicate RD87 to be protective against NMDA induced toxicity at any concentration, comparison of means shows that all conditions of RD87 increased cell survival compared to the 100uM NMDA condition. In addition, data was transformed to assess the ability of each treatment group to increase cell survival above that of the NMDA condition by mean comparison, and indicated that 5uM and 10uM RD87 did so most effectively, with percent cell survival being increased by 8% and 9% respectively.

3.4 NMDA and RD82's Effect on Intracellular Calcium Levels

A pilot study was conducted to assess changes in baseline levels of calcium due to exposure to 100uM NMDA and various RD82 treatment groups. Data was collected as described previously and normalized to the control condition, and no inferential statistics were conducted. Preparation for conduction of an intracellular calcium assay began with growth of primary neurons in culture for 2 weeks prior to stimulation with 100uM NMDA and three selected concentrations of RD82 including 1uM, 5uM, 10uM, and 20uM (Figure 7). Initial data indicates an increase in baseline calcium levels due to exposure to 100uM NMDA, as the NMDA condition showed a 6% increase in calcium flux compared to the control condition. Data also indicates a trend of RD82 decreasing calcium flux when added to cells independently in culture, excluding the 20uM treatment. 1uM, 5uM, and 10uM RD82 all decreased calcium levels from the control by 7%, 16%, and 9% respectively. Similarly, when the RD82 treatment groups were combined with the 100uM NMDA, 1uM, 5uM, and 10uM all reduced calcium levels compared

to the NMDA condition. Further, 5uM and 10uM RD82 reduced calcium levels compared to the NMDA condition and the control condition. The most significant effect was seen with 5uM RD82 which decreased calcium levels when stimulated with NMDA by 11% from the control and 17% from the NMDA condition.

Following the initial intracellular assay conducted (Figure 7), the cultures were then stimulated with glutamate, and a second assay was conducted to assess changes in calcium levels due to the addition of this stressor, as described previously (Figure 8). All data was normalized to the control condition, and no inferential statistics were conducted. Following stimulation with glutamate, calcium levels were 10% higher in the 100uM NMDA condition than the control condition. 1uM and 5uM RD82 without NMDA both decreased calcium levels from the control condition when glutamate was added. However, only 5uM and 10uM RD82 combined with 100uM NMDA decreased calcium levels when glutamate was added. As for the 20uM RD82 treatment condition, calcium levels increased for both the control and NMDA conditions when glutamate was added.

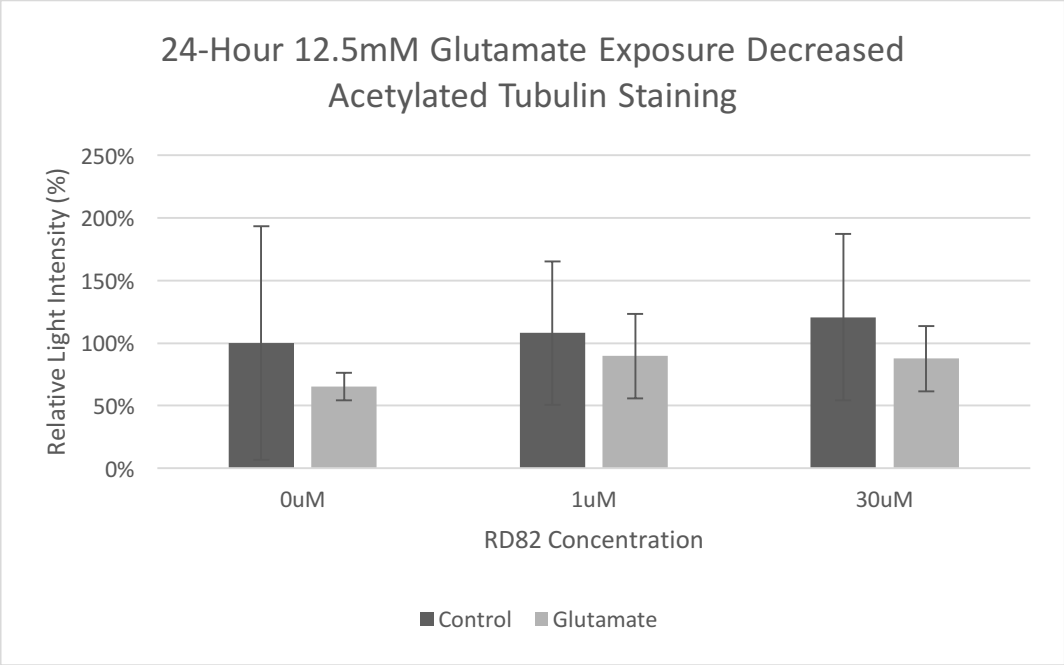
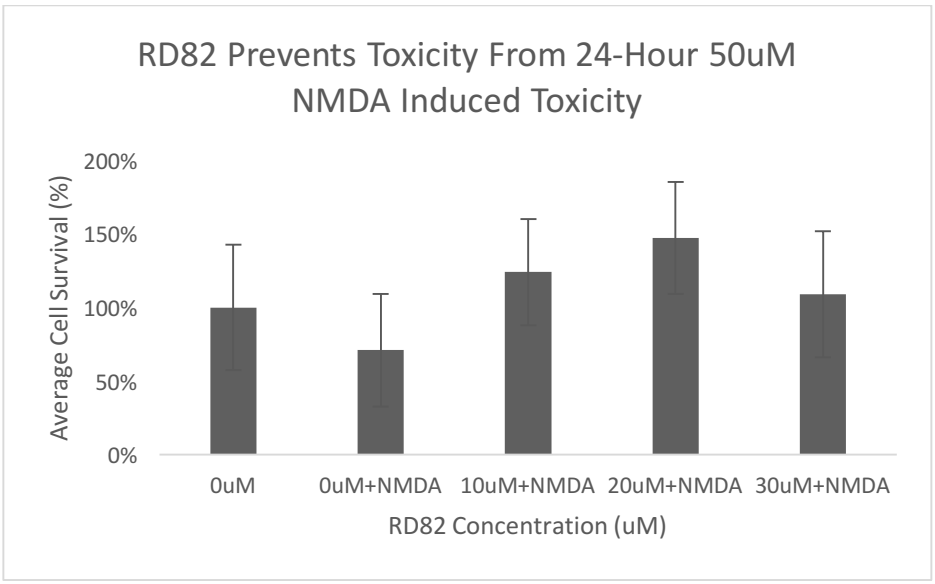
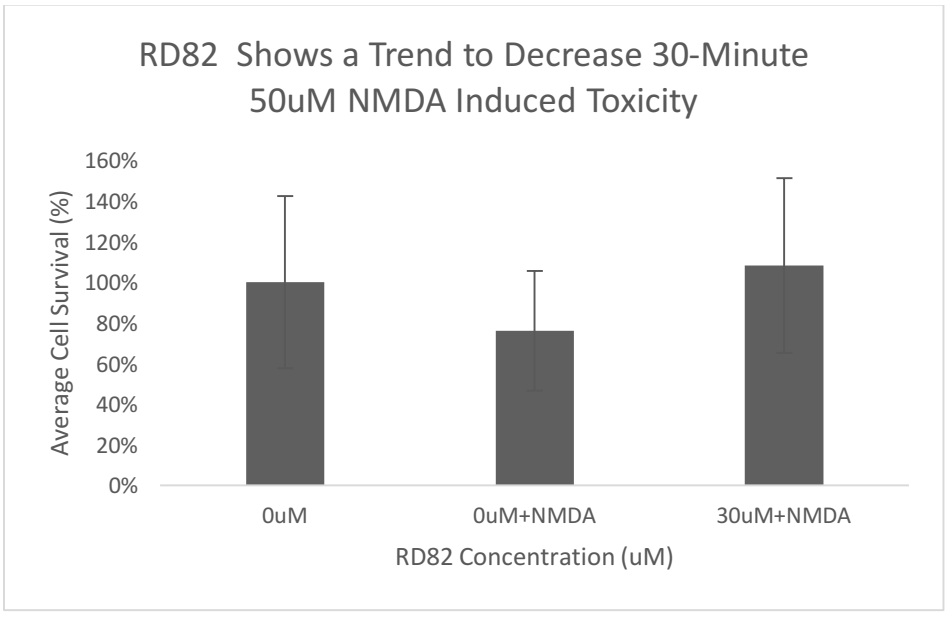


Figure 3. 12.5mM Glutamate stimulation. 24-hour stimulation of 1 week primary neuronal cultures with 0uM, 1uM, and 30uM of RD82 ($N = 120$, $n = 20$). Experimental conditions were stimulated with 12.5mM glutamate and a designated concentration of RD82 while control conditions were stimulated with RD82 alone for assessment of compound toxicity. Cells were stained for acetylated tubulin with primary and secondary antibodies as described previously in methods. Average light intensities were quantified for each condition to assess microtubule stability. According to the result of a 2x3 two-way ANOVA on light intensity by presence of glutamate and dose of RD82, glutamate is the only statistically significant effect ($F_{1,114} = 10.08$, $p = 0.002$). Average light intensity was statistically significantly lower with glutamate (M=62.36, $SD = 27.14$), than without glutamate ($M = 84.25$, $SD = 46.17$).



(A)



(B)

Figure 4. 50uM NMDA Stimulation. A 24 Hour stimulation of 2 week primary neuronal cultures with 0uM, 10uM, 20uM, and 30uM RD82 (N=60) (A). An MTS Assay was conducted and data was normalized to the control at 100% with error bars indicating standard deviation. According to the results of a one-way ANOVA, there is a statistically significant difference between at least two of the treatment group sin the MTS assay, on average ($F_{4,25} = 5.69$, $p < 0.002$). In addition, according to the results of the post-hoc LSD test, the control condition and the 50uM NMDA condition were statistically significantly lower than RD82 treatment groups, on average, with no other statistically significant differences detected. A 30-minute stimulation of 2 week primary neuronal cultures were stimulated with 50uM NMDA and 30uM RD82 (B). Results of a one-way ANOVA on average percent cell survival by treatment indicated that after only 30 minutes, there was no effect of treatment ($F_{2,15} = 2.96$, $p = 0.08$), although there was a trend. Post-hoc LSD testing of the trend indicated that treatment with 30uM RD82 increased average percent cell survival compared to control and negative control conditions.

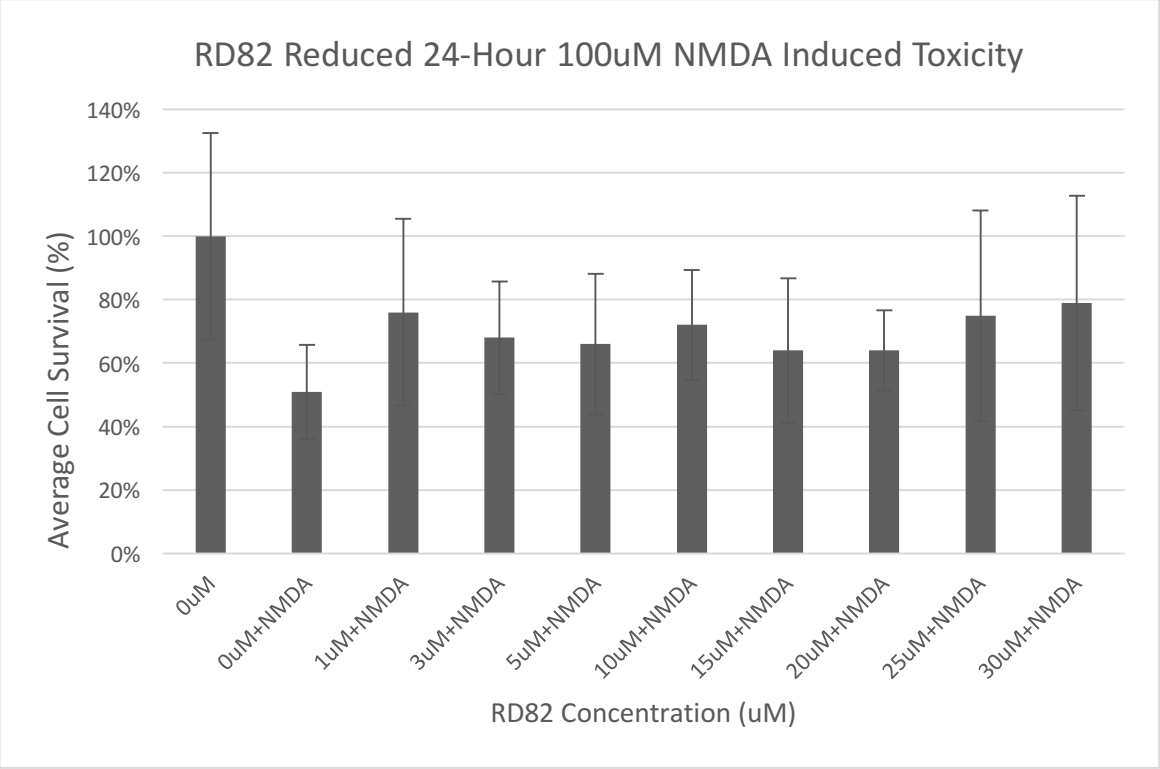


Figure 5. Concentration Curve of RD82 with 100uM NMDA Induced Toxicity. 24 Hour stimulation of 2 week primary neuronal cultures with 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM and 30uM of RD82 ($N = 120$, $n = 12$). MTS Assays were conducted, all conditions were normalized to the control at 100%, and data was compiled into one data set for statistical analysis with error bars indicating standard deviation. According to the results of a one-way ANOVA on presence of NMDA and dose of RD82, there was a statistically significant difference between groups ($F_9 = 3.21$, $p = 0.002$).

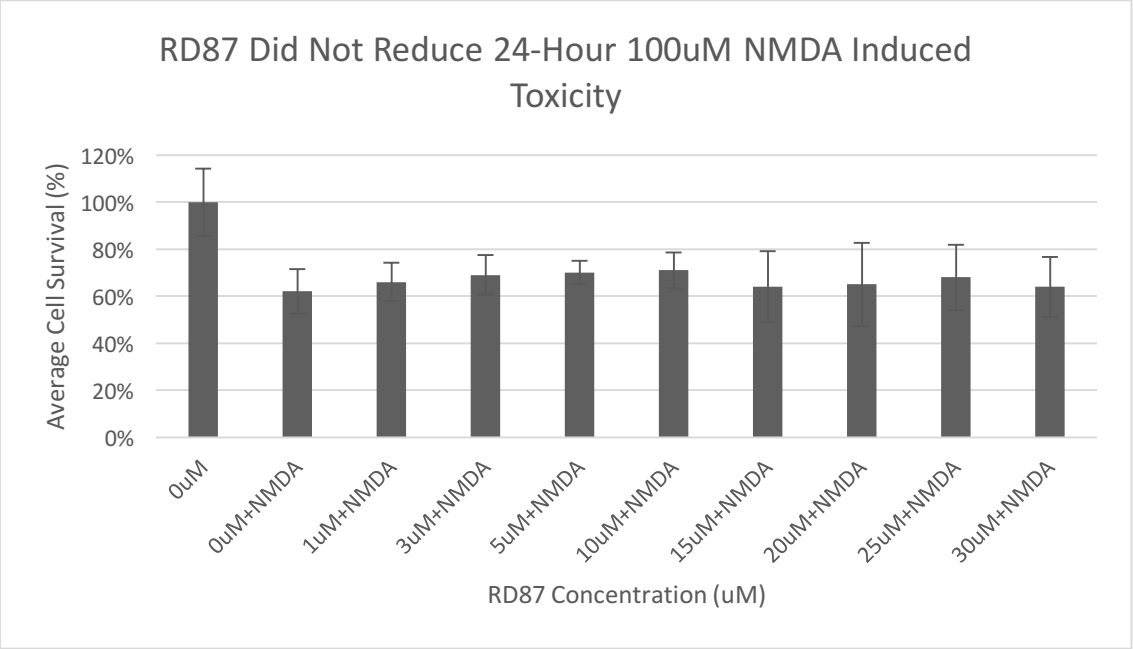


Figure 6. Concentration Curve of RD87 with 100uM NMDA Induced Toxicity 24 Hour stimulation of 2 week primary neuronal cultures with 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM and 30uM of RD87 ($N = 60$, $n = 10$). An MTS Assay was conducted and data was normalized to the control at 100% with error bars indicating standard deviation. According to the results of a one-way ANOVA, all groups differed statistically significantly from the control group ($F_{9,50} = 5.105$, $p < 0.0005$).

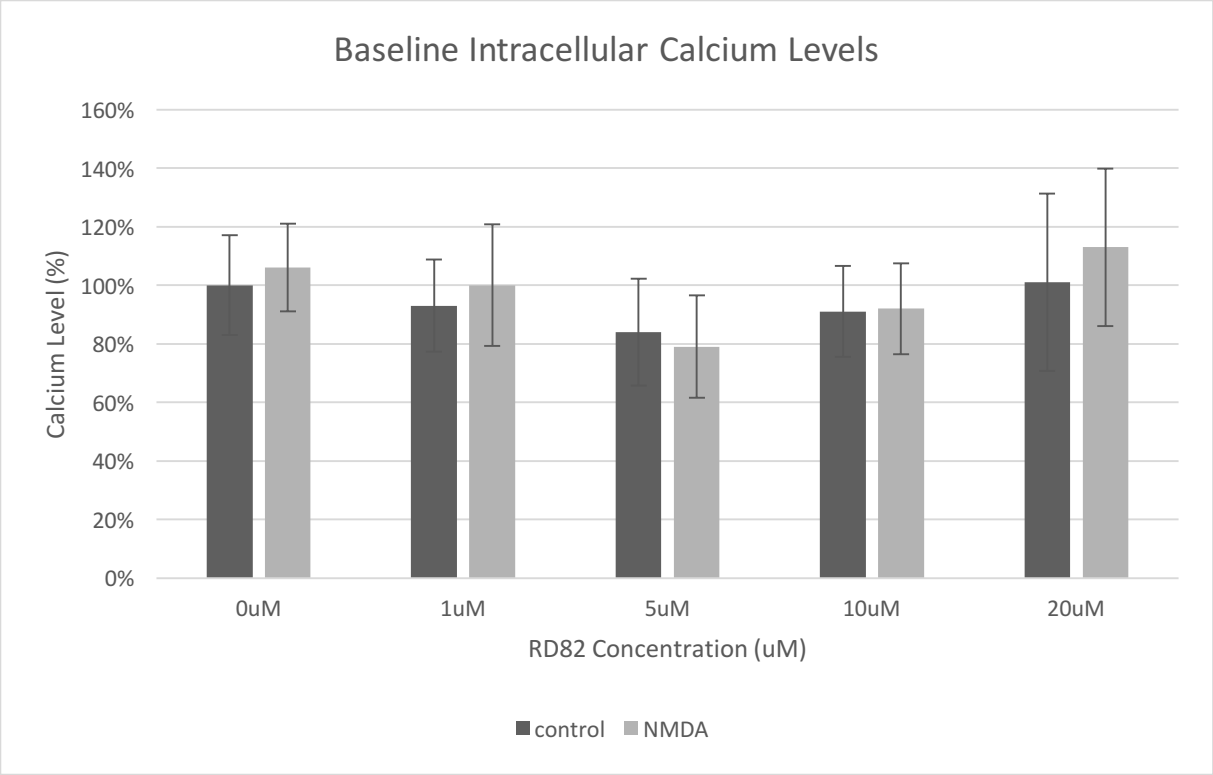


Figure 7. Baseline Calcium Levels. Two-week old primary neuronal cultures were incubated with an intracellular calcium dye for 30 minutes and then stimulated with 1uM, 5uM, 10uM, and 20uM RD82 and toxicity was induced with 100uM NMDA. Immediately following stimulation, the microplate reader was used to measure changes in Ca+2 flux due to stimulation. Analysis indicates a trend for a decrease in Ca+2 due to RD82, most significantly for the 5uM RD82 condition.

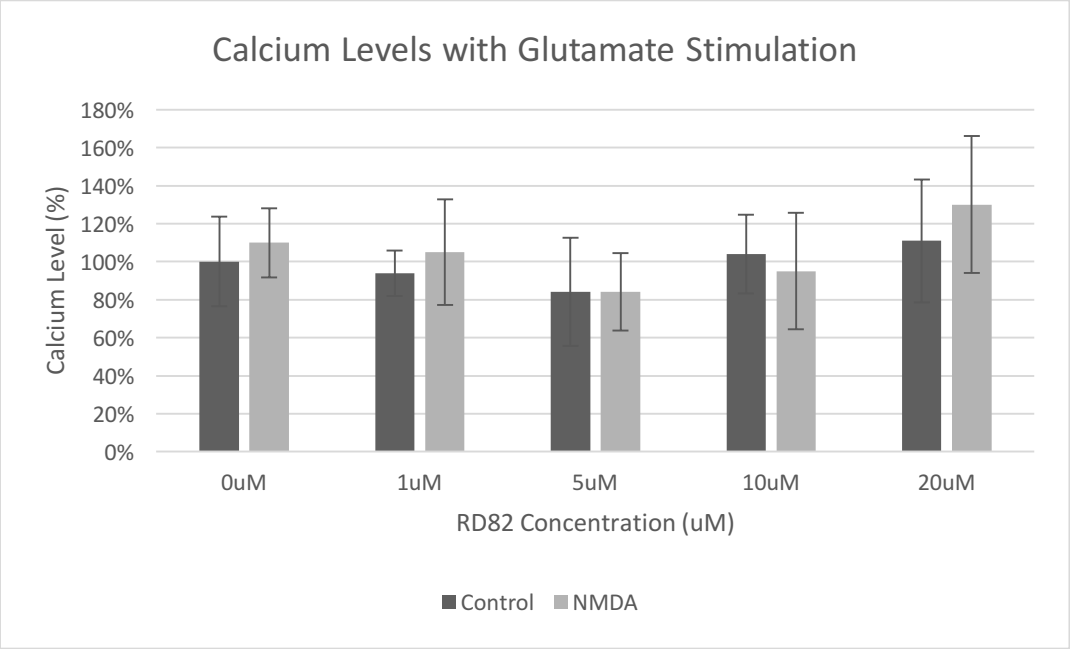


Figure 8. Intracellular Calcium Levels Following Glutamate Stimulation. Immediately after completion of the baseline calcium level assay, 10uL of 50mM glutamate was added to each well to induce additional toxicity and the assay was conducted again.

4. Discussion

The first hypothesis being tested in this research aimed to validate the use of primary neuronal cell culture work as a model system for neurodegenerative diseases by inducing cell death using stressors of the glutamate system. To test this hypothesis, neurons were harvested from E18 rats and were grown in culture for two weeks, excluding one initial experiment where cells were grown in culture for one week (Figure 3). Two weeks was preferable as it permitted more cellular differentiation. After two weeks of growth, the cultures were stimulated with either NMDA or glutamate, both of which are agonists of the glutamate system and therefore can be used to induce cellular stress. Following stimulation, cells sat in culture for most experiments for 24 hours, and then MTS assays and immunocytochemistry were performed to assess the extent of cellular damage induced by the stressors. The MTS assay measures the amount of formazan product that is produced in living cells. Thus, this assay is a good approximation of the number of cells that are alive at any given time. However, for a neuron to functional, it needs axons and dendrites to extend away from the cell body to form the connections necessary to both receive and release signals. The immunocytochemistry assay used in this study was based upon an antibody raised against acetylated tubulin, which is present in stabilized microtubules. So this assay samples information from both the cell body and the axons and dendrites. However, because the majority of the volume of a neuron is in its axons and dendrites, this results from this assay give greater weight to the axons and dendrites rather than the number of living cells.

Combing the results from MTS and immunocytochemistry assays, we found that both glutamate and NMDA could be used to induce toxicity in the cultures to an extent that cell viability was compromised, resulting in cell death (Figures 3,4). We also found that density of

axons and dendrites were decreased due to 24-hour exposure to glutamate (Figure 3). We began our research using glutamate as a stressor, as we were confident that it would induce toxicity and enable us to see if RD82 had any type of effect in preventing damage to our cultures as a result of induced toxicity (Figure 3). We chose 12.5mM glutamate, which is a relatively high concentration due to prior research we had conducted in the lab for the course Cellular and Molecular Neurobiology with Roger Knowles (data not shown). Additionally, this experiment used cells that had only grown in culture for one week, so a higher concentration stressor was necessary to induce induce toxicity that could be reproducibly measured, but not so great that the degree of cell death and elimination of axons and dendrites would be so great that it would be unreasonable to suspect that a preventative agent would be able to have a measured effect. A priori, we set that a loss of between 20-50% of cells and/or similar loss of axons and dendrites as a reasonable model system to use to test potential neuroprotective agents. Using a 2x3 two-way ANOVA we confirmed statistical significance of glutamate as a stressor, validating our model system. In this experiment we did a pilot test to determine whether RD82 was neuroprotective under these conditions, and while the results did not reach the level of statistical significance, there appear to be a trend towards protection. Base on this trend, we shifted our focus to using NMDA, a more specific stressor to our research. The rationale for that shift was due to the expected site of action of the compounds RD82 and RD87. By using glutamate as our stressor, we most likely would be saturating the receptors that the compounds were designed to modulate. Most likely any effect that would be seen by the compounds would occur after the concentration of glutamate lowered in the media. In contrast, NMDA does not directly bind to mGlu4, and therefore while the mechanism of the toxicity of NMDA might be similar to exposure to glutamate, the compounds would have the ability to modulate those receptors without

interference from the stressor. We conducted a pilot study using 50uM NMDA as our stressor and found that it did induce toxicity, as indicated by a 2x4 two-way ANOVA (Figure 4). In this experiment we found there to be a statistically significant interaction between the presence or absence of NMDA and the concentration of RD82 applied to culture ($F_{3,40} = 2.938$, $p = 0.045$). More specifically, we found that RD82 was not toxic at any of the concentrations applied when applied independently to culture, and that the 20uM concentration statistically significantly improved average percent cell survival when added to cells also stimulated with 50uM NMDA, validating our model system.

In the experiment mentioned previously (Figure 4), while it is important that we found RD82 to be protective against NMDA induced toxicity, it is also important to note that we did not see any toxicity produced by applying RD82 to cultures independently, supporting our second hypothesis that there would be a range of concentrations safe to apply to primary neurons. As mentioned previously, we applied a range of concentrations with the maximum concentration being 30uM, but saw the highest effectiveness in this experiment at 20uM. This compound however was in its racemic mixture form, and was not purified into enantiomers. When we chose these concentrations, we used literature (Bruno et al., 2000) that applied the purified enantiomers of similar compounds attempting to identify the active enantiomer (Bruno et al., 2000). Because RD82 was not a single enantiomer, (Figure 4), we suspect that had RD82 been resolved a similar activity would have been seen at half the concentration.

Following the 50uM NMDA pilot study we ran a concentration curve to better understand the optimal concentration of NMDA that could be used to induce cell death without entirely obliterating our cultures (data not shown). Literature that we considered indicated usage of 100uM NMDA to induce toxicity in primary neuronal cell cultures to test the activity of

compounds that we suspect would act similarly to ours (Bruno et al., 2000), so we used this concentration as the middle of our range. We found 100uM to more consistently induce toxicity in our cultures and chose to continue all experimentation with this concentration, though we did see toxicity using concentrations as low as 50uM and as high as 200uM (data not shown). Throughout the duration of our experimentation we did see some variability in the extent of toxicity induced by 100uM NMDA and we attribute this to NMDA solution preparation. For this experiment we did not create new stock solution every time that we conducted an experiment and instead would create stock solution that could be used for several experiments. We suspect that this might have affected the concentration of our stock, as it left the potential for degradation of the NMDA in solution. Future research conducted using NMDA as a stressor should include creating a new stock solution every time an experiment is conducted to eliminate the potential of degradation.

Our third hypothesis that we tested in this research was that exposure of neurons to RD82 and RD87 would lower induced toxicity in cell culture in a concentration dependent manner. Our initial research only included RD82 and we used the concentrations 1uM and 30uM, and stimulated with 12.5mM glutamate, and we did not observe statistically significant reduction in neuronal toxicity (Figure 3). However, in that experiment, in which we examined acetylated tubulin staining, the data by its nature focused more on the amount of coverage of axons and dendrites than surviving neuronal cell bodies. Also, as explained earlier, we suspected that glutamate as a stressor may occlude effects of RD82 because glutamate would be binding mGlu4 receptors to the level that any “positive modulation” would be inconsequential. Even though we did not see significant improvements with this experiment, a comparison of means suggested that maybe there would be an effect with a different experimental design. We then

switched stressors and completed a small concentration curve, including concentrations of 10uM, 20uM, and 30uM, paired with 50uM NMDA (Figure 4). We performed an MTS assay and found that all three concentrations protected cells from NMDA induced toxicity, with the 20uM condition increasing cell survival above the control by 47% and above the NMDA condition by 76%, as indicated by comparison of means. As mentioned previously, a two-way ANOVA indicated that the 20uM RD82 condition statistically significantly increased cell survival. The data from both of these initial studies led us to believe that we needed to test more concentrations of RD82 between 1uM and 30uM, as we saw a visible effect of treatment at 1uM (Figure 3), and saw that treatment appeared to begin to diminish at 30uM (Figure 4). We ran a fuller concentration curve of RD82 including 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM, and 30uM paired with 100uM NMDA (Figure 5). We conducted an MTS assay and found a main effect of treatment, with the 1uM, 10uM, 25uM and 30uM treatments producing the most significant results. We did expect to see an effect of treatment; however, these results were surprising to us as there did not seem to be a normal curve or pattern to the performance of RD82. We attribute the inconsistency in performance of each concentration of RD82 to our methods for preparing RD82 stock. After conduction of many of these experiments, we learned that RD82 degrades rapidly in DMSO, even when frozen. We suspect that due to the rapid degradation of RD82, we were not actually successfully applying the exact concentrations of the compound as intended.

As for RD87, we ran a pilot study to assess the compound's protective abilities against NMDA induced toxicity. For this study we applied the same concentration curve that we used for RD82, including 1uM, 3uM, 5uM, 10uM, 15uM, 20uM, 25uM, and 30uM treatment groups (Figure 6). Toxicity was induced with 100uM NMDA, and an MTS Assay was used for quantitative analysis. Inferential statistics indicated that all treatments, including the 100uM

NMDA negative control and entire concentration curve of RD87 paired with NMDA, were statistically significantly different than the control condition ($F_{9,50} = 5.105$, $p < 0.0005$). These results showed us that there were not any statistically significant differences of RD87 treatment, suggesting that RD87 is not protective in culture. However, by comparison of means the data from this study can suggest that RD87 does have the ability to lower toxicity induced in cell culture, as all treatment concentrations increased the percent cell survival above that of the 100uM NMDA condition. Further, the 5uM and 10uM RD87 treatment groups most successfully decreased toxicity, improving cell survival above the NMDA condition by 8% and 9% respectively. As this was only a pilot study, we cannot comment on the consistency of RD87 treatment concentrations.

Our fourth hypothesis that we tested was that there would be differences in the activity of RD82 and RD87 due to structural differences of the compounds. To address this hypothesis, we compared the concentration curves we conducted for both RD82 and RD87 (Figures 5-6). We conducted statistical analysis for both concentration curves and found for RD87 that the control was statistically significantly different than all other treatments, which did not suggest protective abilities of RD87 by inferential statistics (Figure 6). However, for RD82 a one-way ANOVA and Tukey HSD Post-hoc indicated that specific conditions of RD82 statistically significantly improved percent cell survival above that of the control condition, including 5uM, 15uM, and 20uM RD82 (Figure 5). The comparison of the inferential statistics for the concentration curves of RD82 and RD87 when paired with 100uM NMDA suggest that there are differences in the activity of the two compounds, with RD82 indicating protective abilities that RD87 appeared to lack. In order to better understand the nature of the differences in activity of the two compounds, we have included the polarity values (CLogP, calculated using

ChemBioDraw) for each compound (Figure 9). A higher CLogP value indicates that the compound is more lipophilic. RD82, which has a CLogP value of 3.32, is ten times more lipophilic than RD87, which has a CLogP value of 2.23. We suspect that the differences in the polarity as indicated by the ClogP value would influence the activity of each of these compounds in our model system. The polarity of a compound can affect the binding constant of the compound to a receptor, and thus affect the derived biological effect.

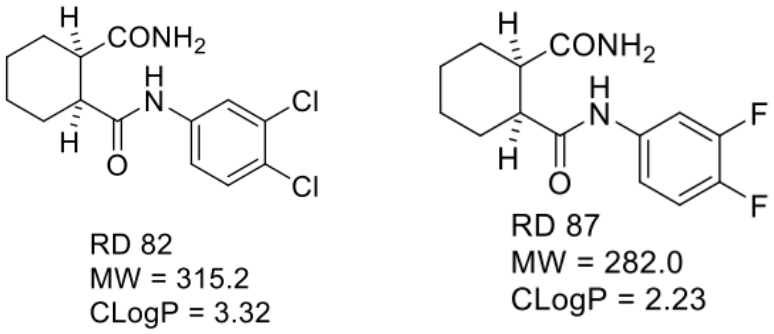


Figure 9. Calculated polarity of RD82 and RD87 (CLogP).

Our final goal of this research project was to examine whether there was evidence that intracellular calcium concentrations were related to the effects of RD82. To explore this aim, we used an intracellular calcium assay to assess the impact of RD82 on baseline calcium levels as well as changes in calcium levels due to the addition of glutamate. During our initial NMDA study in which we used 50uM NMDA and 10uM, 20uM, and 30uM treatment groups of RD82, we ran an additional 30-minute stimulation to assess the timing of the effect seen with RD82 and NMDA (Figure 4B). This stimulation included only a control condition, a 50uM NMDA condition, and a treatment condition including 50uM NMDA and 30uM RD82. We chose 30uM at this point because we had previously seen that it produced a significant effect when we ran our pilot study using 12.5mM glutamate (Figure 3). In this 30-minute assay we found that the effects

of NMDA and RD82 on primary neuronal cultures can we measured in relatively short amount of time, indicating that the mechanism by which their effects are occurring is fast acting (Figure 4B). While we did not see statistically significant differences in between the treatment conditions, we came to the conclusion by comparison of means that a 30-minute stimulation sufficiently impacted the viability of our cultures to suspect that there was a relatively quick mechanism of action occurring. This was evident when considering the NMDA condition, as the 50uM NMDA induced toxicity decreased the cell survival of exposed neurons from the control condition by 24%. In addition, the 30uM RD82 treatment group increased percent cell survival 8% above the control condition and 32% above the 50uM NMDA condition. With these results in mind, we conducted our intracellular calcium assays which involved an even shorter stimulation time. For the initial baseline calcium assay we incubated our cultures with the calcium dye alone for 30 minutes, added our reagents (which included 100uM NMDA, 1uM RD82, 5uM RD82, 10uM RD82, and 20uM RD82) and immediately conducted the assay (Figure 7). In this initial baseline measure we expected to see that stimulation with RD82 would reduce the calcium levels from the 100uM NMDA control condition. We also expected to see that cells treated with 100uM NMDA in addition to RD82 treatments would exhibit higher levels of calcium than conditions stimulated with RD82 treatments alone. The first portion of this hypothesis was supported by our data, excluding the 20uM RD82 treatment group, as 1uM, 5uM, and 10uM RD82 all reduced calcium levels independently and paired with NMDA. The greatest effect was seen in the 5uM RD82 condition, where 5uM RD82 paired with 100uM NMDA decreased calcium levels 11% from the control condition and 17% from the 100uM NMDA condition. It was interesting that the 5uM NMDA condition exhibited the best results for this assay as this is inconsistent with our previous MTS data (Figure 5). In addition, the 20uM RD82

condition increased calcium levels above the control condition independently and when coupled with 100uM NMDA. This was not what we anticipated for this concentration of RD82, as our previous data indicated that 20uM RD82 successfully decreased NMDA induced toxicity (Figure 4). We attribute the inconsistencies in RD82 performance again to the degradation of our compound in DMSO solution, and the lack of resolution of the compound.

Following conduction of the baseline level calcium assay (Figure 7) we added glutamate to each condition and immediately ran the assay a second time to assess the impact of the addition of a stressor (Figure 8). We anticipated that adding glutamate would increase calcium permeability and calcium flow and that we would see an effect of RD82 concentration on the change in calcium levels following adding glutamate. This is because modulating mGlu4's should lead to further decrease release of endogenous glutamate. Consistent with the baseline calcium assay, we saw that the 5uM RD82 treatment outperformed the others, reducing the calcium levels 16% below the control and 26% below the 100uM NMDA condition. Also consistent with the baseline assay, the 20uM RD82 condition increased calcium levels when glutamate was added. Again, this was surprising to us compared to our initial RD82 MTS data, and we attribute this to the potential decay of our RD82 stock in DMSO. However, in both the baseline data and the glutamate stimulation condition, we also cannot rule out the possibility that RD82's mechanism of protecting neurons is independent of modulating steady state intracellular calcium concentrations. It is also possible that since this was a single experiment, the neurons in the wells with 20uM RD82 were simply more stressed than the other wells due to some slight variation in plating. Future research should be able to distinguish these possibilities.

Taking all of the data that has been collected in this research, we would suggest the following model to explain RD82's protective effects due to NMDA exposure. When neurons

are treated with NMDA, neurons are abnormally excited and begin to increase their rate of firing action potentials. When those action potentials arrive at the presynaptic terminals, they cause a release of neurotransmitter. A significant proportion of these neurons are releasing glutamate, which causes further excitation. This further excitation excites those target neurons even more, causing more action potentials, and more release of neurotransmitter. The resulting cycle is a feed forward loop which causes these neurons to reach high levels of intracellular Ca+2 for prolonged periods of time, leading to the initiation of cell death pathways. When RD82 is introduced, this chemical binds to mGlu4 receptors, and when glutamate is released, these receptors are enhanced. These receptors are on the presynaptic terminals and when active they act via second messengers to decrease the ability of the neurons to release further glutamate. This action dampens down the feed forward cycle, protecting some of the neurons from reaching toxic levels of intracellular Ca+2. Most likely RD82 would not be able to protect neurons from a catastrophic release of glutamate due to something like a stroke, but it is possible that RD82 could limit the ability of that catastrophic release to cause further damage due to secondary excitation of more glutamate neurons. In a disease like AD, in which damage to neurons is most likely due to several factors, of which excess glutamate is only one, RD82 would most likely have a modest effect on disease progression. However, at this point since there are no drugs that have conclusively demonstrated the ability to slow down the progression of the disease, even modest improvements in the rate of degeneration would be extremely welcome.

Taking into consideration the compiled results from each of the experiments conducted in this study we would argue that use of the RD compounds should be further considered as treatment options in neurodegenerative disease research. Upon completion of this research, we want to acknowledge future directions that should be considered in future work.

First, as mentioned previously, we used the racemic mixtures of both RD82 and RD87. For this reason, we do not have an accurate of idea the single enantiomer biological activity of these compounds because each enantiomer may possess a different profile of biological activities. Future research should involve resolving the racemic mixture into separated enantiomers in order to identify the biological effect of each enantiomer. Following isolation of the desired enantiomer, it should be used to test and understand concentration dependent effects of the compounds. In addition to resolution of the compounds, we think that future research should include utilization of all of the RD compounds (RD82, RD83, RD84, RD85, RD86, RD87) to gain a better understanding of how the structural and physical properties of the compounds can influence their effectiveness as neuroprotective agents.

In addition to expanding this research in the future to gain more knowledge on the individual compounds themselves, we believe that future studies should also use different model systems for neurodegeneration. For example, our model system primarily focused on the ability of these compounds to protect against induced neurotoxicity by manipulation of the glutamate system. However, many neurodegenerative diseases have components that contribute to changes in the glutamate system and further contribute to neurodegeneration. For example, in Alzheimer's Disease it is argued that amyloid beta interacts with the glutamate system, resulting in neuronal loss (Revett et al., 2013). Future model systems could include using amyloid beta to induce toxicity, both *in vitro* and *in vivo*.

Finally, future research should focus on developing a better understanding on the specific mechanism of how these compounds work. We initiated this research by conducting a pilot experiment measuring calcium levels after applying NMDA, RD82, and glutamate to our cultures. Future research could include manipulation of cultures to generate changes in calcium

concentrations in conditions that mimic cell survival to look for corresponding trends that could potentially suggest a mechanism of action.

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