Drew University

College of Liberal Arts

Investigating the Effects of RD100, a Potential Group III mGluR Positive Modulator, in a Primary Rat Cortical FAB/NMDA Model of Alzheimer's Disease

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By

Kelly Maegerlein

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ABSTRACT

Past research into therapeutic compounds for Alzheimer's disease which have often focused on targeting amyloid beta, tau, and post-synaptic NMDA receptors, have shown largely underwhelming results. An alternative approach is targeting pre-synaptic glutamate release, achieved by modulating pre-synaptic metabotropic glutamate receptors. Group III mGluRs, mGluR4 in particular, through inhibition of second messenger pathways such as adenylyl cyclase, regulate the release of the glutamate from the pre-synaptic neuron. This activity may mitigate excitotoxic glutamatergic transmission and provide neuroprotection, as seen in studies using the mGluR4 positive allosteric modulator, PHCCC, and the mGluR4 agonist, L-AP4. The present pilot study seeks to investigate a potential mGluR4 PAM, known as RD100, in a FAB/NMDA primary rat cell culture model of AD. The goals of this study are to identify the effects of RD100 on cell survival and oxidative stress and identify its mechanism of action. Elucidation of RD100's mechanistic pathway will be conducted with co-application of MSOP, a broad group III mGluR antagonist. This study also seeks to optimize use of the FAB/NMDA model system by identifying appropriately neurotoxic concentrations, and assess its effects on oxidative stress. To assess oxidative stress, MitoSOX red mitochondrial superoxide indicator will be utilized as a marker of superoxide. The most useful and reflective measurements of MitoSOX fluorescence under the compound microscope will also be determined.

At 5uM, RD100 does not have a significant effect on cellular survival. FAB/100 uM NMDA elicits a concentration dependent negative effect on cellular survival, with at

least 33.3% FAB significantly reducing survival. For assessment of superoxide levels, correction of relative intensity by binary area (intensity/area) should be conducted as the most reflective measure of MitoSOX fluorescence. Neither RD100 nor FAB/NMDA have any significant effect on superoxide at 24 hrs after application. It was unable to be determined whether RD100 acts as a positive modulator of mGluR4 due to RD100's and MSOP's lack of significant effects on cell survival, though joint toxicity of RD100 and MSOP suggests so. Recommendations for further studies include determining the effects of PHCCC in the FAB/NMDA model, investigating the neuroprotection of other RD compounds, and further elucidation of the timing of ROS influenced by FAB/100 uM NMDA.

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ABBREVIATIONS

Aβ – amyloid beta

AC – adenylyl cyclase

AD – Alzheimer's disease

AMPAR – AMPA receptor

APP – Amyloid precursor protein

cAMP – cyclic AMP

FAB/NMDA – FeSO4, amyloid beta, L-buthionine, and 100 uM NMDA

GM – growth media

mGluR – metabotropic glutamate receptor

NMDAR – NMDA receptor

NAM – negative allosteric modulator

OS - oxidative stress

PAM – positive allosteric modulator

PKA – protein kinase a

PKC – protein kinase c

PLC – phospholipase c

PM – plating media

ROS – reactive oxygen species

GLOSSARY

Aβ –a protein that forms extracellular aggregations known as plaques

Agonist - an agent that binds directly to a receptor at its orthosteric site and promotes its activity

Antagonist – an agent that binds directly to a receptor at its orthosteric site and prevents/inhibits its activity

Antioxidant – a compound that combats oxidative stress by scavenging free radicals/ROS

cAMP – produced via activation of adenylyl cyclase and modified from ATP

Depolarizing – a change in the electric potential of a neuron that makes its voltage more positive, which is excitatory and promotes neurotransmitter release

Glutamate – a neurotransmitter involved in excitatory neurotransmission

Hyperpolarizing – a change in the electric potential of a neuron that makes its voltage more negative, which is inhibitory and prevents neurotransmitter release

L-AP4 – a general group III mGluR agonist

MSOP – a general group III mGluR antagonist

PAM – activates a receptor by binding in the allosteric site in the presence of the orthosteric ligand

Synapse – consisting of the pre-synaptic and post-synaptic regions, this is where a neuron communicates to other neurons via chemical messengers, neurotransmitters, which act on receptors located on synaptic membranes

Tau – a protein that forms intracellular aggregates known as tangle

INTRODUCTION

Executive Summary

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases across the world, and is marked by devastating cell death in the memory center of the brain, the hippocampus[22]. This leads to progressive deterioration of one's memory to the point that sufferers will lose all recollection of themselves and loved ones in just a few short years after diagnosis, as there is no effective treatment[20]. Aggregations of the proteins amyloid beta-42 (Aβ42)[34] and tau[36] are two markers seen in post-mortem AD brains implicated in this toxicity, as both correlate with areas of degeneration in AD brains[34,32]. However, despite years of clinical trials for therapies targeted against Aβ and tau, results and prognoses for AD patients remain the same, and largely disappointing[29].

This calls for investigation into other mechanisms within AD brains that contribute to neurotoxicity, such as oxidative stress (OS) and glutamate toxicity[22]. OS is a process where reactive oxygen species (ROS) produced through dysfunctional cell metabolism attack membrane biomolecules and lead to membrane fragmentation[17,8]. Glutamate toxicity results from overly heightened levels of the excitatory neurotransmitter, glutamate, particularly in the hippocampus, acting on post-synaptic glutamate receptors[22]. There are multiple kinds of glutamate receptors implicated in this process, primarily within the broader ionotropic and metabotropic categories[40].

NMDA receptors (NMDARs), a kind of ionotropic receptor, are especially pertinent in glutamate toxicity. These receptors open to allow most importantly Ca^{2+} into

neurons[41]. Excessive stimulation of post-synaptic NMDARs by high levels of glutamate release onto the post-synaptic neuron may induce drastically high influxes of Ca^{2+} into cells, which is implicated in mitochondrial dysfunction and cognitive decline in AD[41]. Because of the potential for post-synaptic NMDAR stimulation to be implicated in glutamate toxicity, therapies such as memantine have targeted and blocked activity of these receptor to mitigate these toxic responses[15]. While effective for a short period of time, memantine does not halt symptom progression and cognition will continue to decline, leading to continued poor prognoses[35].

An alternative approach is to target the pre-synaptic release of glutamate, which can be achieved through modulation of pre-synaptic metabotropic glutamate receptors (mGluRs)[14]. In particular, group III mGluRs show promise as natural regulators of glutamate release through their inhibition of the adenylyl cyclase (AC) cascade[14]. Activation of these receptors blocks this pathway, which prevents Ca^{2+} influx and allows K^+ efflux to reduce sensitization of pre-synaptic neurons to reduce neurotransmitter release.

Of group III receptors, mGluR4 is a particular target of interest in studies investigating neuroprotection against glutamate toxicity. The general group III mGluR agonist L-AP4 acting on mGluR4 has been shown to reduce glutamatergic transmission in cerebellar neurons through depression of excitatory post-synaptic potentials[6], reduction of pre-synaptic Ca^{2+} influx[1], and general reduction in glutamate release[2]. The more targeted mGluR4 positive modulator PHCCC in particular has shown neuroprotection in NMDA and Aβ-mediated toxicity[25]. The present study seeks to elucidate the effects of

another a potential mGluR4 positive modulator, RD100, in a primary rat cortical FAB/NMDA model of AD.

Overview

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases, affecting around 30-35 millions individuals across the world[42]. AD is characterized by neurodegeneration due to cellular toxicity primarily in the hippocampus and eventually afflicting other cortical areas[22]. The hippocampus functions in the retrieval and storage of memories, and consolidation of memory from short-term to long-term[18]. Degeneration in this region first impairs the retrieval of short-term memory encompassing occurrences within the previous seconds to minutes, and often involves semantic knowledge such as naming and verbal fluency[18]. Early memory disruption also impairs problem solving, judgment, executive functioning, multitasking, and abstract thinking, and leads to disorganized behavior[20]. This memory loss progresses as degeneration advances in the hippocampus, impairing new memory formation and later developing into long-term memory impairment involving memories stored from prior days, weeks, and years[18]. In later stages of AD with degeneration progressing into other cortical regions, this often results in apathy, social withdrawal, agitation, and psychosis[20]. Eventually, the patient progresses to the extent where they can no longer remember loved ones and identify themselves, often becoming incontinent and unable to care for themselves in the latest stages[20]. Other than this devastating neurodegeneration, the major markers seen in AD brains are extracellular plaques of amyloid beta (Aβ) and intracellular tangles of tau

protein, which are identified post-mortem[22]. Both amyloid beta and tau are implicated in the cellular toxicity seen in AD, and research suggests they may be both involved in generating toxicity and also result from this toxicity.

Amyloid beta and tau

Amyloid beta is a 37 to 49 amino acid protein that results from cleavage of the amyloid precursor protein (APP)[9]. APP is cleaved by α, β, or γ secretases in a specific location that determines whether the fragment will become amyloidogenic or nonamyloidogenic. APP is either initially cleaved by α secretase in the nonamyloidogenic path or β secretase in the amyloidogenic path to form α- or β- C-terminal fragments (CTFs)[9]. These α- and β-CTFs are further cleaved by γ secretase to form either P3 or Aβ peptides respectively. Aβ peptides are further processed to become either Aβ40 or Aβ42, which have different structure and function within the cell[9]. It is Aβ42 that is predominately found within plaques, aggregates faster, and is more neurotoxic. The activity of these secretases is influenced by mutations within the APP gene both in familial and sporadic forms of the disease. These mutations typically influence the activity of γ secretase and skew the ratio of Aβ40:AB42 to favor Aβ42[9].

Monomers of Aβ form into oligomers which are soluble inside the brain, but when oligomers join together they form large insoluble fibrillated plaques[9] Aβ plaques are associated with areas of neurodegeneration in AD brains, though increases in Aβ plaques do not necessarily correlate with increased severity of cognitive impairment[34]. Plaque formation is suggested to be more highly correlated in the early pathology of AD, as their

appearance correlates in these early stages alongside early cognitive disruption[30]. As AD progresses these Aβ depositions increase in load and eventually reach a plateau, while cognition continues to decline[30]. More current research suggests instead that it is rather the soluble non-fibrillated Aβ monomers that contribute to primarily degeneration, as cognitive decline does occur before fibrillated plaque formation and more directly correlates with the detection of soluble non-fibrillated monomers[12]. Overall, Aβ42 has been shown to be involved in a number of pathways that contribute to degeneration and toxicity in AD brains[34].

Soluble Aβ42 monomers have also been determined to increase aggregation of intracellular tau tangles through their interaction on the P75 receptor[36]. Tau is a protein found within normal neurons that functions to stabilize microtubules inside the axons of neurons to allow for cellular communication[36]. However, in AD tau becomes hyperphosphorylated, which destabilizes the protein's structure and causes it to aggregate into tangles and cease its proper function in microtubule stability[36]. This interrupts signaling along the axon and contributes to neurodegeneration. Tau has been seen to correlate strongly with areas of degeneration in AD brains[32]. It is generally understood that soluble Aβ appears early in AD progression and may induce the development of tau pathology later in disease progression[5], though tau may become hyperphosphorylated in the absence of interaction with Aβ. Additionally, soluble Aβ and tau also have a synergistic effect in increasing neurodegeneration[5].

AB and tau therapies

There are many therapies in development that target aspects of Aβ and tau pathology[9]. Regarding Aβ, therapies seek to reduce levels of oligomeric Aβ, such as by inhibition of oligomerization, oligomer neutralization with immunotherapy, encouraging breakdown of Aβ with overexpression of Aβ-degrading enzymes, hydrolyzation of plaques with catalytic Aβ antibodies, blocking Aβ channels, and inhibition of secretases[9]. Regarding tau, therapies target inhibition of oligomerization and aggregation, controlling phosphorylation, stabilizing microtubules, increasing degradation of tau, and immunotherapy[9]. Though a few of these therapies are undergoing clinical trials, most have failed to provide the expected benefit to patients with mild to moderate AD[29]. Despite the evidence of their contribution to neurotoxicity, it is still debated whether Aβ and tau truly drive AD pathology or if they are downstream products that have synergistic effects on pre-existing toxicity. Thus, alternate avenues of therapeutic intervention in pathways that also contribute to neurodegeneration in AD are important to explore, such oxidative stress (OS) and excitotoxic glutaminergic transmission[22].

Oxidative stress overview

Oxidative stress (OS) is a contributor to many neurodegenerative diseases, including AD. OS can develop through a number of mechanisms, including mitochondrial dysfunction, redox-active metal accumulation, amyloid beta aggregation, and hyperphosphorylated tau tangles[11]. During OS, heightened levels of reactive oxygen species (ROS) attack and oxidize molecules within cells and lead to cellular toxicity and

neurodegeneration[17]. ROS are radically reactive oxygen-containing species that are mostly produced during cellular respiration in the mitochondria[8]. They are highly unstable and reactive due to the presence of unpaired electrons in their atomic orbitals, and will either give up an electron or receive an electron from other compounds to become stabilized[24]. These species are combatted and balanced by levels of antioxidants within the body, which neutralize, or scavenge, radicals by donating an electrons to stabilize the unpaired radical electron[24]. Common antioxidant molecules within the body include glutathione (GSH), glutathione peroxidase, superoxide dismutase, catalase, and some proteins[31]. In the context of AD, oxidation by ROS of membrane biomolecules such as lipids (cholesterol), membrane proteins (including receptors and transporters) and nucleic acids leads to membrane fragmentation[8].

Oxidative stress and aging

Other than the involvement of oxidative stress in neurodegenerative diseases such as AD, increases in ROS and OS may also be associated with normal aging. According to the theory of "inflamm-aging", levels of ROS and other redox active molecules rise as we get older, and these molecules influence the ability of immune cells to control inflammation, which then negatively impacts homeostasis and accelerates aging[43]. Additionally, the body's natural defenses to OS may decline with normal aging, and the balance between ROS and antioxidants may tip in the favor of ROS[31]. In one example, as we age there is a shift in the GSH:GSSG ratio, which describes the shift in the oxidative state of the antioxidant glutathione from reduced (GSH) to oxidized (GSSG)[33]. GSH is

just one antioxidant that scavenges free radicals to prevent the oxidative destruction of ROS[33]. Considering that risk for AD increases with age, these changes in defense to OS and ROS production likely explain why increases in ROS associated with AD cause such devastation.

Oxidative stress and amyloid beta

AB is thought to be a contributor to OS in AD. Regions of the brain rich in Aβ depositions correlate with higher levels of OS, while regions low in Aβ depositions show little to no OS[8]. Additionally, metal ions such as Cu^{2+} and Zn^{2+} are found in high concentrations within Aβ plaques, and these metal ions may contribute to ROS production as they have been identified to associate with Aβ, which may potentially lead to their reduction to generate ROS[37]. Fe^{2+} has also been identified to bind to Aβ within these plaques, and additionally Fe^{2+} accumulations that develop with aging interact with water and oxygen in the brain to directly generate ROS[39]. Aβ's involvement in the overactivation of extrasynaptic NMDARs has also been shown to increase ROS levels and contribute to OS due to the excessive Ca^{2+} influx through NMDA receptors leading to mitochondrial dysfunction[19]. In addition to Aβ contributing to the development of OS, OS also reciprocally contributes to the formation Aβ plaques[8]. Additionally, the redox-active metal ions found in cell membranes and are suggested to play a role in binding and aggregating Aβ plaques[8]. Additionally, oligomeric Aβ has been shown to induce increases in proinflammatory cytokines like nitric oxide, TNFα and TNFβ[9].

Glutamate excitotoxicity overview

Glutamate excitotoxicity is thought to develop in AD as a result of sensitization of NMDA receptors, reduced glutamate uptake, and increased glutamate release at synapses[22]. Glutamate is involved in excitatory neurotransmission, and glutaminergic synapses are found in the hippocampus amongst other areas and are essential for learning and memory. At glutaminergic synapses in the hippocampus, memories are encoded and retrieved through the transmission of action potentials from pre-synaptic neurons to post-synaptic neurons (Fig. 1A). This process occurs when pre-synaptic neurons (at their axon terminals) release glutamate into the synapse, which then activates different types of glutamate-gated receptors on the post synaptic neuronal membrane (commonly the dendritic membrane) to induce excitatory post-synaptic potentials. These post-synaptic potentials sum together to induce larger changes in voltage known as action potentials in the post-synaptic neuron, which pass down the length of the neuron and induce neurotransmitter release at its own axon terminals to continue transmission.

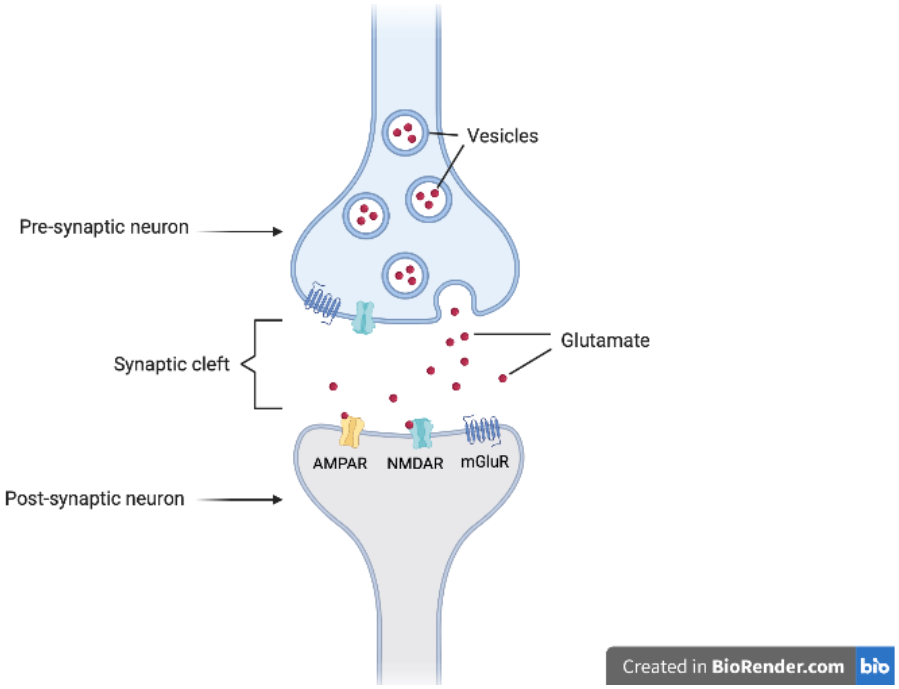

Figure 1A. Glutamatergic synapse. The pre-synaptic neuron releases vesicles of the neurotransmitter glutamate into the synaptic cleft, which diffuse across the synapse and activate glutamate receptors on the membrane of the post-synaptic neuron. Created with BioRender.com

There are two major classes of glutamate receptors involved in glutaminergic transmission: ionotropic glutamate receptors and metabotropic glutamate receptors[40]. Ionotropic glutamate receptors open in response to glutamate binding and allow most importantly K^+, Na^+, and Ca^{2+} ions to enter or exit the post-synaptic cell to cause either depolarizing (excitatory) or hyperpolarizing (inhibitory) graded potentials. Metabotropic glutamate receptors activate in response to glutamate binding and induce conformational changes in associated G-proteins bound to the intracellular portion of the receptor, and these G-proteins trigger a cascade of second messenger molecules inside the cell which have multiple widespread targets.

Ionotropic receptors: AMPARs

AMPA receptors (AMPARs) are a type of ionotropic glutamate receptor responsible for the majority of fast excitatory transmission at glutaminergic synapses and are located primarily at the post-synapse membrane[10]. These channels open and allow Na+ to enter and K^+ to leave to depolarize the post-synaptic cell[7] (Fig. 2A). They are responsible for the majority of depolarization occurring at the post-synaptic cell, and their activation underlies the mechanisms important to learning and memory in the hippocampus, particularly in their influence in restructuring dendritic spines[3]. In both human AD brains and neuronal cultures treated with Aβ, AMPARs are seen to be drastically reduced in number and increasingly tagged for degradation via ubiquitin[44]. Aβ alters the level of enzymes responsible for controlling the ubiquitination of AMPARs, which leads to increases in their ubiquitin-mediated degradation[44]. Alongside general reduction in the number of AMPARs, the weakening of hippocampal synapses results from reduced current through AMPARs leading to reductions in the number of dendritic spines, which is associated with the appearance of Aβ and tau pathology[3].

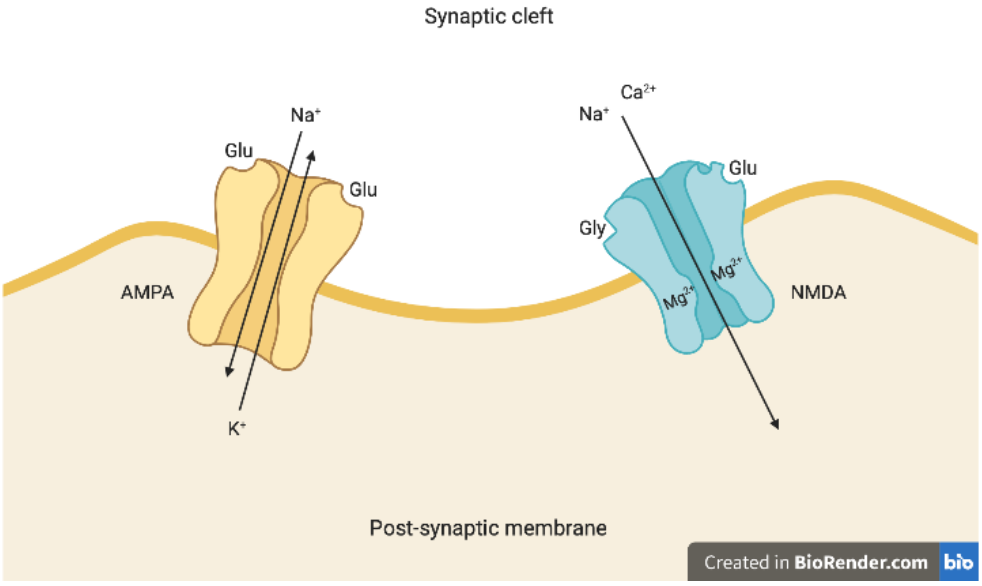

Figure 2A. Ionotropic glutamate receptors. AMPA receptors open in response to glutamate binding and allow Na^+ ions to enter the neuronal membrane. NMDA receptors open in response to glutamate and glycine binding and contain a voltage sensitive Mg^{2+} ion removed only by attaining a certain voltage. Opening of these receptors allow Na^+ and Ca^{2+} ions to enter neurons. Created with BioRender.com

Ionotropic receptors: NMDARs

Alongside AMPARs, NMDA receptors (NMDARs) play a large role in learning and memory through influencing the restructuring of synapses, which is known as synaptic plasticity[40]. NMDARs allow Na^+, K^+, and Ca^{2+} to flow through to induce depolarization, and Ca^{2+} in particular is essential for the plastic responses NMDARs preside over[40] (Fig. 2A). They contain a voltage sensitive Mg^{2+} block and show slower kinetics, and continued activation and depolarization from AMPRs allows the proper voltage to be achieved to remove the Mg^{2+} block to allow Ca^{2+} to enter the cell[41]. In AD, excessive activation of NMDARs, particularly those located away from the post-synaptic

membrane (extra-synaptic), contributes to excitotoxicity and is implicated in cell death[41]. This cytotoxicity is largely due to excessive influx of Ca^{2+} into the cell, as these rising Ca^{2+} levels correlate with the onset of cognitive decline[41].

NMDARs and amyloid beta

AB modulates NMDAR dysfunction in AD through complex interactions. In one manner, Aβ is involved in the reduction of synaptic NMDARs, which contributes to the weakening of plastic responses and loss of synaptic connections[23]. Additionally, Aβ is seen to activate extrasynaptic NMDARs that contain the GluN2B subunit, and Aβ also shows increased accumulation around NMDARs containing this subunit[23]. Activation of NMDARs may also increase the production and secretion of Aβ[23]. Aβ also induces glutamate release from astrocytes[38]. Astrocytes are a glial cell that provide support and protection for neurons through the blood brain barrier and at synapses, where they regulate molecular transport, metabolic activity, glutamate clearance[4]. The influence of AB may cause them to shift from their normally neuroprotective functions in favor of pro-inflammatory behaviors, such as altering the clearance and balance of glutamate at synapses[4].

Disruption in Ca^{2+} homeostasis is also implicated in neurodegeneration seen in AD, though this can result from a number of different mechanisms. As mentioned, increases in Ca^{2+} can in one way be ascribed to increased activation of NMDARs. These disruptions can also be generated by other pathways leading to excessive influx of Ca^{2+}

from the extracellular environment, and also are due to the release of Ca^{2+} into the cytosol from intracellular stores[13].

Post-synaptic NMDAR therapy

Due to the involvement of post-synaptic NMDARs in contributing to glutamate excitotoxity and oxidative stress, drug therapies for AD have explored methods of reducing the responsiveness of post-synaptic NMDARs to the increasing levels of glutamate. One method of doing so is application of an antagonist, which prevents the activity of the NMDARs in response to glutamate binding. One notable example of a post-synaptic NMDAR antagonist which has been approved for use in treating AD is memantine. Memantine is an uncompetitive antagonist that binds to post-synaptic NMDARs and prevents their activation only when NMDARs are bound by glutamate[15]. However, since its clinical approval Memantine has shown only modest effects in improving cognition and behavior, and is mainly used in moderate to severe AD[35]. Memantine cannot prevent the onset of cognitive decline in AD patients even if it does reduce symptoms for a short period of time. Side effects of memantine are reportedly uncommon, but include headache, fatigue, irritability, agitation, and most concernedly, increased confusion[35].

Though memantine blocks NMDARs only when bound to glutamate when levels are high within the synapse, the necessity of post-synaptic NMDARs in the functioning of synapses involved in memory processing makes antagonism of these receptors a point of concern and may even explain the confusion experienced by some patients. The potential

for disruption of memory is counterintuitive to treating a memory-loss disease such as AD. Thus, due to the limited effectiveness and concerning potential effects, a related therapeutic intervention to target excitotoxic glutaminergic transmission should be investigated. One method would be to modulate the release of glutamate from the pre-synaptic neuron rather than to reduce the responsiveness of the post-synaptic cell to increasing levels of glutamate. Controlling the release of glutamate by reducing the likelihood of glutamate release allows for glutaminergic synapses to function without impairing their normal roles in learning and memory, as this would only reduce the excessive release of glutamate down to normal baseline levels.

NMDAR, amyloid beta, and oxidative stress AD model systems

Therapeutics that target either Aβ or post-synaptic NMDARs independently have proven mostly disappointing, so perhaps a useful application of these targets involves models of AD for research. Aβ42 has a number of interactions within the neurons to contribute to neurotoxicity and oxidative stress, as described above. NMDA can be applied as an agonist of post-synaptic NMDARs to induces NMDAR toxicity, which mimics the conditions in AD where excessive stimulation of post-synaptic NMDARs causes cellular toxicity[41]. These effects suggest that combination Aβ and NMDA application may provide a model system that may target multiple pathways for generating the neurotoxic stress found in AD, which is a model system that has already been employed. In addition to Aβ and NMDA models, another commonly used model involving Aβ is FAB, which also includes FeSO4 and L-Buthionine. FeSO4 is an

oxidizing agent that triggers OS by oxidizing membrane and extramembrane intracellular molecules to generate ROS[21]. The application of this compound, which dissociates into Fe^{2+} and $So4^{2-}$ in aqueous solution also mirrors conditions found within the AD brain where iron ions interact with water molecules to produce ROS. Additionally, Fe^{2+} may also interact with the Aβ to generate ROS. L-Buthionine prevents the synthesis of GSH to weakens neuronal defense to OS, which mirrors the conditions of the aged brain[21]. The FAB model itself provides a multifaceted approach to create oxidative stress and neurotoxicity. However, due to the importance of glutamate toxicity in AD pathology, the addition of NMDA to create a combination FAB/NMDA model will provide a comprehensive model system with both oxidative stress and glutamate toxicity present for study.

Targeting pre-synaptic glutamate release: metabotropic glutamate receptors (mGluRs)

An alternative to post-synaptic NMDAR therapies involves exploring the reduction or limitation of pre-synaptic glutamate release. Targets that may lead to a reduction in the likelihood of pre-synaptic glutamate release are certain pre-synaptic metabotropic glutamate receptors. Metabotropic glutamate receptors (mGluRs) are involved in a variety of processes essential to neuronal function, and there exist 8 types, mGluR1-8. Located at the membrane of either the pre- and/or post-synaptic cell, each mGluR is composed of a large N-terminal extracellular "Venus flytrap" domain (ECD), a 7-pass membrane spanning region (7TM), and a C-terminal intracellular domain (ICD) which associates with the G-protein[14](Fig. 3A). The orthosteric ligand, glutamate, binds in the

ECD, while allosteric ligands bind in the cysteine-rich region that links the ECD and 7TM or in the 7TM[14]. An allosteric ligand binds to a site other than the orthosteric site[28]. MGluRs are divided into three groups based on the intracellular signaling pathway associated with their G-protein; Group I includes mGluR1 and 5; group II includes mGluR2 and 3; and group III includes mGluR4, 6, 7, and 8. Primarily, Group I is linked to the phospholipase C (PLC)/ Ca^{2+} cascade while groups II and III are linked to the adenylyl cyclase (AC)/cyclic AMP/PKA cascade[14].

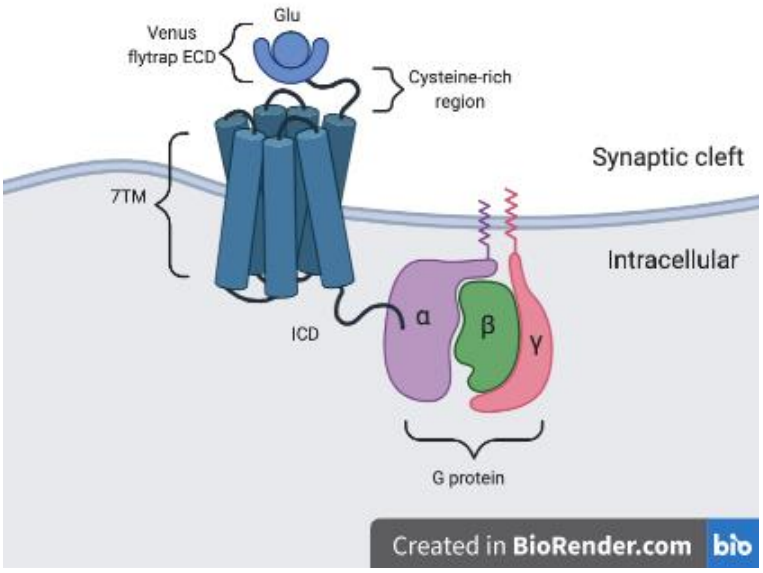

Figure 3A. Structure of metabotropic glutamate receptors. These receptors contain an extracellular "Venus flytrap" domain where glutamate binds, a cysteine-rich region where allosteric ligands bind, a 7-pass membrane spanning region, and the intracellular domain that associates with a G-protein. The G-protein consists of an alpha, beta, and gamma domain, of which the alpha and gamma are associated with the membrane. Created with BioRender.com

Group I mGluRs

Group I receptors are located in the post-synaptic neuron, and their activation of the PLC cascade increases excitability of the post-synaptic membrane[14](Fig. 4A). When

activated, the associated G-protein induces PLC to cleave membrane-bound PIP3 into DAG and IP3. IP3 can then open IP3-gated Ca^{2+} channels in the membrane of the endoplasmic reticulum to release Ca^{2+} stores into the cytosol. Alongside Ca^{2+} acting as a signaling molecule on its own, Ca^{2+} can bind and activate protein kinase C, which when bound to the membrane-associated DAG, can enact its own downstream effects. Group I mGluRs contribute the plastic responses modulated by NMDARs by increasing their current and trafficking to the membrane[14]. Dysfunction of mGluR5 in particular may be implicated in AD especially those found in hippocampal astrocytes, as astrocytes localized near Aβ plaques overexpress mGluR5, as this is associated with dysregulated Ca^{2+} levels and synaptic death[14].

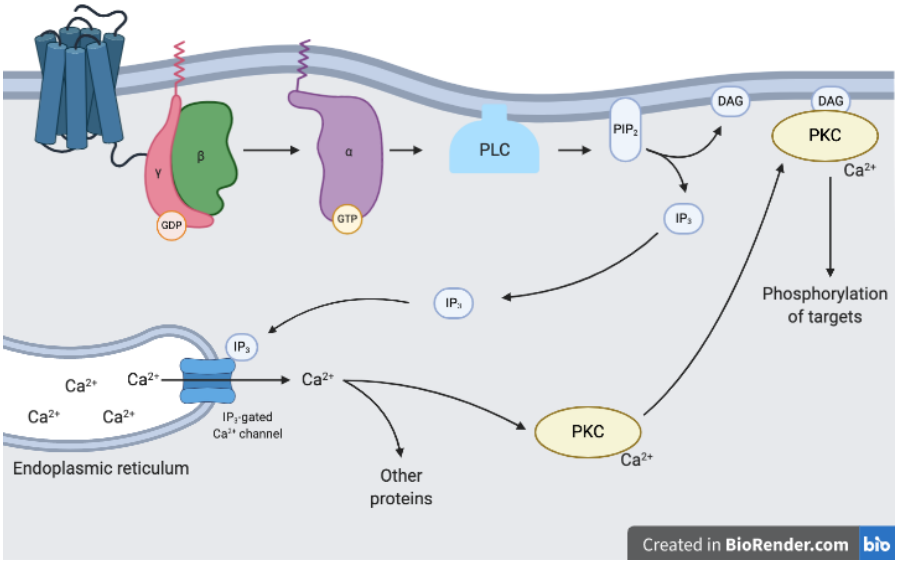

Figure 4A. Phospholipase C pathway. Activation of the G-protein coupled receptor activates the associated G-protein, which releases its alpha domain. The alpha domain activates phospholipase c (PLC), which cleaves PIP3 into DAG and IP3. IP3 opens IP3-gated Ca^{2+}channels located on the endoplasmic reticulum membrane, which releases Ca^{2+} into the cytosol. Ca^{2+}can bind to and activate protein kinase c (PKC), which binds to DAG on the membrane and allows target molecules to become phosphorylated by PKC. Created with BioRender.com

Group II mGluRs

Group II receptors are located on the pre-synaptic cell and their activation inhibits the adenylyl cyclase (AC) cascade to reduce excitability in the pre-synaptic cell[14] (Fig. 5A). In the absence of stimulation by these receptors, AC converts ATP into cyclic AMP (cAMP), which then activates protein kinase A (PKA). PKA has a number of targets, notably Ca^{2+} channels which PKA opens to increase Ca^{2+} influx and thus the probability of neurotransmitter release in the pre-synaptic cell, as well as K^+ channels which PKA closes to prevent K^+ efflux thus also increasing excitability. Activation of Group II receptors inhibits this process, opening K^+ channels and closing Ca^{2+} channels to reduce excitability and the probability of neurotransmitter release respectively[14]. These type II receptors are implicated in potential neuroprotective abilities due to their effects on the AC cascade in reducing the probability of neurotransmitter release, which in the case of glutamate excitotoxicity in AD would prove beneficial.

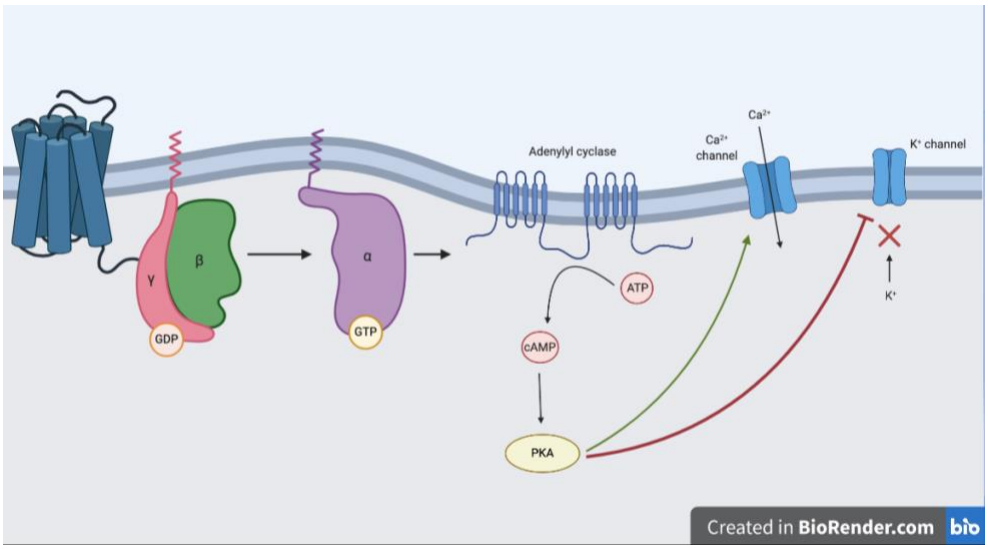

Figure 5A. Adenylyl cyclase (AC) pathway. When activated, the associated G-protein can release its activated alpha domain. The alpha domain activates adenylyl cyclase, which allows the formation of cyclic AMP (cAMP) from ATP. cAMP can activate protein kinase a (PKA), which opens Ca^{2+} channels to allow Ca^{2+} into the neuron while also blocking certain K^{+} channels to prevent K^{+} from leaving the neuron. Created with BioRender.com

Group III mGluRs

Group III receptors also are coupled to and inhibit the AC cascade and are also located on the pre-synaptic neuron, and they function at glutamatergic synapses as negative regulators of glutamate release[14]. This group of receptors play an essential role in the regulation of vesicle release and their regulation is dependent on the activity at the synapse, so they function to reduce neurotransmitter release in response to high levels of excitation and vice versa[27]. Expression of group III receptors is common across the brain, in particular the cortex, hippocampus, and basal ganglia[14]. Dysfunction of these receptors is not highly implicated in AD pathology, but their function in reducing excitability and probability of glutamate release in the hippocampus and cortex, regions highly affected in

AD, has led pharmacological manipulation of these receptors to be a topic of great interest and potential for AD therapy.

Group III mGluRs: mGluR4

MGluR4 in particular is a group III receptor that shows promise in neuroprotection. In addition to inhibition of AC, their potential for neuroprotection is also thought to be linked to inhibition of the MAPK and JNK pathways, which aids particularly in oxidative stress conditions. MAPK and JNK both serve as activators of procaspase-8/9/3, which are factors involved in the initiation of the cellular death process known as apoptosis[45]. This occurs via alterations in the balance of Bcl-2 (which prevents release of cytochrome c from the mitochondria to prevent apoptosis) and Bax (which induces cytochrome c release to trigger apoptosis)[45]. There are a few documented positive allosteric modulators and agonists of mGluR4 seen to show neuroprotective benefits. Positive allosteric modulators (PAM) bind in allosteric sites and require the presence of the orthosteric ligand to bind, and they potentiate the responses initiated by the orthosteric ligand, which in this case is glutamate[28]. Conversely, negative allosteric modulators (NAM) inhibit the response initiated by the orthosteric ligand[28]. Agonists, however, bind directly to the receptors without regard for the orthosteric ligand to activate the receptor.

Group III mGluR agonist: L-AP4

L-AP4 is a general group III receptor agonist that has been investigated for its neuroprotective abilities. L-AP4 has been used for research particularly in regard to

Parkinson's disease, where areas affected include the basal ganglia and cerebellum[6]. However, studies have shown that L-AP4 does have the potential to induce neuroprotection in other regions, such as those more pertinent to AD like the hippocampus. In a study of mGluR4 function in mouse and rat cerebellar parallel fiber-purkinje cells from cerebellar slices, L-AP4 was seen to significantly depress excitatory post-synaptic currents (EPSCs) by 5.8%[6]. These effects were confirmed to be due to mGluR4 as this is the only group III receptor present in purkinje cells pre-synapse, and co-application with the broad group III antagonist MSOP reduced L-AP4 depression of EPSCs significantly to 3.6%[6]. L-AP4 was also shown to depress ESPCs in cultured mouse hippocampal neurons by decreasing the probability of neurotransmitter release[16]. One important aspect of this synaptic depression is influencing Ca^{2+}levels in the pre-synaptic cell, which Abitbol et al. elucidate is due to mGluR4's effect on all types of pre-synaptic voltage-gated Ca^{2+} channels (VGCCs)[1]. They determined this in their study of purkinje cells in cerebellar rat slices, where L-AP4 reduced pre-synaptic Ca^{2+} influx by 2.3%, and inhibitors of each different VGCC attenuated the effect of L-AP4 on Ca^{2+} current to an extent[1]. The reduction in EPSCs would imply a reduction in neurotransmission at these synapses, as EPSCs are evoked by mechanisms such as glutamate released by the pre-synaptic cell acting on post-synaptic channels to induce these post-synaptic currents; reduction in Ca^{2+} current would allow this to occur as Ca^{2+} is needed to localize neurotransmitter vesicles to the pre-synaptic membrane. In fact, L-AP4 has been shown to directly impact glutamate release. L-AP4 was shown to induce a concentration-dependent reduction in release of D-aspartate in rat subthalamic nuclei,

maximally by 4.5% at 30uM[2]. D-aspartate was used in this study as an approximation of glutamate, as it is a glutamate analog and shares the same transporter as endogenous glutamate. The effect of L-AP4 was confirmed to be due to mGluR4 activity as pre-application of the selective group III mGlu antagonist CPPG inhibited the effect of L-AP4 on aspartate release[2].

mGluR4 PAM: PHCCC

PHCCC (Fig. 6A) is an mGluR4 PAM and, though less widely researched, it has shown neuroprotective benefits. PHCCC has been described to provide neuroprotection during ischemic brain damage, where blood flow is reduced to the brain[26]. This was discovered as knockout mGluR4 mice showed 25% to 30% increased infarction (reduction of blood supply), and injection of PHCCC reduced ischemic damage by 35% to 45%[26]. Rats treated with PHCCC also showed better recovery from ischemic brain damage[26]. More relevantly, Maj et al. describe that PHCCC provides neuroprotection against NMDA and Aβ toxicity in mouse cortical neuronal culture, which is blocked by application of MSOP[25].

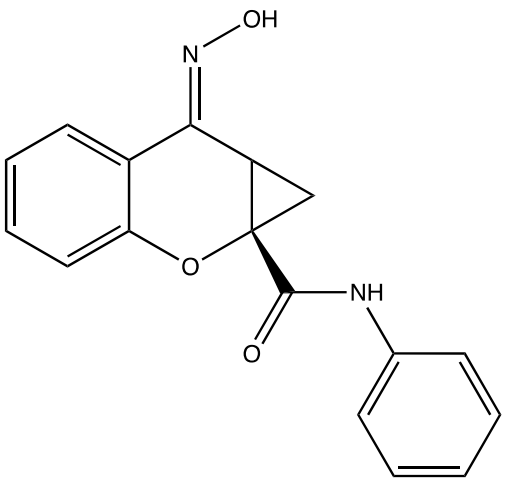

Figure 6A. Structure of PHCCC. Created with ChemDraw.

Project scope

Evidently, there is promise in the investigation of mGluR4 in providing neuroprotection. Due to the limited nature of research into mGluR4 PAMs in the context of AD, the present study aims to investigate a new potential PAM of mGluR4 known as RD100, named after its synthesis in Dr. Ronald J. Doll's lab of the Drew University RISE department (Fig. 7A). This pilot study investigates whether RD100 (5uM) provides neuroprotection in an FAB/NMDA model of AD in cultured mixed rat cortical neurons, and seeks to elucidate whether its potential mode of action is via PAM of mGluR4. The mode of action will be investigated with co-application of the group III receptor antagonist MSOP (1 mM). This study also seeks to optimize the utilization of the FAB/NMDA model system in cell culture by determining appropriately neurotoxic concentrations and oxidative stress markers activated by its application. Oxidative stress will be evaluated with application of MitoSOX red mitochondrial superoxide indicator.

Additionally, this study seeks to optimize which data collection measures are most useful and reflective for the MitoSOX indication of superoxide and oxidative stress.

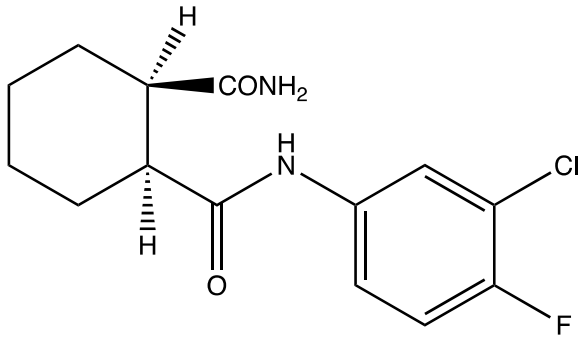

Figure 7A. Structure of RD100. Created with ChemDraw.

Hypotheses and predictions

It is predicted that RD100 will provide neuroprotection against FAB/NMDA induced toxicity as shown through increased cell survival using MTS assays, and that FAB/100 uM NMDA will induce concentration-dependent negative effects on cell survival. It is hypothesized that FAB dilutions in 100 uM NMDA will produce concentration-dependent markers for oxidative stress as indicated by MitoSOX red mitochondrial superoxide indicator, and it is predicted that application of RD100 will reduce the relative intensity and binary area of MitoSOX signal visualized under a compound microscope. It is predicted that application of MSOP will negate the neuroprotection provided by RD100, leading to decreased cell survival using MTS assay.

METHODS

Model System and Dissection

Dissections of E18 rat embryos were performed under sterile technique. Embryonic rat heads were first dissected to remove the intact brain from the skull and surrounding facial tissues. The dura was punctured and peeled off, and the intact embryonic brain was removed while excess tissues were discarded. The hind- and mid-brain sections were removed, leaving only the two frontal lobes. The outer meningeal layer was removed from both lobes, followed by dissection to remove any vessels running throughout the cortical tissues. The remaining vessel-free frontal lobe tissue was dissociated for further processing to be plated.

Plating Conditions

Dissociated cells were pipetted first into trypsin, followed by two washes of HBSS, and then plating media (PM) enriched with Fetal Bovine Serum. Cells were diluted in PM to a concentration of 1.0 x 10^6 cell/mL. Cells were plated into 24 and 96-well plates. 100uL of the cell solution was plated in each well for a total of 60 wells in the 96-well plate. 25uL of cells were plated in each well of the 24-well plate. Cells were maintained in a 40°C incubator at 5 % CO2 and 5 % O2, and fed by replacing half the media with growth media (GM) every 3-4 days.

Preparation of Stressors

Stressors were applied on days 12-14 in culture and removed after 24hr incubation. FeSO4, amyloid beta, L-buthionine (FAB) and NMDA were utilized to simulate the cytotoxic conditions of Alzheimer's disease. FAB in its highest concentration (100%) was prepared using 7.95mg FeSO4, 133.5mg buthionine, and 1uM Aβ into 50mL GM. Serial dilutions of FAB in GM were prepared, and include 100%, 50%, 33.3%, 25%, and 20% FAB. To each concentration was added 30mM stock NMDA prepared in DMSO in order to yield 100 uM NMDA in each dilution. The FAB/100 uM NMDA dilutions were stored at -20°C.

Preparation of Stimulations

Stimulations were applied on days 12-14 in culture and removed after 24hr incubation. RD100 was prepared in a 30mM stock in DMSO, and concentrations of 5 and 10 mM were prepared in GM. The stock and dilutions were stored at -20°C. MSOP was prepared in a 10mM stock in GM. One dilution of 1 mM was prepared in GM. The stock and dilution were stored at 4°C.

Assays

On day 13-15 in culture, MTS (Microtubule Staining), ICC (Immunocytochemistry), and MitosSOX assays were performed. MTS dye was prepared in GM and incubated on 96-well plates for one hour. A microplate reader from which the absorbance values at 490 nm were obtained was used to calculate mean cell count values.

ICC was carried out on 24-well plates and select 96-well plates. Cells were fixed in 4% formaldehyde for 20 mins and washed with phospho-buffered saline (PBS) and 0.5% Triton-100 (detergent). Cells were then incubated with primary anti-tubulin mouse antibody for one hour, washed with PBS, and incubated for one hour with secondary fluorescent CY3 or FITC anti-mouse antibodies. Cells were visualized under a compound fluorescent microscope.

MitoSOX™ Red Mitochondrial Superoxide Indicator was prepared in a 30mM stock in DMSO and shielded from light exposure. The dye was incubated with cells for 10 minutes and then replaced with HBSS. Cells were fixed with 4% formaldehyde for 20 mins and washed with PBS, then visualized under a compound fluorescent microscope. Data collected included relative whole image intensity (calculated by obtaining mean – min whole image intensity), binary area (area of the image only reflecting neuronal matter with the background subtracted), and relative intensity/binary area.

Data collection

For MTS trials, a microplate reader with SoftMax Pro 6.2.1 was used to collect data values. In some trials, the Amersham Imager 600 was used to collect images, which were then analyzed by ImageQuant TL by GE. NIS elements software was used to collect and analyze images of cells under CY3 and FITC fluorescent filters for the MitoSOX trials.

Data analysis

All data was normalized within each trial to the control GM-only condition. Data is represented with error bars showing standard error of the mean (SEM). Statistical analysis included two-way ANOVA and one-way ANOVA with Tukey post-hoc analysis. Data is reported with p values and N indicating sample sizes.

RESULTS

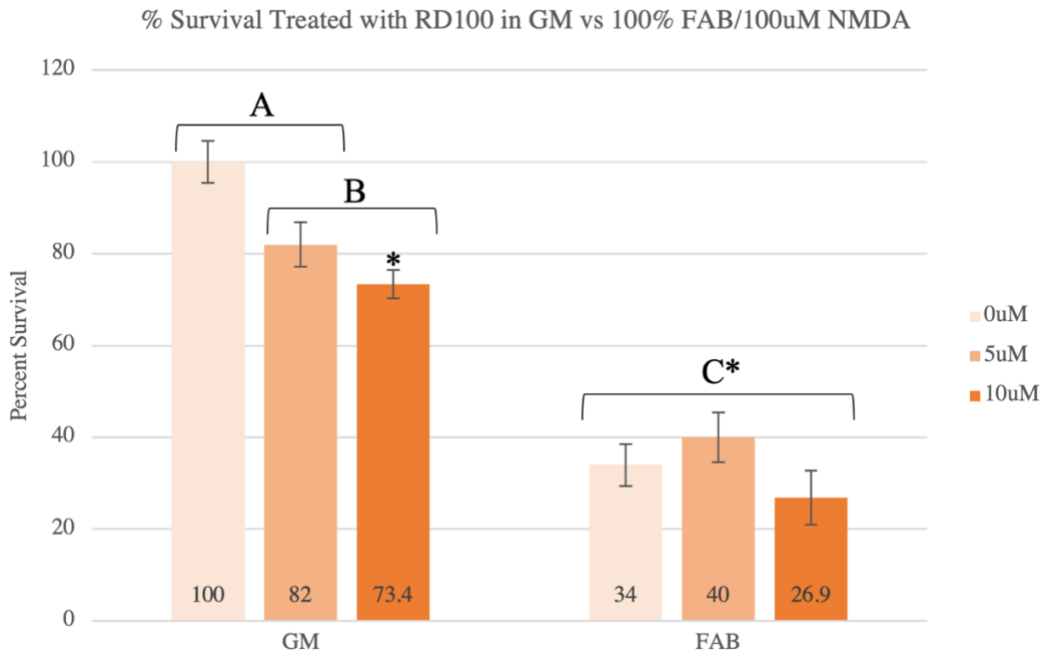

Figure 1B. Percent cell survival in GM or stressed with 100% FAB/100 uM NMDA and treated with 0uM, 5uM, or 10 uM RD100. Data is represented in percent survival and normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. For each group, N=10. Groups statistically different from the control by $p<0.05$ are indicated with *. Groups that are statistically equivalent are indicated with brackets and designated with either A, B, or C for equivalent groups.

To explore whether RD100 exerts any effect on cell survival, primary mixed cortical rat neurons were stimulated with 5 uM and 10 uM RD100 in either GM or stress with 100% FAB/100 uM NMDA (see Methods) (Fig. 1B). There is a significant main effect on cell survival of stress (GM or FAB) and of RD100 ($p<0.0005$, $p=0.003$). There is also an interaction between stress and RD100, indicating that the effect of RD100 varies dependent on whether it is applied in GM or 100% FAB/100 uM NMDA ($p=0.033$). The average survival of all 100% FAB groups is significantly reduced compared to all GM groups, indicating the significant effect of FAB in decreasing

survival ($p<0.0005$). RD100 shows a dose-dependent decrease in cell survival in GM, where only 10 uM RD100 significantly reduces cell survival compared to the control ($p=0.003$). RD100 does not significantly affect survival in 100% FAB/NMDA.

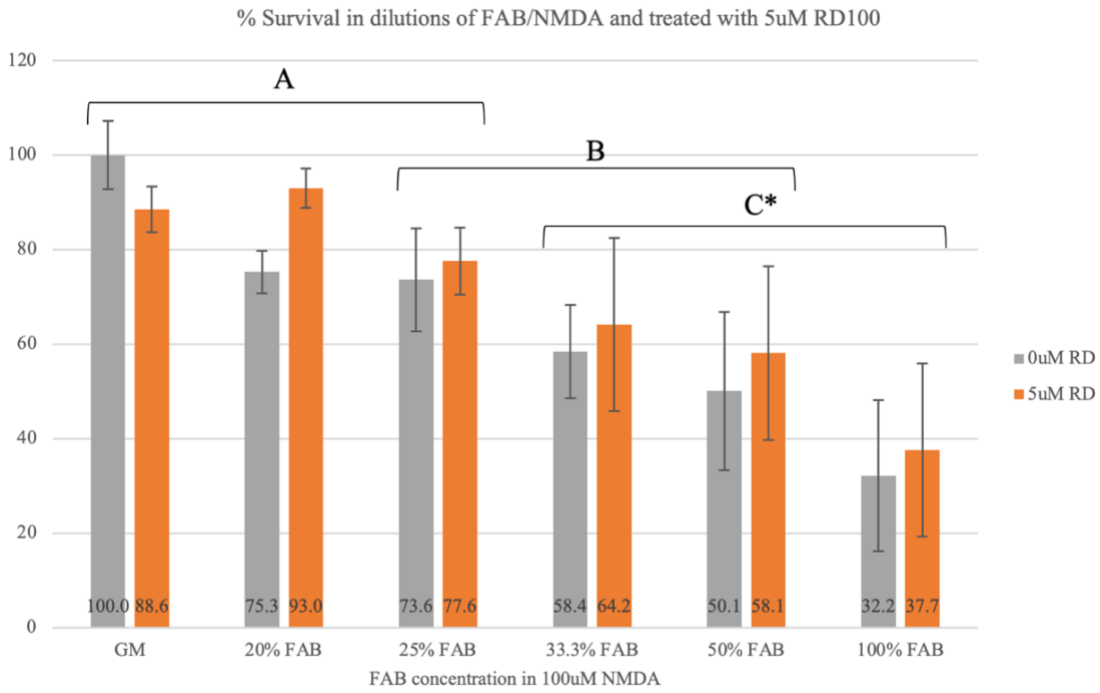

Figure 2B. Percent cell survival in GM or stressed with dilutions of FAB/100 uM NMDA and treated with 5 uM RD100. Data is represented in percent survival and normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. For each GM and 100% group, N=15. For all other groups, N=5. Groups statistically different from the control by $p<0.05$ are indicated with *. Groups that are statistically equivalent are indicated with brackets and designated with either A, B, or C for equivalent groups.

To elucidate any effect of FAB/100 uM NMDA on cell survival, concentrations of FAB in 100 uM NMDA were applied to cultured mixed cortical rat neurons (see Methods) (Fig. 2B). Any effect of 5 uM RD100 was also investigated at each of these FAB dilutions. There is no effect of RD100, as at each concentration of FAB RD100

does not significantly alter cell survival ($p= 0.451$). There is a main significant effect of the concentration of FAB on cell survival ($p<0.0005$). Increasing FAB concentration induces a dose-dependent decrease in cell survival, where 33.3%, 50%, and 100% FAB/100 uM NMDA significantly reduce cellular survival ($p=0.038$, $p=0.003$, and $p<0.0005$, respectively). The percent survival for each of these FAB dilutions are statistically equivalent.

Figures 3B, B4, 5B Overview

To investigate whether FAB/NMDA effects markers of oxidative stress, MitoSOX superoxide radical indicator was applied to cultured mixed rat cortical neurons stressed with dilutions of FAB/100 uM NMDA in GM (see Methods). Any effect of 5 uM RD100 on MitoSOX was also evaluated. Increased oxidative stress is indicated by increased intensity of MitoSOX signal or increased image area covered by neuronal processes showing MitoSOX fluorescence. Image intensity was assessed by calculating relative intensity from the mean whole image intensity and minimum whole image intensity (mean – min) (Fig. 3B). Image area was assessed by binary image area, which is the image area only covered by neuronal matter with the background subtracted out (Fig. 4B). Relative intensity was normalized to binary area to account for any changes intensity as a result of changes in the area covered by neuronal matter and is represented as relative intensity/binary area (Fig. 5B).

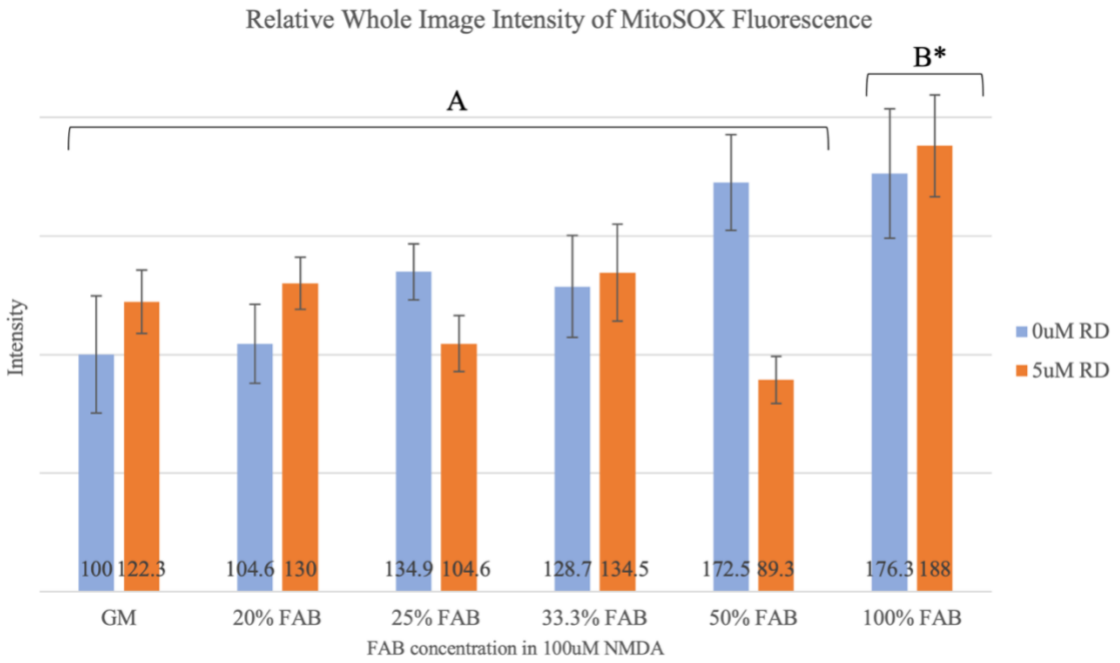

Figure 3B. Relative intensity of MitoSOX signal of cells stressed with dilutions of FAB/100 uM NMDA and treated with 5 uM RD100. Increased oxidative stress is indicated by increased relative intensity. Data is represented as relative intensity, calculated from mean whole image intensity – minimum whole image intensity. Data was normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. For each group, N=6. Groups statistically different from the control by $p<0.05$ are indicated with *. Groups that are statistically equivalent are indicated with brackets and designated with either A or B for equivalent groups.

There is a significant main effect of FAB concentration ($p=0.020$) and an interaction between RD100 and FAB on relative whole image intensity ($p=0.023$) (Fig. 3B). There is no effect of RD100 on relative intensity ($p=0.613$). 100% FAB/100 uM NMDA significantly increases relative intensity compared to control ($p=0.001$) and compared to all other dilutions of FAB and GM ($p=0.004$, 0.005, 0.027, 0.027). In 50% FAB/NMDA, 5 uM RD100 decreases relative intensity compared to 0uM RD100 ($p=0.082$), the only concentration of FAB where there is a trend for RD100.

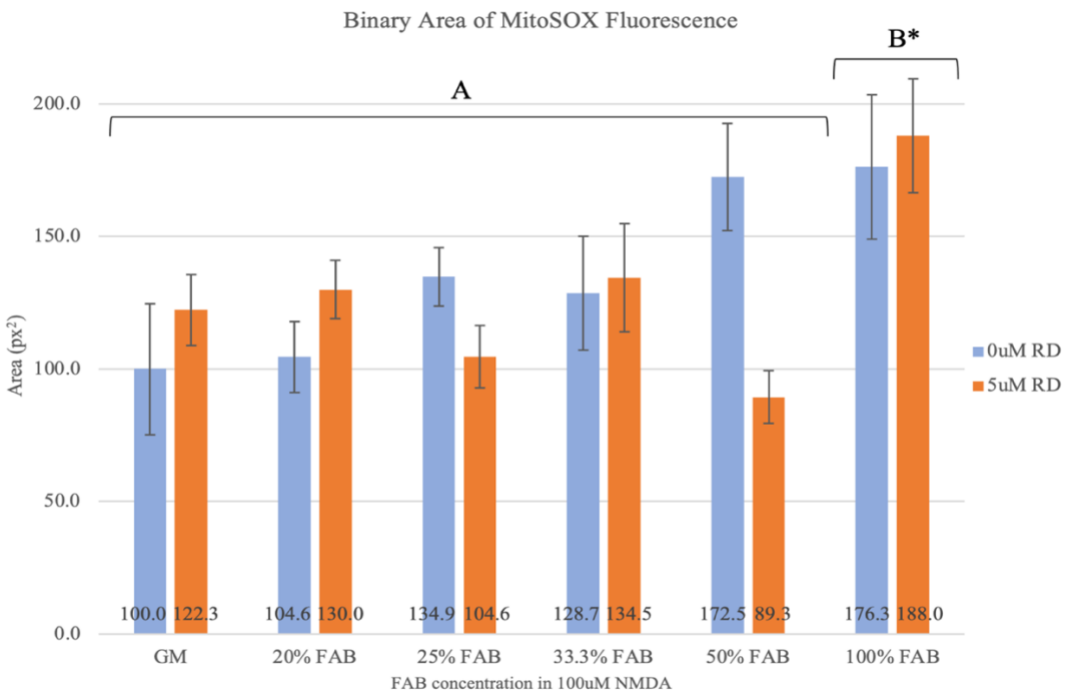

Figure 4B. Binary area of MitoSOX signal of cells stressed with dilutions of FAB/100 uM NMDA and treated with 5 uM RD100. Increased oxidative stress is indicated by increased binary area. Data is represented as binary area in px^2, which represents only the region of the images covered by neuronal processes with the background subtracted. Data was normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. For each group, N=6. Groups statistically different from the control by $p<0.05$ are indicated with *. Groups that are statistically equivalent are indicated with brackets and designated with either A or B for equivalent groups.

There is a significant main effect of FAB concentration (p=0.006) (Fig. 4B). There are no significant individual effects of RD100 on binary area across all dilutions (p=0.486) and no interaction between RD100 and FAB on binary area (p=0.053). 100% FAB/100 uM NMDA significantly increases relative intensity compared to control ($p<0.0005$) and compared to all other dilutions (p=0.001, 0.002, 0.010, 0.009).

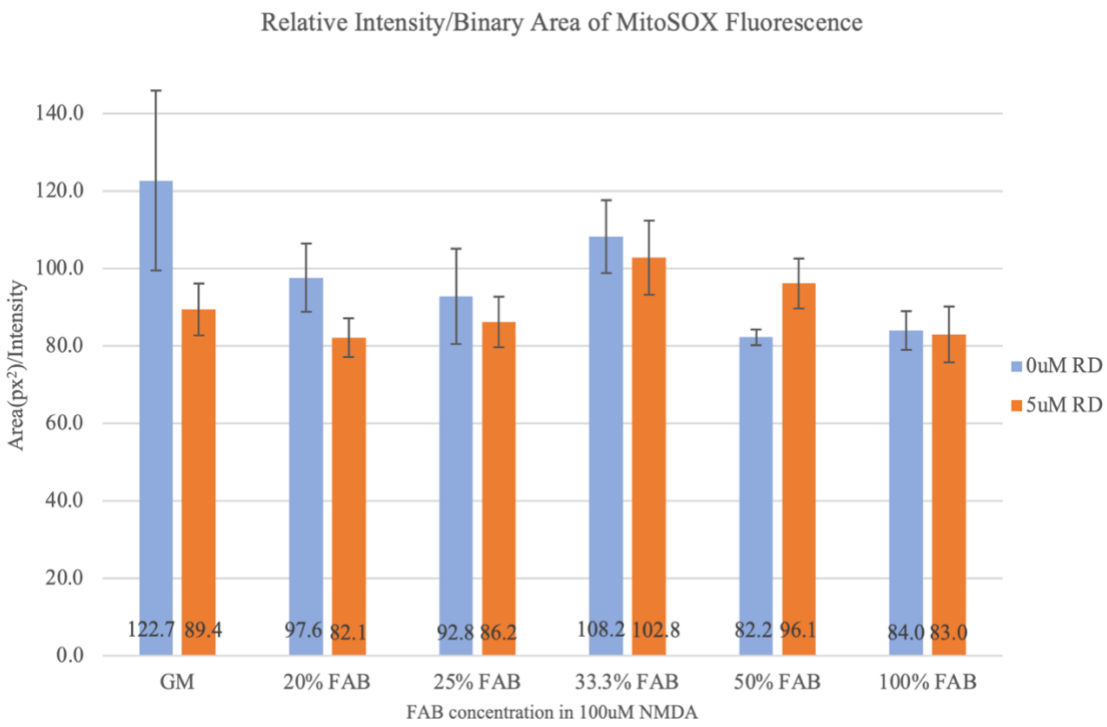

Figure 5B. Relative intensity/binary area for MitoSOX signal of cells treated with dilutions of FAB/100 uM NMDA and treated with 5 uM RD100. Data is represented as relative intensity/binary area and was normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. For each group, N=6. The means of all groups are statistically equivalent.

When relative intensity is normalized to binary area, the effects of relative intensity and binary area are negated, and there is no longer an effect of FAB concentration ($p=0.114$) nor RD100 ($p=0.191$) and no interaction ($p=0.308$) (Fig. 5B).

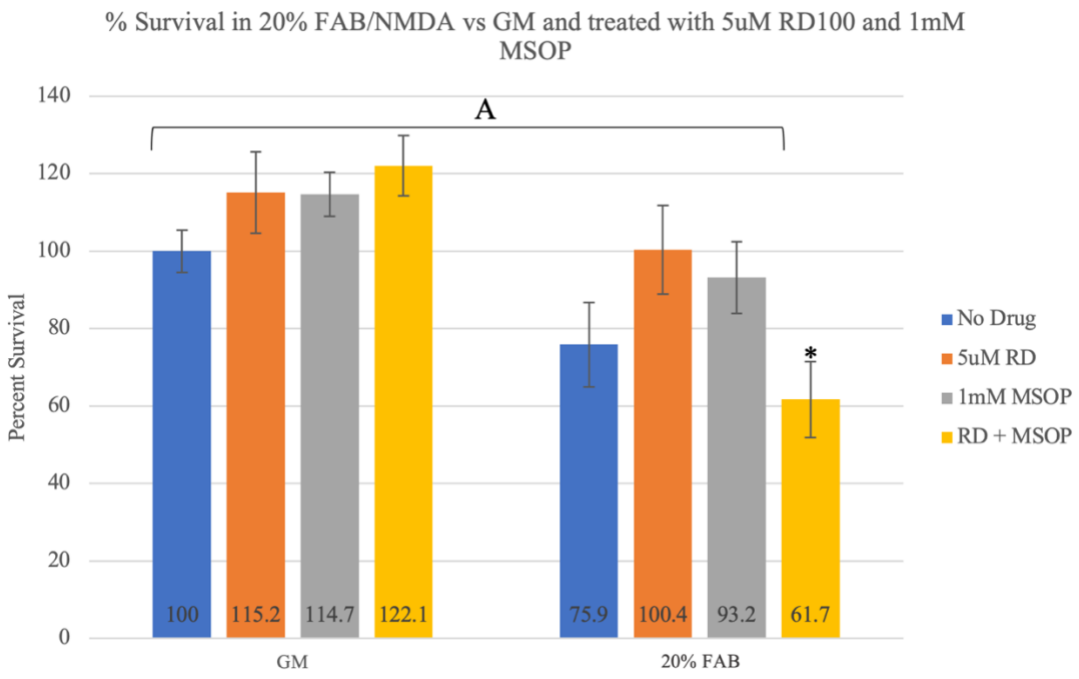

Figure 6B. Percent cell survival in GM or stressed 20% FAB/100 uM NMDA and treated with 5 uM RD100 and 1 mM MSOP. Data is represented in percent survival and normalized to the control condition of 0uM RD100 in GM. Error bars represent SEM. Control N=36, all other groups N=12. Groups statistically different from the control by $p<0.05$ are indicated with *. Groups that are statistically equivalent are indicated with brackets and designated as A.

To investigate whether RD100 acts on mGluR4, the broad group III antagonist MSOP (1 mM) was applied alongside 5 uM RD100 in 20% FAB/NMDA to mixed rat cortical neurons (see Methods) (Fig. 6B). There is a significant effect of 20% FAB on cellular survival ($p<0.0005$), as the average cellular survival of all FAB groups are statistically reduced compared to the averages of all GM groups. However, compared to control, 20% FAB on its own does not significantly reduce cellular survival ($p=0.358$). There is no effect of RD100 or MSOP alone ($p=0.610$ and 0.884, respectively). Though individually RD100 and MSOP show no effect on survival, there is a significant

interaction between RD100 and MSOP ($p=0.023$), as combination RD100 + MSOP in FAB significantly decreases survival compared to control ($p=0.018$).

DISCUSSION

Optimizing the FAB/NMDA model of AD

Before elucidating the effects of RD100, it was first important to create an environment with characteristics akin to AD to appropriately stress cells. The FAB model proves to be a multifaceted model, with FeSO4 to induce oxidation of molecules to generate ROS, L-buthionine to weaken cell defense to OS through reduced GSH as seen in aged brains[21], and amyloid beta as a contributor to OS through interactions with metal ions[37] and extrasynaptic NMDARs[19]. The addition of the agonist NMDA induces glutamate toxicity similar to AD with elevated post-synaptic NMDAR stimulation[41]. While understood to be a model providing many characteristics of AD, due to the extent of the neurotoxicity afforded by 100% FAB/100 uM in the initial trial (Fig. 1B), a concentration of FAB/100 uM NMDA where neurotoxicity was significant, but yet not overly toxic to where cell viability was unable to be rescued with therapeutic intervention as with the 100% solution had to be determined. Thus, a concentration curve with serial dilutions of FAB in GM while retaining a concentration of 100 uM NMDA was conducted (Fig. 2B).

It was determined that, in alignment with initial hypotheses, FAB/100 uM NMDA does have a dose-dependent negative effect on cell survival, with the 33.3%, 50%, or 100% FAB/100 uM NMDA solutions each providing significant neurotoxicity (Fig. 2B). 100% FAB/NMNDA shows the most severely progressed neurotoxicity, an effect identified previously (Fig. 1B). Though a strong effect, it may not always be optimal to examine effects where nearly 70% of all cells are dead. Thus, identifying two additional

concentrations which provide a modest but significant effect with around 40-50% cell death is a very useful finding for future use of FAB/NMDA in this lab and for additional work identifying the effects of other neuroprotective compounds. This grants the ability to examine facets of this AD model in varied states of cell death progression.

Identifying the effects of RD100 in FAB/NMDA

It is suggested due to the degree of similarity in structure between RD100 and the mGluR4 PAM PHCCC that positive activity on mGluR4 might be one of the primary functions of RD100. If this is so, a possible explanation for potential neuroprotection provided by RD100 is the reduction in successive glutamate release though activation of mGluR4 and subsequent blockage of the AC pathway. Blocking this pathway may prevent excessive glutamate release by inhibiting the downstream effects of PKA, which normally allows Ca^{2+} into the pre-synaptic cell and prevents K^{+} efflux to sensitize neurotransmitter release[14]. This may prevent the development or progression of glutamate excitotoxicity. This protective effect is suggested based on the observation of PHCCC affording neuroprotection against NMDA and AB-mediated toxicity in mouse cortical neurons[25], which is a similar model to what is employed in the present study. Activity on these receptors is additionally suggested to have beneficial promise due to the effects of L-AP4, the group III agonist, which was shown to depress EPSCs[6], reduce pre-synaptic Ca^{2+} influx[1], and reduce D-aspartate (an approximation for glutamate) release[2] when acting on mGluR4 in Parkinson's studies using cerebellar neurons.

In the initial trial evaluating the neuroprotection of different concentrations of RD100 (5 uM vs 10uM), it was determined that there is no concentration of RD100 that provides significant neuroprotection in 100% FAB/100 uM NMDA (Fig. 1B). However, the FAB/NMDA solution utilized may have been so neurotoxic that RD100 may not have been capable of providing a protective effect due to severely progressed cell death, with nearly 70% of cells impacted. Due to the degree of cell death, cell count may have been severely reduced to the extent where effects are being evaluated in by the survival of just tens to hundreds of cells. In GM as well, no concentration of RD100 provided protection, and conversely to what was predicted, 10 uM RD100 induced neurotoxicity. Thus, 5 uM RD100 was chosen for further study, as though it did not significantly improve survival in undiluted FAB/NMDA, it did not worsen toxicity as 10 uM RD100 did.

The unexpected finding of toxicity using 10 uM RD100 in GM may be explained. If RD100 were to act as a PAM on mGluR4 as is thought, a possible explanation for the induction of neurotoxicity is by over-inhibition of glutamate release in glutamatergic synapses when cells are otherwise functioning at normal glutamate levels. Lack of appropriate stimulation in this case could induce atrophy of cells and lead to cell death due to the depression of glutamate release and levels.

To investigate any effect of 5 uM RD100 in less neurotoxic conditions than the extreme effects noted with 100% FAB/NMDA, effects of RD100 were also evaluated in FAB dilutions to assess whether in less progressed neurotoxicity RD100 might provide significant effects (Fig. 2B). It was confirmed that in each of these dilutions, RD100 does

not provide significant neuroprotection, effectively rejecting the hypotheses regarding RD100's potential neuroprotection in the FAB/NMDA model system.

Evaluating effects on oxidative stress and optimizing data collection

Once optimal concentrations of FAB/NMDA were identified (Fig. 2B), it was next sought to identify if FAB impacts ROS, markers of OS. The effects of FAB/NMDA on levels of the ROS superoxide within cells were investigated, with MitoSOX used as an indicator of superoxide presence (Fig. 3B, Fig. 4B, Fig. 5B). Though RD100 was confirmed not have an effect on cell survival across all dilutions of FAB/NDMA (Fig. 2B), it was also explored if RD100 might have an effect on superoxide, as effects on this molecule may implicate that RD100 could impact cell viability or integrity through OS, since it does not significantly impact survival.

Before this could be done, it was necessary to optimize the data collection methods for MitoSOX to determine which measure accurately reflects superoxide presence in cells imaged under the compound microscope. Initially, multiple measures of MitoSOX were collected from images of cells treated with FAB/NMDA dilutions, including binary image intensity, whole image intensity, mean image intensity, minimum image intensity, whole image area, and binary image area. After collecting and processing this data, it was determined that utilizing relative intensity (calculated by subtracting mean-min whole image intensities) and binary area (the area of the image covered only by cell processes) provide the most meaningful representations of the presence of superoxide within cells.

In utilizing the measures of relative intensity and binary area, for both of these measures across the dilutions of FAB/NMDA the trends for relative intensity and binary area aligned very closely. FAB/NMDA had a significant effect on MitoSOX with an interaction between RD100 and FAB/NMDA (Fig. 3B, Fig. 4B). RD100 alone had no effect on MitoSOX. For both measures, 100% FAB/NMDA significantly increased both relative intensity and binary area, while at the 50% dilution of FAB/NMDA, application of RD100 induced a harsh drop in the averages of both measures when compared to 0uM RD100. Due to the close alignment of these trends, it was thought to correct and normalize relative intensity to the binary area, as fluctuations in intensity could directly be a result of more or less MitoSOX due to the number of cells covering the image rather than as an effect of the compound applied. Thus, relative intensity was corrected by dividing by the binary area, to represent intensity by area (Fig. 5B).

Once this was completed, the effects of FAB and RD100 on MitoSOX in both measures were nullified, losing the previously noted significance (Fig. 5B). Based on these findings after correction, neither FAB/NMDA nor RD100 impact superoxide levels, however the timing of superoxide assessment must be addressed. Superoxide levels were measured 24 hrs after FAB/NMDA and RD100 application, so these results only show that at 24 hrs after application FAB/NMDA and RD100 do not have an effect on superoxide. It is unable to be confirmed if either compounds have effects at any other point in time before 24 hrs. Possible effects on other ROS involved in OS were also unable to be investigated, So, the initial hypotheses regarding FAB/NMDA and RD100's

individual effects on oxidative stress markers are unable to be rejected or confirmed, but can be rejected when assessing superoxide levels 24 hrs after application.

Involvement of group III mGluRs

Though RD100 does not have any significant effects on cell survival (Fig. 1B, Fig. 2B) nor superoxide levels (Fig. 5B), it was necessary to determine the potential mode of activation of this compound by utilizing the broad group III mGluR antagonist, MSOP (1 mM). It was hypothesized that RD100 acts as PAM of mGluR4 or another group III mGluR based on its similarity in structure to PHCCC. Due to RD100's apparent lack of effect in FAB/NMDA (Fig. 2B) and its neurotoxicity in GM (Fig. 1B), identifying the mechanism of action of RD100 may be important to confirm as this mechanism may potentially be harmful to cells under normal, non-toxic conditions. If RD100 were acting as a positive modulator a group III receptor as proposed by its structure, application of a compound that negates these effects such as MSOP would reverse the impact RD100 has on cell survival, as MSOP is an antagonist of these receptors. This process was utilized in the prior PHCCC studies to elucidate the mechanism of PHCCC[25].

After doing so, it was demonstrated again that FAB/NMDA significantly reduces survival (Fig. 6B). However, neither RD100 nor MSOP in FAB/NMDA independently altered cell survival, contrary to what was hypothesized. The lack of effect of MSOP was unexpected. On its own at 1 mM, MSOP should have induced neurotoxicity within cells due to its antagonism of group III receptors inducing high of glutamate release as seen in Maj et al's study in NMDA and AB-mediated toxicity[25]. Co-application of RD100 and

MSOP significantly reduced survival compared to control as predicted, which may suggest that RD100 acts on mGluR4 as predicted. However, since neither RD100 nor MSOP significantly affected cell survival, inference as to what this combination neurotoxicity may mean in terms of mechanistic pathways for RD100 is not conclusive. The hypothesis regarding RD100's function as a PAM of mGluR4 is unable to be rejected nor supported by this data. The unexpected results for both RD100 and MSOP calls into question the ability of mGluR4 in this model system to influence cell survival in any capacity.

CONCLUSIONS

This study concludes that FAB/100 uM NMDA induces a dose dependent negative effect on cell survival. It is recommended to use dilutions of 33.3%, 50%, or 100% FAB/100 uM NMDA to induce significant neurotoxicity for assessment of any future neuroprotective compounds. 100% FAB/NMDA may be excessively neurotoxic, and thus hard to elucidate protective effects within. FAB/100 uM NMDA does not influence levels of superoxide 24 hrs after application as visualized with MitoSOX dye, so it is unclear if superoxide is a mechanism contributing to cellular toxicity relating to OS in this model at other time points. When assessing superoxide levels in imaged cells using MitoSOX, it is recommended for measures the most reflective of superoxide to normalize relative intensity to binary area (by intensity/area) so that any changes in intensity as a result of cell density are corrected. This study also determined that RD100 does not have any neuroprotective effect against FAB/NMDA-mediated neurotoxicity. Additionally, RD100 does not impact levels of superoxide 24 hrs after application, but effects at other time points were not investigated. This study could not confirm whether RD100 acts as a PAM of mGluR4, though the joint toxicity of RD100 and MSOP together may suggest this is so.

Limitations and variability

Throughout all stages of this study, there were many opportunities for the introduction of variability in data generation and collection. Beginning at the level of plating cells, if the poly-l lysine used to prepare the well-plates did not cover the wells

completely, this could have affected the adherence of cells to the wells and influence their survival. The largest issue this may have contributed to is mass cell death localized to single wells across plates. Additionally, the author was not the sole individual preparing plates for cells as others often shared this responsibility, which introduces natural variability to the preparation of plates. When culturing and stimulating cells, multi-pipettors were often employed, which generate more force than individual pipettors well by well and impact the ability to precisely apply stimulations, which could have impacted cell viability. The use of multi- vs single pipettors interchangeably also introduced variability in the stimulation and feeding process. Others in the lab were also involved in feeding cells, which introduced further variability in the survival of cells.

There were generally very few replications of each group and condition across all trials and experiments, which creates small sample and population sizes and influences the statistical significance of any effects and results elucidated. Contamination, leading to the mass loss of multiple replications and plates of cells, also contributed to limited replications of trials and small sample sizes, which is to an extent avoidable with proper aseptic technique but unpredictable in the occurrence. When assessing data, the ImageQuant TL software was very sensitive to small changes in the grid utilized to identify individual wells, where small shifts changed the data values for the intensities of all wells unevenly across each different experimental group. The lack of the ability to standardize application of grids for this software to analyze intensities also introduced much variability that may have affected the results and trends identified.

FUTURE STUDIES

The ability for group III receptors like mGluR4 to have a neuroprotective effect in the FAB/NMDA model system should first be confirmed by assessing the effects of PHCCC. Maj et al's study of PHCCC used a model system of NMDA and Aβ only[25], so without first applying PHCCC in the FAB/NMDA model, neuroprotective effects via mGluR4 can only be hypothesized. The outcomes of PHCCC application in this model may also explain the lack of effects of both RD100 and MSOP. If PHCCC does not provide neuroprotection, this can explain why RD100 did not show the projected effects based on its similarity in structure to PHCCC. If PHCCC does afford neuroprotection, this would suggest RD100 may not be acting as a PAM of mGluR4 and that the original hypotheses regarding RD100's proposed effect and mechanism were incorrect. If MSOP does not afford neurotoxicity nor negate effects of PHCCC, then another group III mGluR or mGluR4 antagonist should be utilized in this model. After confirming PHCCC's effects, it is not recommended to utilize RD100 for future studies of neuroprotection in the FAB/NMDA model, and rather it is recommended to explore other RD compounds for their potential neuroprotection.

The manner in which FAB/NMDA induces cellular death should also be determined, such as by identifying how these compounds impact the oxidative stress pathway. An evaluation of superoxide production over different points in time should be conducted to determine if there is a certain time in which FAB/NMDA impacts levels of superoxide, starting from just minutes after application of this stressor. Other downstream ROS from superoxide may also be included in this evaluation, such as hydrogen peroxide

and hydroxyl radical. Doing so will elucidate more thoroughly the effects of this stressor on OS.

Correcting for variability in future studies should also be considered. This could be done such as by ensuring one individual only is preparing and culturing cell plates, conducting multiple replications of trials with increased sample sized per condition, ensuring use of an automated software for data collection, and standardizing the use of multi-pipettors vs single-pipettors for stimulation and feeding, which may help to draw more meaningful conclusions utilizing similar methodology in the present study.

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