THE ROLE OF OXIDATIVE STRESS IN AMPA RECEPTOR TRAFFICKING FOLLOWING ISCHEMIC STROKE

By

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Abstract

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Stroke is a leading cause of disability and results in 140,000 deaths in the United States each year. Ischemic stroke occurs when blood flow to brain tissue is decreased or absent due to vessel occlusion, leading to infarction. While it is necessary to reintroduce blood flow to the infarcted area, this act also results in further damage to surrounding brain tissue by inflammation and oxidative stress, leading to delayed neuronal death within vulnerable neuronal populations, including hippocampal pyramidal neurons. During ischemia, the lack of energy available disrupts ATP-dependent processes that maintain ionic gradients, which are critical to cellular survival. Disrupting the ionic balance leads to excessive release of neurotransmitters, like glutamate, which are unable to effectively cleared from the synapses. Excessive stimulation of *N*-methyl-D-aspartate receptors (NMDARs) by glutamate is a contributing factor to delayed neuronal death, and multiple studies report that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) also contribute to delayed neuronal death. As a result of ischemia/reperfusion, AMPARs undergo a subunit composition switch from calcium-impermeable, GluA2-containing AMPARs, to calcium-permeable, GluA2-lacking AMPARs.

This allows the AMPAR to conduct calcium which, in combination with excessive NMDAR stimulation, exacerbates cell death. We determined that there is a sex difference in the trafficking of AMPARs following oxygen glucose deprivation/reperfusion (OGD/R), an *in vitro* model for ischemia/reperfusion. GluA2-containing AMPARs are degraded by the lysosome in a calcium-dependent manner following OGD/R in male hippocampal slices, but AMPARs are not degraded in female hippocampal slices with OGD/R. Pharmacological inhibition of NADPH oxidase 1 rescues the OGD/R-induced degradation of GluA1 and GluA2 AMPAR subunits in males. Preventing the degradation of GluA2-containing AMPARs following ischemia/reperfusion has therapeutic implications in the treatment of stroke patients, so understanding the mechanisms at play are of utmost importance. In this dissertation I will discuss how OGD/R affects AMPARs in an *ex vivo* aged rodent acute hippocampal slice model and the role oxidative stress plays in the trafficking of these receptors.

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CHAPTER ONE: INTRODUCTION

Ischemic Stroke

Approximately 140,000 people in the United States die each year from stroke. Nearly 800,000 people suffer from stroke annually in the United States, of which approximately 87% are ischemic strokes [Virani *et al.* 2020; Yang *et al.* 2017]. The remainder percentage of strokes are classified as hemorrhagic strokes. The Merriam-Webster dictionary defines stroke as a "sudden impairment or loss of consciousness, sensation, and voluntary motion that is caused by rupture or obstruction (as by a clot) of a blood vessel supplying the brain, and is accompanied by permanent damage of brain tissue." Ischemic strokes occur when a blood vessel is occluded and blood flow to the brain is decreased [Virani *et al.* 2020]. When a blood vessel ruptures in the brain, this form of stroke is characterized as hemorrhagic stroke. There are several types of hemorrhagic stroke, but this literature review and subsequent chapters will solely focus on ischemic stroke. For a review of hemorrhagic stroke, see [Righy *et al.* 2016; Sekerdag *et al.* 2018].

There are four different classifications of ischemic stroke: thrombotic, embolic, systemic hypoperfusion, and cerebral venous sinus thrombosis. When a locally formed blood clot, such as an atherosclerotic plaque, blocks a blood vessel in the brain, this is referred to as a thrombotic stroke. When a blood clot originating from elsewhere in the body travels to and blocks a blood vessel in the brain, this is referred to as an embolic stroke. The debris that travels to the brain and blocks a blood vessel can be a number of things: blood clot, plaque, air, or cancer cells, for example. A heart attack commonly results in systemic hypoperfusion, or a general decrease in blood supply to the brain. When a blood clot prevents drainage of blood from the brain through

the dural venous sinuses, this form of ischemic stroke is referred to as cerebral venous sinus thrombosis [Virani *et al.* 2020].

The severity of ischemic stroke depends on a wide variety of factors including: length of time from ischemic onset to medical care, which area of the brain is being affected, sex, age, race, and lifestyle. A complete lack of oxygen is referred to as anoxia. Hypoxia occurs when there are low levels of oxygen. Hypoxia, combined with low glucose levels, is referred to as ischemia. These events are all damaging, but reperfusion, or the reintroduction of blood flow to an ischemic area, results in further brain damage. The general treatment for ischemic stroke is to restore blood flow, or reperfuse the infarcted brain area, as quickly as possible. This is an interesting conundrum, as it's necessary to reintroduce blood supply to the damaged brain area, but reperfusion damages this brain area through inflammation, oxidative stress, and cell death. Although reperfusion causes further damage, nearly all stroke treatments focus on reintroducing oxygen to the affected brain area(s) as quickly as possible [Falluji *et al*. 2011; Virani *et al.* 2020].

The brain damage that results from ischemia and subsequent reperfusion can be categorized into two distinct areas of damage, the ischemic core, also referred to as the necrotic core, and the penumbra. Cells within the ischemic core are localized closest to where blood flow is occluded, where they undergo necrosis, leading to irreversible damage [Schaller and Graf, 2004]. Necrosis, a form of unorganized cell death or bursting of cells, occurs during the early phase of ischemic insult [Lei, *et al.* 2017] near the area of blood flow occlusion. This cellular debris causes infiltration of immune cells, such as microglia, and ultimately results in tissue scarring. The brain tissue within the ischemic core is irreversibly damaged and there is no ability for these cells to recover.

The penumbra is the brain area immediately surrounding the ischemic core. This tissue is damaged, but still viable for a short time following ischemia/reperfusion, and therefore is a therapeutic target (Schaller & Graf, 2004). The manner in which cells within the penumbra die is distinct from the way in which cells perish in the ischemic core. Cells within the penumbra typically undergo programmed cell death, or apoptosis, a short period of time after ischemia/reperfusion [Sekerdag *et al.* 2018]. Therapies to target this cell death within the penumbra are lacking. Numerous clinical trials have occurred, but an effective pharmacotherapy to prevent ischemia/reperfusion-induced cellular damage has yet to receive approval from the Federal Drug Administration (FDA) and be available to the general public.

Therapies for ischemic stroke consist of various reperfusion strategies. The current pharmacotherapy is thrombolysis utilizing recombinant tissue plasminogen activator (tPA), which breaks apart the blood clot [Chen and Wang, 2016; Jeanneret *et al.* 2019]. tPA may also be neuroprotective from ischemia through a nonproteolytic action [Flavin and Zhao, 2001], but this mechanism needs to be investigated further. This treatment is only available within 4.5 hours of symptom onset [Marier, 1995]. Other criteria for tPA administration following ischemic stroke leave only about 5% of patients eligible for this treatment [Hacke, and Lichy, 2008], and there's an increased risk of hemorrhage following tPA administration [Marier, 1995]. Other pharmacological treatments following reperfusion are needed to treat ischemic stroke. The mechanisms underlying the cell death that occurs with reperfusion of the ischemic brain tissue have not been fully elucidated and understanding these molecular mechanisms will be crucial in developing effective therapeutic strategies for ischemic stroke treatments beyond the current reperfusion treatments.

Sex Differences and Ischemia/Reperfusion

In the United States each year about 55,000 more women compared to men suffer from a stroke [Kleindorfer *et al.* 2010], resulting in women having a higher lifetime risk of stroke. In younger age groups, the incidence rate of ischemic stroke is substantially lower in females compared to males. However, as age increases, the incidence rates for stroke in females is equal, or higher, than in males [Reeves *et al.* 2008; Hollander *et al.* 2003; Lewsey *et al.* 2009; Rothwell *et al.* 2005; Sealy-Jefferson *et al.* 2012; Vega *et al.* 2009]. 1 in 5 women between the ages of 55 and 75 years of age have a stroke, compared to 1 in 6 men in this age group [Seshadri *et al.* 2006].

Since females appear to have intrinsic protection from ischemic stroke for most of their life compared to males, it was thought perhaps hormone replacement therapy could be effective for either stroke prevention, treatment, or perhaps both [Rosamond *et al.* 2008; Turtzo and McCullough 2008]. Unfortunately, hormone replacement therapy has been ineffective for the treatment of stroke and chronic estrogen therapy results in higher incidences of stroke in women [Wassertheil-Smoller *et al.* 2003; Anderson *et al.* 2004]. These results suggest that there may be a hormonal-independent mechanism that affords females increase protection compared to males with ischemia/reperfusion injury.

Numerous studies have demonstrated sex differences in the cell death pathways activated following ischemic stroke (Diagram 1.1) [Hagberg *et al.* 2004; Du *et al.* 2004; McCullough *et al.* 2005; Renolleau *et al.* 2007; Liu *et al.* 2009; Yuan *et al.* 2009; Li and McCullough 2009]. Caspase-independent cell death in males with ischemic injury induces poly-ADP-ribose polymerase (PARP-1) cell death, via increased neuronal nitric oxide synthase (nNOS) and

apoptosis-inducing factor (AIF) [Lang and McCullough, 2008]. Inhibition or genetic depletion of nNOS is neuroprotective in male mice following ischemia, but exacerbates damage in female mice [McCullough *et al.* 2005]. The well-known apoptotic caspase-dependent cell death pathway occurs in females following ischemic stroke [Liu *et al.* 2009], although this pathway does occur in both sexes [Le *et al.* 2002; Cho *et al* 2004]. Inhibition of the caspase cascade, with the pharmacological inhibitor QVD-OPH, prevents ischemia-induced cell death in females, but not in male mice [Renolleau *et al.* 2007; Liu *et al.* 2009].

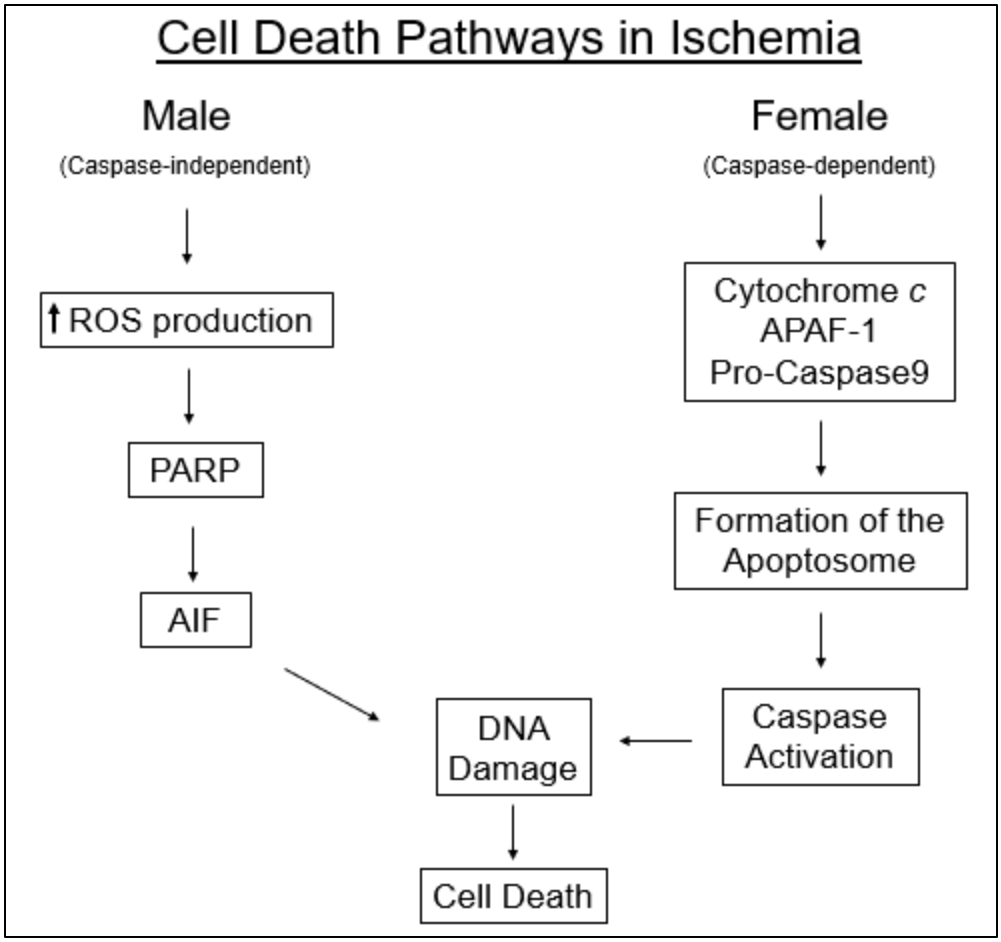

Diagram 1.1: Model for male and female cell death pathways with ischemia. Cell death following ischemia stroke in males is likely caspase-independent, and cell death in females is likely mediated in a caspase-dependent manner. An increase in reactive oxygen species (ROS) following ischemia/reperfusion induces PARP-1-dependent cell death in males. In females, release of cytochrome *c* from the mitochondria results in apoptosome formation and subsequent caspase activation, leading to DNA damage and ultimately cell death.

By understanding these sex differences in the ischemic cell death cascade, new sex-specific therapies may be developed and improve the outcomes for stroke patients.

Ischemic Cascade

There has been much progress in elucidating the underlying molecular mechanisms of ischemia/reperfusion, but effective therapeutics have yet to make it to the clinic. If there is any hope for identifying new therapeutic targets for ischemic stroke treatment, it is imperative to understand and identify the molecular signaling cascades that occur during ischemia/reperfusion.

The most fundamental upstream consequence of ischemia is reduced blood supply, and thus reduced levels of adenosine triphosphate (ATP). Once total ATP levels decrease, neurons depolarize and lose their ionic homeostasis. Once this happens, there is a series of molecular events that take place referred to as the ischemic cascade.

Ionic imbalance in neurons leads to neurotransmitter release including the amino acid, glutamate, the most abundant excitatory neurotransmitter in the central nervous system (CNS). Additionally, excitatory amino acid transporters (EAATs) localized on neurons and astrocytes are unable to clear glutamate from the synapse during ischemia [Camacho and Massieu, 2006; Inui *et al.* 2013]. Glutamate binds to postsynaptic ionotropic glutamate receptors, AMPA receptors, NMDA receptors, and Kainate receptors, resulting in a massive influx of intracellular calcium. This cytotoxic calcium overload activates catabolic phospholipases and proteases, resulting in degradation of cellular membranes and proteins. This process is referred to as glutamate excitotoxicity [Lipton, 1999]. This accumulation of intracellular calcium following ischemia/reperfusion results in neurodegeneration and cell death [Arundine & Tymianski 2003]. Aside from the contribution of calcium from AMPA receptors, NMDA receptors, and Kainate

receptors, which raises the intracellular calcium levels, calcium is also released from the mitochondria and endoplasmic reticulum because of metabotropic glutamate receptor activation via the inositol triphosphate receptor (IP3R) [Arundine & Tymianski, 2003].

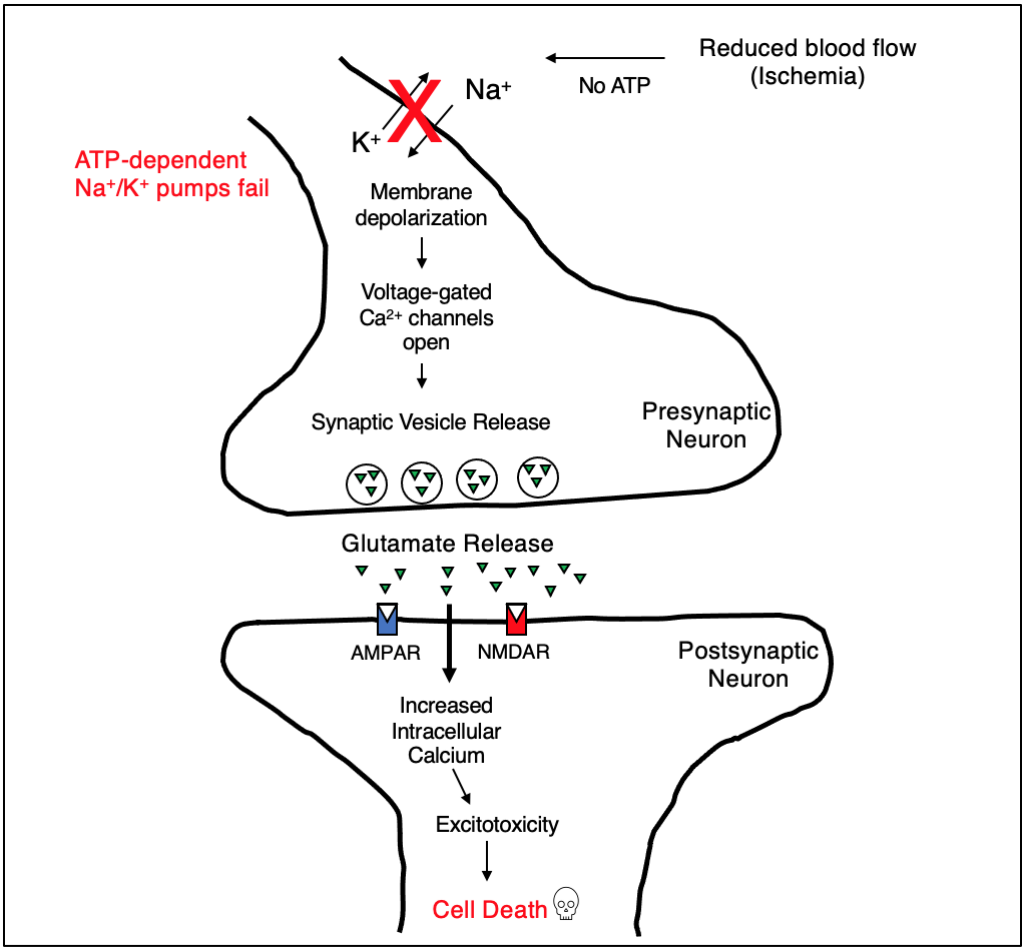

Diagram 1.2: The molecular signaling underlying the ischemic cascade in neurons. Loss of ATP during ischemia results in disruption of Na^+/K^+ ATP-dependent pumps which are crucial for maintaining neuronal membrane homeostasis. Upon membrane depolarization, voltage-gated calcium channels open and there is a massive presynaptic release of neurotransmitters, including glutamate. This glutamate stimulates postsynaptic AMPA and NMDA receptors, leading to an excessive influx of intracellular calcium in the postsynaptic neuron. This cytotoxic calcium overload, excitotoxicity, initiates cell death signaling cascades, eventually resulting in neurodegeneration and neuronal death.

One of the major consequences of excitotoxicity is the calcium-dependent opening of the mitochondrial permeability transition pore, resulting in release of cytochrome *c*, and initiating the apoptosis, programmed cell death, cascade [Stavrovskaya and Kristal, 2005; Tafani *et al.* 2002]. Cytochrome *c* release from the mitochondria activates cysteine-dependent aspartate-directed proteases (caspase)-dependent apoptosis cascades. Upon release of cytochrome *c* from the mitochondria, it binds to apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, forming the apoptosome [Dejean *et al.* 2006]. Pro-caspase-9 is cleaved to its active form, caspase 9, which then cleaves pro-caspase-3 to caspase 3. Caspase-3-dependent proteolysis occurs and has a critical role in apoptosis [Stavrovskaya and Kristal, 2005] following ischemia/reperfusion.

Apoptosis cascades independent of caspases are also involved in ischemia/reperfusion-induced cell death [Nizuma *et al.* 2010]. A well-studied cell death pathway following ischemic stroke revolves around AIF. AIF is released from the mitochondria as a result of calcium overload and translocates to the nucleus, resulting in DNA fragmentation and apoptosis [Culmsee *et al.* 2005]. Endonuclease G is also released from the mitochondria due to increased intracellular calcium concentrations following ischemia/reperfusion and translocates to the nucleus, where subsequent DNA fragmentation occurs, resulting in apoptosis [Lee *et al.* 2005].

Several research studies and clinical trials have attempted to target glutamate excitotoxicity as a potential therapeutic [Nakajima *et al.* 2018; Landucci *et al.* 2018; Mazzocchetti *et al.* 2020; Niu *et al.* 2018; Carpanese *et al.* 2014; Nayak & Kerr 2013], however, pharmacologically targeting NMDA and AMPA receptors have failed to improve patient outcomes [O'Collins *et al.* 2006; Chen & Wang, 2016]. While antagonists of AMPA and NMDA receptors may not be viable therapeutic targets, targeting the signaling pathway downstream

from these receptors and regulating their trafficking/signaling, may provide new pharmacological targets with better translatability to the clinic for ischemic stroke patients.

AMPA Receptors

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are ionotropic glutamate receptors that mediate fast excitatory neurotransmission in the brain. Other ionotropic glutamate receptors include Kainate receptors, Delta receptors, and *N*-methyl-D-aspartate receptors (NMDARs). There are also metabotropic glutamate receptors (mGluRs) (Diagram 1.3). All of these glutamate receptors have important roles in proper synaptic function. NMDARs are glutamatergic calcium channels that are both voltage and ligand gated. mGluRs are G protein-coupled receptors (GPCRs) that act through various effector proteins to modulate synaptic transmission. This body of work will focus on AMPA receptors. For more information on NMDARs and mGluRs, with specific attention to neuropathology, see [Wu and Tymianski, 2018; Baskys and Blaabjerg, 2005; Ribeiro *et al.* 2010].

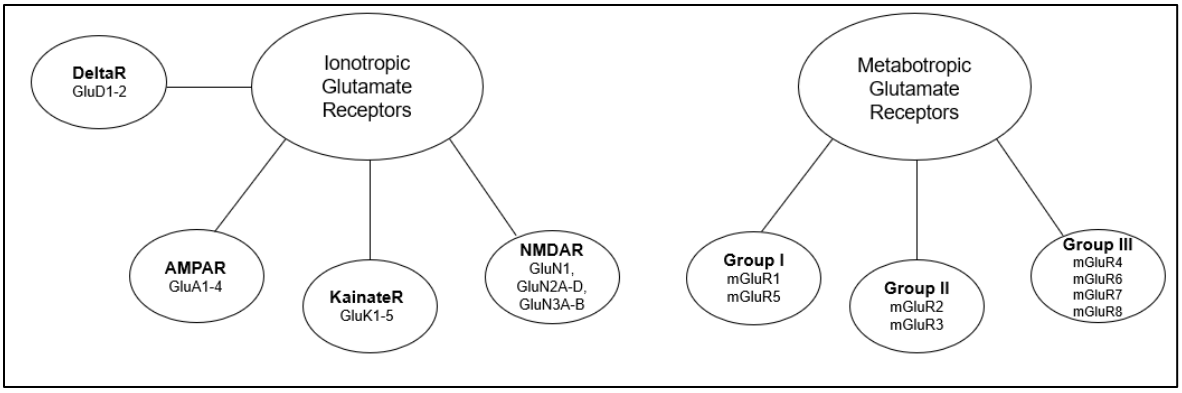

Diagram 1.3: Family of Glutamate Receptors. There are four ionotropic glutamate receptors: Delta receptors (GluD1-2 subunits), AMPA receptors (GluA1-4 subunits), Kainate receptors (GluK1-5), and NMDA receptors (GluN1, GluN2A-D, GluN3A-B). There are three groups of metabotropic receptors: Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3), and Group III (mGluR4, mGluR6, mGluR 7, and mGluR8).

AMPARs are composed of four subunits, GluA1-4, that combine as tetramers to form cation channels with different functional properties depending on their subunit composition [Hollmann and Heinemann, 1994]. AMPARs are developmentally regulated, and their expression varies depending on its localization in the brain. All the AMPAR subunits can be alternatively spliced. These alternatively spliced regions are known as flip and flop, located in the ligand-binding domain [Sommer *et al.* 1990], which can alter the channel's properties and are also developmentally regulated [Monyer *et al.* 1991]. Upon glutamate stimulation, the flop AMPARs rapidly desensitize and are less responsive to cyclothiazide, a positive allosteric modulator of AMPARs that prevents desensitization [Sommer *et al.* 1990]. The flip splice variant is expressed early in postnatal development, and the flop variant is predominantly expressed later in development [Shepherd and Huganir, 2007].

The majority of AMPARs in the hippocampus are GluA1—GluA2 or GluA2—GluA3, with small pools of GluA2-lacking AMPARs [Wenthold *et al.* 1996; Reimers *et al.* 2011]. AMPAR subunit expression varies considerably depending on neuroanatomical location [Passafaro *et al.* 2001]. Each AMPAR subunit contains three transmembrane domains and one re-entrant loop [Bennett and Dingeldine, 1995]. The extracellular domains form the ligand-binding domain, which contains the ligand, glutamate, binding site (Diagram 1.4) [Borges and Dingledine, 1998].

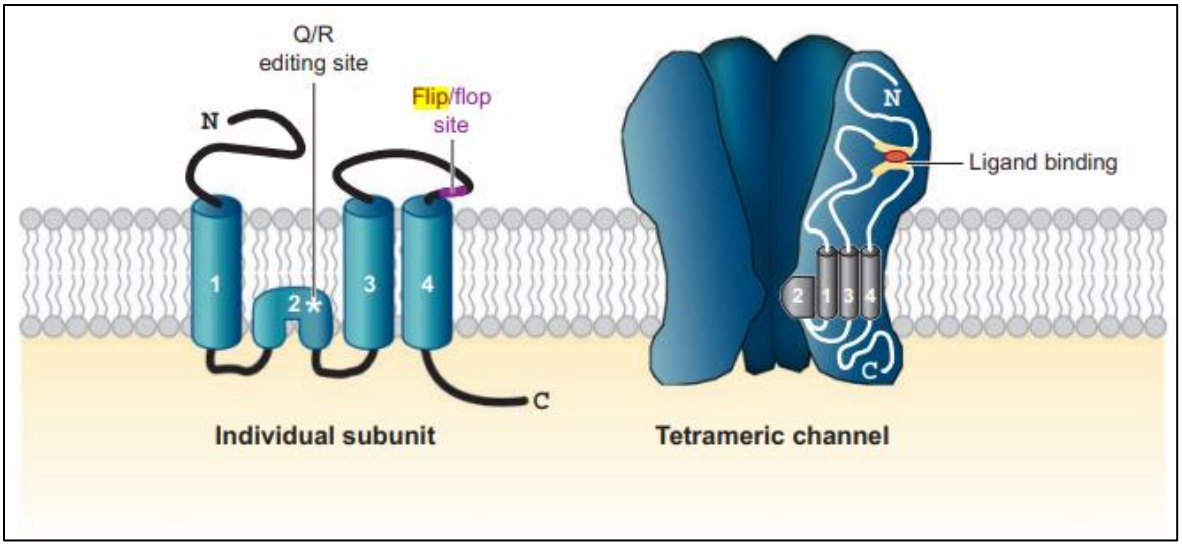

Diagram 1.4: Schematic diagram of AMPAR subunit and complex. The AMPAR subunits are composed of four transmembrane domains, the Q/R editing site is on the second transmembrane domain. The flip/flop site is also shown. The tetrameric receptor is composed of two dimers, four subunits total. Diagram is from [Shepherd and Huganir, 2007].

Once the AMPAR subunits were cloned, the genomic GluA2 subunit sequence was discovered to differ from the GluA2 complementary deoxyribonucleic acid (cDNA) sequence by one amino acid in the ion channel pore [Sommer *et al.* 1991]. It was discovered that the GluA2 subunit undergoes post-transcriptional RNA editing; the glutamine at position 607 is edited to an arginine. This site is referred to as the Q/R editing site, and the edited GluA2 subunit is referred to as GluA2Q/R (Diagram 1.4). The Q/R editing site is crucial for normal neurotransmission. Brusa *et al.* (1995) demonstrated that impaired Q/R editing in mice results in epilepsy and death within 14 days of birth. Indeed, the Q/R editing of GluA2 is crucial for normal synaptic function, and the impairment of this post-transcriptional editing affects long-term potentiation (LTP), strengthening of synapses, and results in epilepsy [Konen *et al.* 2020]. The RNA-editing enzyme adenosine deaminase enzyme 2 (ADAR2) is responsible for the substitution of the adenosine base to inosine, resulting in the post-transcriptional editing of the Q/R editing site of GluA2[Bass, 2002]. Unedited GluA2 subunits are typically retained in the endoplasmic reticulum, but GluA1 and GluA2Q/R subunits form tetramers and can efficiently exit the endoplasmic reticulum [Mansour *et al.* 2001; Greger *et al.* 2002; Greger *et al.* 2003]. This regulation at the endoplasmic reticulum favors GluA1—GluA2Q/R assembly over other AMPAR subunit compositions in the hippocampus.

This post-transcriptional editing of the GluA2 AMPAR subunit renders the AMPAR impermeable to calcium. GluA2-lacking AMPARs are calcium-permeable and blocked by endogenous polyamines [Traynelis *et al.* 2010]. GluA2-containing AMPARs are non-rectifying and GluA2-lacking AMPARs are rectifying, at positive voltages they allow only small outward current due to the polyamine block. Unedited GluA2 and GluA2-lacking AMPARs have been

implicated in many different pathologies [Pellegrini-Giampietry *et al.* 1997; Wright and Vissel, 2012], including: amyotrophic lateral sclerosis [Kwak and Weiss, 2006], epilepsy [Rakhade *et al.* 2008; Lippman-Bell *et al.* 2013], Parkinson's disease [Sekar & Taghibiglou, 2020], and ischemic stroke [Noh *et al.* 2005].

AMPA Receptor Trafficking

There is a highly organized endocytic trafficking system in eukaryotic cells which regulates the endocytosis and exocytosis of various molecules, including proteins. There are over 60 Rab genes in the human genome [Stenmark and Olkkonen, 2001], and these Rab proteins are GTPases which are thought to regulate much of this endocytic trafficking system. Rab GTPases are inactive when bound to guanosine diphosphate (GDP) and are active when bound to guanosine triphosphate (GTP). The cycling between these active and inactive forms of Rabs is catalyzed by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (Diagram 1.5). Rab proteins associate with membranes because of a posttranslational prenylation on their C-terminus. Rab proteins exert their functions through effector proteins which enables them to perform a diverse array of functions [Stenmark and Olkkonen, 2001]. For a complete review of Rab proteins, their regulation and function, see [Homma *et al.* 2020].

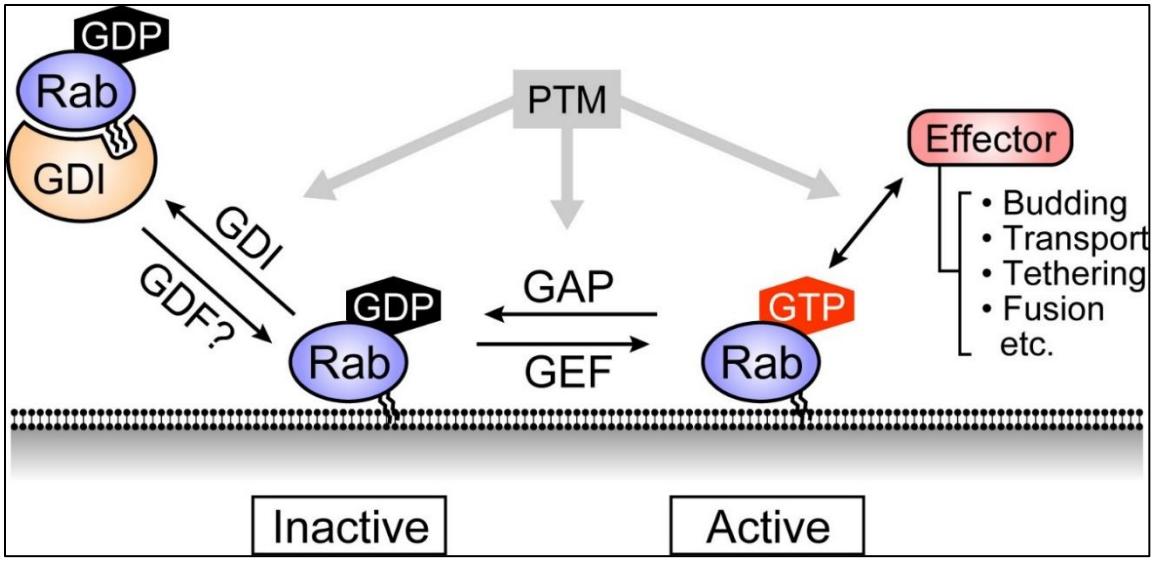

Diagram 1.5: The regulation of activated and inactivated Rab GTPases. The conformation states of Rab proteins are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Post-translational modifications (PTM) can regulate the activation of GAPs and GEFs. GEFs catalyze the reaction of GDP-bound Rabs to GTP-bound (active) Rabs. GAPs catalyze the inactivation of GTP-bound Rabs to GDP-bound (inactive) Rabs. Inactive Rabs are bound to GDP dissociation inhibitor (GDI) and retains inactive Rabs in the cytosol. Diagram from [Homma *et al.* 2020].

Rab proteins play a critical role in regulation of endocytosis, exocytosis, recycling, and degradation of AMPARs. Newly synthesized AMPARs exiting the endoplasmic reticulum are regulated by Rab39B through an interaction with protein interacting with C-kinase 1 (PICK1) [Mignogna *et al.* 2015]. Rab5 regulates the early endocytosis of AMPARs with long-term depression (LTD) in the hippocampus [Brown *et al.* 2005] and in oxygen-glucose deprivation/reperfusion (OGD/R) in U251-MG cells [Achzet *et al.* 2021a]. Furthermore, p38 mitogen-activated protein kinase (p38MAPK) enhances Rab5 activity during LTD to increase the internalization of AMPARs [Cavalli *et al.* 2001; Huang *et al.* 2004; Zhong *et al.* 2008]. Following internalization, AMPARs can be trafficked to Rab4-positive endosomes, sorting endosomes [Ehlers, 2000]. From the Rab4-positive endosome, or directly following internalization, AMPARs can be sorted toward Rab11-positive recycling endosomes [Fernandez-Monreal *et al.* 2012; Gu *et al.*2016; Esteves *et al.* 2015; Lise *et al.* 2006; Wang, *et al.* 2008; Park *et al.* 2004] or Rab7-positive late endosomes, destined for lysosomal degradation [Fernandez-Monreal *et al.* 2012; Achzet *et al.* 2021a]. Rab proteins play a large role in the regulation of AMPAR endocytic trafficking, but there are other proteins that are also critical in regulating the trafficking of AMPARs.

AMPARs, through SNARE-dependent exocytosis [Lu *et al*. 2001], may be inserted directly into the synaptic membrane (Gerges *et al.* 2006) or may be inserted in the extrasynaptic membrane, then laterally diffuse to the synapse (Adesnik *et al.* 2005). The rates at which GluA1 and GluA2 AMPARs are inserted into the plasma membrane differ considerably. Synaptic insertion of GluA1 is slow under basal conditions, but increases upon NMDAR activation [Hayashi *et al.* 2000]. GluA2 AMPARs are constitutively inserted into the synaptic membrane

[Passafaro *et al.* 2001]. GluA1—GluA2 AMPAR complexes predominantly rely on GluA1 activity-dependent trafficking, whereas GluA2—GluA3 AMPAR complexes predominantly rely on the constitutive trafficking of GluA2 [Shepherd and Huganir, 2007]. The long C-terminus tail of GluA2 also binds to N-ethylmaleimide-sensitive fusion protein (NSF), regulating GluA2 AMPAR exocytosis [Nishimune *et al.* 1998].

Phosphorylation and dephosphorylation also have a large role in the trafficking of AMPARs to and from the synapse. Phosphorylation allows AMPARs to selectively bind to or dissociate from a wide assortment of proteins that are involved in endocytosis, exocytosis, recycling, and endocytic sorting. For example, dephosphorylation of the Ser845 reside of GluA1 is associated with removal of AMPARs from the synaptic membrane [Lee *et al.* 2000] and may play a role in the degradation of these receptors [Fernandez-Monreal *et al.* 2012].

The protein kinase C α (PKCα)-dependent [Xia *et al.* 2000] phosphorylation of the Ser880 residue of GluA2 AMPAR subunit mediates receptor internalization [Chung *et al.* 2000] through its interactions with glutamate receptor interacting protein 1 (GRIP1), PICK1, and AMPA receptor binding protein (ABP) [Dong *et al.* 1997; Srivastava *et al.* 1998]. Phosphorylation of the Ser880 residue of GluA2 promotes dissociation of the GluA2—GRIP1 complex. The interaction between GluA2 and GRIP1 is crucial for the stabilization of GluA2 at the synaptic membrane [Matsuda *et al.* 1999]. The disruption of the GluA2—GRIP1 complex by phosphorylation of the Ser880 residue of GluA2 promotes GluA2 binding to PICK1 [Chung *et al.* 2000], mobilizing AMPARs and leading to increased internalization (Diagram 1.6) [Terashima *et al.* 2004; Terashima *et al.* 2008; Lu and Ziff, 2005].

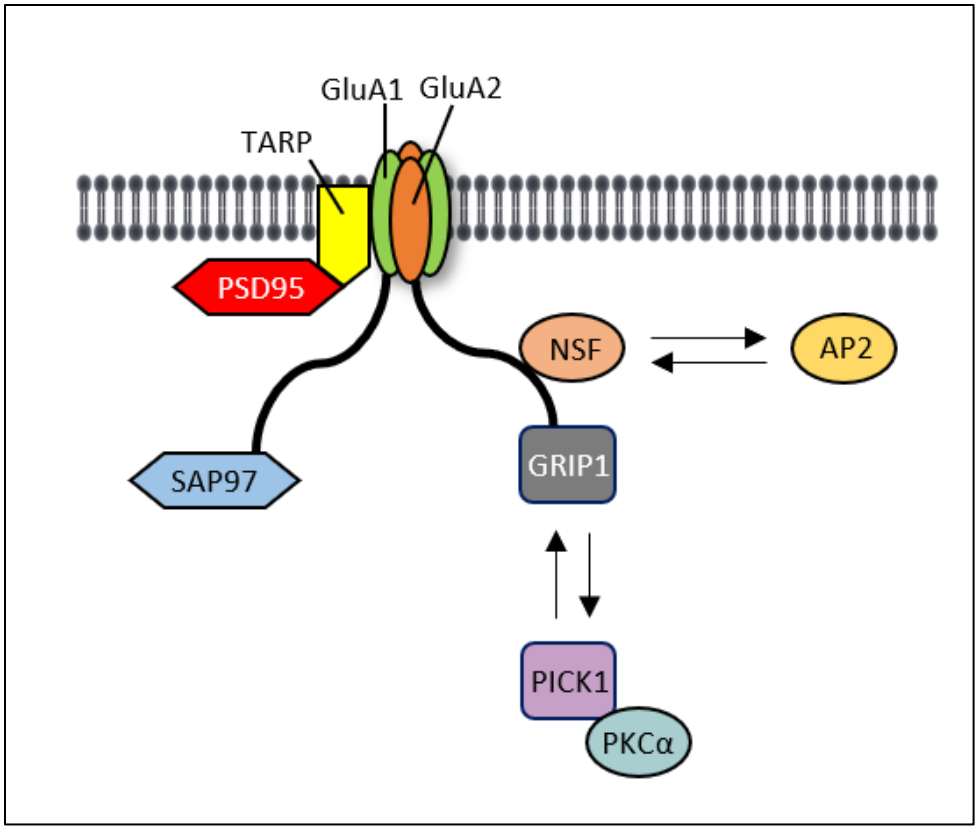

Diagram 1.6: Interaction of GluA1 and GluA2 AMPAR subunits with scaffold proteins. N-ethylmaleimide sensitive fusuon protein (NSF) facilitates exocytosis of AMPARs, with glutamate receptor interacting protein 1 (GRIP1) stabilizing GluA2 at the membrane surface. During internalization, protein interacting with C kinase 1 (PICK1) displaces GRIP1 and mobilizes AMPARs to endocytosis through interactions with adaptor protein 2 (AP2). Transmembrane AMPAR regulatory proteins (TARPs) complex with AMPARs and tether them to the postsynaptic density through interactions with scaffold proteins such as post-synaptic density protein 95 (PSD-95) and synapse associated protein 97 (SAP-97).

The phosphorylation of the Tyr876 residue of GluA2 may also play a role in mediating AMPAR internalization, although this precise mechanism is unclear. Tyr876 phosphorylation may not play a role in LTP, but may have a role in LTD-dependent AMPAR trafficking and synaptic downscaling [Yong *et al.* 2020]. Widagdo *et al.* [2015] identified a sorting mechanism for AMPARs once they are internalized following agonist stimulation. AMPAR subunits, including GluA1 and GluA2, undergo activity-dependent ubiquitination, another posttranslational modification, that regulates AMPAR endocytic sorting and degradation [Widagdo *et al.* 2015]. This mechanism is discussed more in the subsequent section with regards to LTP/LTD.

Auxiliary proteins, including transmembrane AMPA receptor regulatory proteins (TARPs), also regulate AMPAR trafficking [Tomita *et al.* 2003] as well as alter the channel's kinetics [Bats *et al.* 2012; Ben-Yaacov *et al.* 2017]. For proper LTP within the dentate gyrus of the hippocampus, TARP γ-8 is required, which complexes with AMPARs [Khodosevich *et al.* 2014]. TARP γ-8 is highly expressed in the hippocampus and in mutant TARP γ-8 mice, there is a 70% reduction in synaptic AMPARs in the CA1 region of the hippocampus [Fukaya *et al.* 2006]. TARP γ-2 and γ-7 are required for AMPAR expression in the cerebellum [Kato *et al.* 2007], and TARP γ-7 also promotes AMPAR expression in glial cells [Yamazaki *et al.* 2010]. TARP γ-2 also mediates synaptic targeting of AMPARs in the hippocampus [Chen *et al.* 2000]. Cornichon-2 and cornichon-3 are another class of auxiliary proteins [Schwenk *et al.* 2009] that play a role in AMPAR trafficking, but their roles are less well-defined. Without these cornichon proteins expressed, there is a selective loss of GluA1-containing AMPARs [Herring *et al.* 2013]. Cornichon-2 and TARP γ-8 colocalize in the hippocampus, and cornichon-2 blocks TARP γ-8-

mediated resensitization of AMPARs [Kato *et al.* 2010]. The auxiliary protein GSG1L is inhibitory in nature [Shanks *et al.* 2012] and suppresses the function of calcium-permeable AMPARs [McGee *et al.* 2015]. Shisa9/cysteine-knot AMPAR modulating protein 44 (CKAMP44), another auxiliary protein, modulates AMPAR transmission [Engelhardt *et al.* 2010; Twomey *et al.* 2017] and plasticity through its interactions with PICK1 and PKCα [Kunde *et al.* 2017]. TARPs also act to stabilize AMPARs at the postsynaptic density through interactions with PSD-95 and PSD-93 [Dakoji *et al.* 2003]. Upon glutamate stimulation, Tomita *et al.* [2004] determined that AMPARs detach from TARPs during internalization and TARPs remain stabilized at the plasma membrane. [For a detailed review of TARPs and their regulation of AMPARs, see Bissen *et al.* 2019]. TARPs are a relatively new discovery and have not been fully explored yet. They have been implicated in a few pathologies, including schizophrenia [Drummond *et al.* 2013] and epilepsy [Kennard *et al.* 2011], but there is still much to learn about their role in pathophysiology.

AMPAR trafficking is regulated through a complex interplay between many different proteins and post-translational modifications. Many of these mechanisms aren't completely elucidated, and fewer have been characterized in pathological states, such as ischemic stroke.

Role of AMPA Receptors in Long-Term Depression and Long-Term Potentiation

The hippocampus is a subcortical structure located in the medial temporal lobe. It is part of the limbic system and plays a role in learning and memory formation, as well as memory consolidation. Scoville and Milner [1957] published a case study on a patient, H.M., who had both of his hippocampi removed as treatment for his epilepsy. As a result, H.M. had severe

memory deficits. This case study resulted in the hippocampus becoming popularly used to study synaptic plasticity, the molecular mechanisms underlying learning and memory.

Long-term potentiation (LTP), or strengthening of the synapse, and long-term depression (LTD), or weakening of the synapse, are two well-studied models of synaptic plasticity (Diagram 1.7) and are thought to underlie memory formation in the hippocampus [Collingridge *et al.* 2004].

Protein phosphorylation plays a crucial role in synaptic plasticity. At its most basic level, LTP is an increase in the number of synaptic AMPARs. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays an important role in LTP induction [Lisman *et al*. 2002], by direct phosphorylation of the Ser831 residue of the GluA1 subunit [Barria *et al.* 1997; Mammen *et al.* 1997], and thereby increasing AMPAR conductance [Benke *et al.* 1998]. Absence, or inhibition, of CaMKII blocks the induction of LTP [Silva *et al.* 1992; Malenka *et al.* 1989; Malinow *et al.* 1989], but CaMKII is not required for AMPAR trafficking to the synapse [Hayashi *et al.* 2000]. Protein kinase A (PKA) also plays an important role in LTP. PKA phosphorylates the GluA1 AMPAR subunit at the Ser845 amino acid residue. Phosphorylation of the Ser845 residue of GluA1 induces extrasynaptic delivery of AMPARs, which then travel to the synapse by lateral diffusion [Man *et al.* 2007; Oh *et al.* 2006]. GluA1 seems to play a central role in the exocytosis of AMPARs during LTP. Indeed, GluA1-knockout mice have dysfunctional, or nonexistent, LTP in the CA1 region of the hippocampus [Zamanillo *et al.*, 1999], however LTP can be rescued by overexpression of GluA1 [Mack *et al.* 2001].

At its most basic level, LTD is a decrease in the number of synaptic AMPARs, which is typically achieved by endocytosis of synaptic AMPARs [Beattie *et al*. 2000; Lissin *et al.* 1999].

Stimulation of NMDARs leads to a decrease in surface AMPARs, a process known as chemical LTD [Carroll *et al.* 1999]. The central role of AMPAR endocytosis in LTD has also been confirmed *in vivo* [Heynen *et al.* 2000]. Phosphorylation and dephosphorylation play important roles in LTD as well. The Ser845 residue of GluA1 is dephosphorylated during the induction of LTD [Lee *et al.* 2000].

Phosphorylation of the GluA2 AMPAR subunit at the Ser880 residue by PKCα [Xia *et al.* 2000] results in GluA2 dissociating from GRIP1, which is a scaffold that stabilizes AMPAR at the membrane surface [Chung *et al.* 2000; Matsuda *et al.* 1999]. Ser880 phosphorylation of GluA2 also promotes binding to PICK1, which mobilizes AMPARs for internalization [Chung *et al.* 2000; Matsuda *et al.* 1999; Perez *et al.* 2001; Terashima *et al.* 2004; Terashima *et al.* 2008]. Ser880 phosphorylation decreases synaptic transmission and prevents the synaptic expression of AMPARs in CA1 hippocampal pyramidal neurons [Seidenman *et al.* 2003].

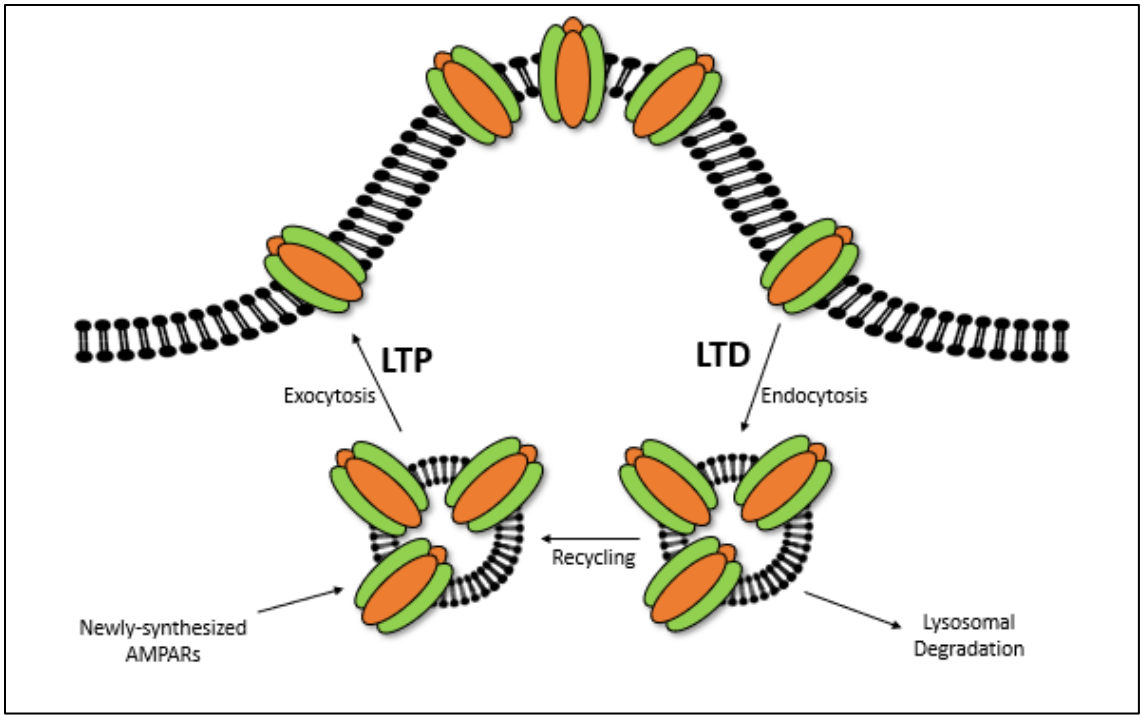

Diagram 1.7: Basic mechanisms underlying LTP and LTD. During LTD, AMPARs are removed from the plasma membrane and are either degraded or recycled. During LTP, newly synthesize and/or recycled AMPARs are inserted into the extrasynaptic membrane and laterally diffuse to the synapse.

Lin and Huganir [2007] have demonstrated that PICK1 is not required for NMDAR-mediated internalization of GluA2 in hippocampal neurons but is instead involved in the endocytic trafficking of GluA2-containing AMPARs. This study suggests that GluA2 is retained in endosomes during NMDAR-dependent LTD in a PICK1-dependent manner. The full mechanism remains to be elucidated, but PICK1 may decrease AMPAR surface levels by increased endocytosis of GluA2-containing AMPARs, preventing the recycling of GluA2-containing AMPARs, or perhaps both.

The phosphorylation of the Tyr876 residue of the GluA2 AMPAR subunit is also important in mediating synaptic plasticity. The Tyr876 residue of the GluA2 AMPAR subunit is phosphorylated by Src-family protein tyrosine kinases, including Fyn and Src [Hayashi & Huganir, 2004; Scholz *et al.* 2010; Kohda *et al.* 2013]. Another form of LTD is mediated by mGluRs, and this form of LTD is associated with tyrosine dephosphorylation of GluA2 [Moult *et al.* 2006; Gladding *et al.* 2009]. However, Yong *et al.* [2020] recently showed that LTP and LTD are not dependent on the phosphorylation of the Tyr876 residue of GluA2. This study also confirmed that phosphorylation of GluA2 at Tyr876 increases GluA2 binding to GRIP1 during synaptic upscaling [Yong *et al.* 2020].

The regulation of AMPAR levels at the synapse are crucial for controlling synaptic plasticity, namely LTP and LTD. The dysfunction of these processes leads to neuropathology, and the internalization and subsequent degradation of GluA2-containing AMPARs plays a detrimental role in ischemia/reperfusion.

AMPA Receptors and Ischemia/Reperfusion

Reintroduction of blood flow to an infarcted area following ischemia is necessary, but this act also results in further tissue damage within the brain, by inflammation, oxidative stress, and delayed neuronal death (DND) within vulnerable neuronal populations, such as CA1 hippocampal pyramidal neurons [Ford *et al.* 1989; Mitani *et al.* 1998; Chip *et al.* 2013; Suarez-Blanco & Hanley, 2014; Han *et al.*; 2016]. A hallmark of ischemic stroke is the massive release of the excitatory neurotransmitter glutamate, which is unable to be effectively cleared from the synapses following ischemia/reperfusion [Camacho and Massieu, 2005]. The excessive stimulation of NMDARs is a contributing factor to DND [Faden *et al.* 1989; Mcintosh *et al.* 1989; Takagi *et al.* 1997; Liu *et al*. 2007], but multiple studies have reported that AMPARs also contribute to DND [Liu *et al.* 2004; Yin *et al.* 2002; Anzai *et al.* 2003; Calderone *et al.* 2004; Noh *et al.* 2005; Liu *et al.* 2006].

The majority of AMPARs expressed in the CA1 region of the hippocampus contain the edited form of GluA2 (Q706R), and therefore are calcium-impermeable [Kuner *et al.* 2001]. Following ischemia/reperfusion, AMPARs undergo a subunit composition switch from calcium-impermeable, GluA2-containing AMPARs [Kuner *et al.* 2001], to calcium-permeable, GluA2-lacking AMPARs. Calcium-permeable AMPARs, in combination with stimulation of NMDARs, exacerbates cell death with ischemia/reperfusion [Kwak & Weiss, 2006].

Overexpression of GluA2 [Liu *et al.* 2004] or inhibition of calcium-permeable AMPARs [Yin *et al.* 2002; Deng *et al.* 2003; Noh *et al.* 2005] increases neuronal viability following ischemia. Complete inhibition of AMPARs also prevents ischemia-induced cell death *in vivo* [Pringle *et al.* 1997; Strasser and Fischer 1995; Montero *et al.* 2007]. Physiological LTD appears

to have similar mechanisms with ischemia/reperfusion with regards to GluA2 AMPAR regulation. The phosphorylation of Ser880 residue of GluA2 plays a role in decreased surface GluA2 levels in ischemia/reperfusion [Liu *et al.* 2006; Zhang *et al.* 2013; Jackson *et al.* 2018]. Ischemia also decreases the association between GluA2 and GRIP1 [Liu *et al.* 2006; Beske *et al.* 2014] and promotes the association of GluA2 with PICK1 [Dixon *et al.* 2009; Beske *et al.* 2014; Achzet *et al.* 2021b]. This results in decreased surface levels of GluA2-containing AMPARs and ultimately excitotoxicity as the balance between GluA2-containing, calcium-impermeable AMPARs and GluA2-lacking, calcium-permeable AMPARs is disrupted (Diagram 1.8) Gerace *et al.* 2014; Han *et al.* 2016]. Koszegi *et al.* [2017] also demonstrated that the hippocampal-specific internalization and degradation of GluA2 AMPARs following ischemia is dependent upon PICK1.

Long-lasting synaptic changes can be triggered by a brief period of ischemia, in a process considered ischemic LTP (i-LTP). It's unknown exactly how the cellular mechanisms differ between i-LTP and LTP, however it is known that calcium-permeable AMPARs play a critical role [Dias *et al.* 2013]. It's been well established that a short period of ischemia can induce a form of LTP that may be responsible for the excitotoxicity in CA1 neurons [Ai and Baker, 2006].

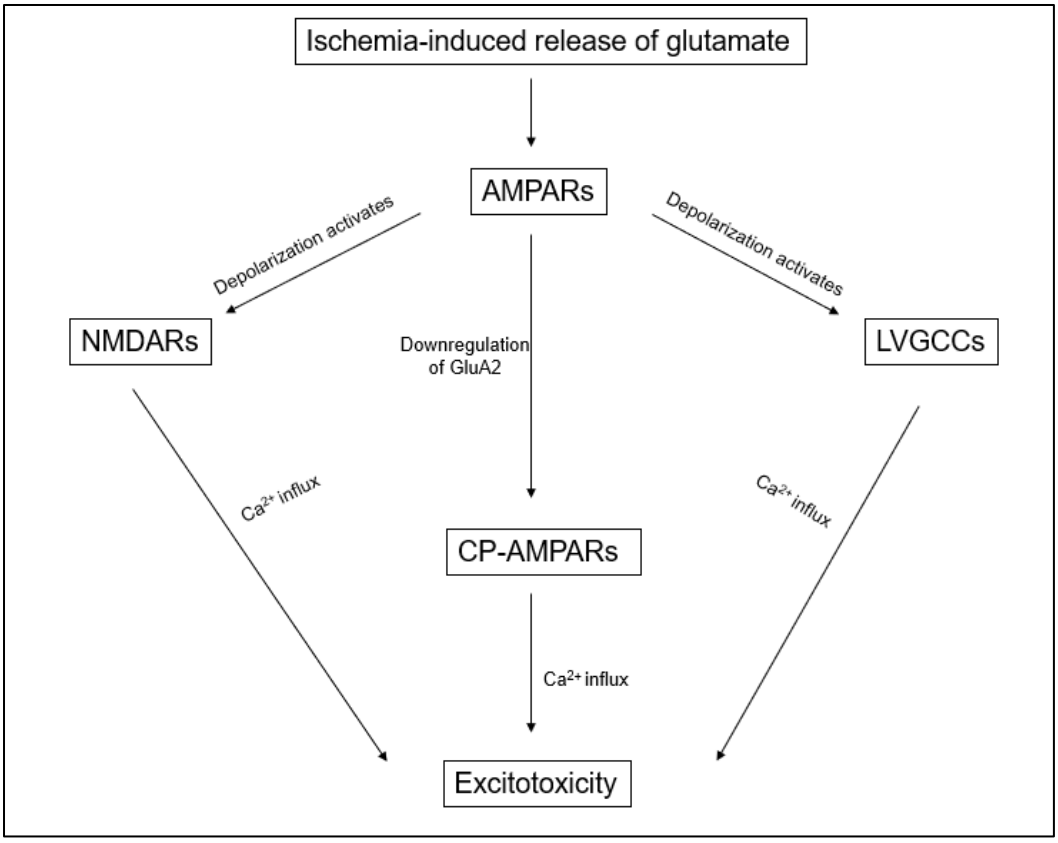

Diagram 1.8: Calcium sources responsible for excitotoxicity during ischemia/reperfusion. Glutamate release stimulates AMPARs, inducing rapid local depolarization of neurons. Depolarization of the postsynaptic dendrites allows NMDARs and L-type voltage-gated calcium channels (LVGCCs) to open, flooding the neuron with calcium. The downregulation of GluA2 results in more calcium-permeable AMPARs at the synaptic membrane, all of which culminates in an overload of intracellular calcium. This increase in intracellular calcium results in excitotoxicity and ultimately cell death.

Dysregulation at the transcriptional and translational levels also contributes to the ischemia/reperfusion-induced decrease in GluA2-containing AMPARs. During ischemia, protein synthesis is reduced by up to 30% [Diuricic *et al.* 1994]. Following ischemia, GRIA2 (GluA2) messenger ribonucleic acid (mRNA) is selectively reduced in the CA1 region of the hippocampus [Pelligrini-Giampietro *et al.* 1997; Ying *et al.* 1997; Fernandes *et al.* 2014]. The repressive transcription factor repressor element silencing transcription factor-1 (REST-1) is increased with ischemia [Calderone *et al.* 2004] and suppresses transcription of GRIA2 with ischemia/reperfusion [Gorter *et al.* 1997]. Knockdown or inhibition of REST-1 rescues the ischemia/reperfusion-induced decrease in GRIA2, increasing neuronal cell viability [Calderone *et al.* 2004; Noh *et al.* 2012; Doeppner *et al.* 2013]. Furthermore, the enzyme responsible for the post-transcriptional Q/R editing of the GluA2 subunit, ADAR2, is decreased with ischemia/reperfusion. The overexpression of ADAR2 or constitutive activation of CREB, which is a positive transcriptional regulator of ADAR2 expression, restores the Q/R editing of GluA2 and ultimately results in neuroprotection [Peng *et al.* 2006].

Preventing the ischemia/reperfusion-induced loss of GluA2-containing AMPARs could be a crucial target for the treatment of DND following ischemic stroke. There are many different proteins involved in this process. A recent study identified that activation of mGluR5 prevents the ischemia-induced downregulation of GluA2-containing AMPARs [Cavallo *et al.* 2020]. Treatment with pertussis toxin, a G-protein blocker, also prevents pathologic calcium influx following ischemic stroke, and thereby reducing apoptosis [Tang, *et al.* 2015]. Inhibition of presynaptic adenosine A1 receptors also prevents hypoxia-induced GluA1—GluA2 AMPAR internalization [Chen, *et al.* 2014], potentially through increased activation of protein

phosphatases, PP2A and PP2B [Stockwell, *et al.* 2016]. Acid-sensing ion channel 1a inhibition may afford neuroprotection following ischemia/reperfusion by preventing the increase in calcium-permeable AMPARs [Quintana, *et al.* 2015].

One limiting factor to elucidating the mechanisms behind the ischemia/ reperfusion-induced degradation of GluA2-containing AMPARs, is the inconsistency of model systems between researchers. It has been well established that ischemic stroke is an age and sex-related disease [Virani *et al.* 2020], however many studies rely upon young male animals, or primary neuronal cultures. Lalonde and Mielke [2014] confirmed that age effects the ischemia/reperfusion-induced vulnerability of the hippocampus. It's important to use a model system that has a high translatability to the clinic, and my Specific Aim 2 and Specific Aim 3 includes sex as a variable, as well as using aged animals to be as physiologically relevant as possible.

The ischemia/reperfusion-induced loss of GluA2-containing AMPARs is affected at many different levels, including both the protein and mRNA levels. These mechanisms all result in decreased surface expression of GluA2-containing AMPARs, which mediates ischemia/reperfusion excitotoxicity, and ultimately DND in vulnerable neuronal populations, including CA1 pyramidal neurons in the hippocampus.

NADPH Oxidase

Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) was first discovered over 50 years ago in neutrophils, where it produces oxidative stress as part of the host response to bacteria [Infanger *et al.* 2006]. NADPH oxidase (NOX) is an enzyme that catalyzes the electron transfer between NADPH and molecular oxygen (O_2) to form superoxide ($O_2^{\cdot -}$) and

$NADP^+$. Flavin adenine dinucleotide (FAD) is a required cofactor for this reaction. It was previously thought that NOX was only involved in the body's immune response, but now it is well established that NOX plays a large role in many cellular functions. There are seven distinct NOX proteins (NOX1-5 and DUOX1/2), all of which produce reactive oxygen species (ROS) (Diagram 1.9) [Brown and Griendling 2009].

NOX is expressed in a variety of tissues, including the CNS. NOX1, NOX2, NOX3, and NOX4 are all expressed in the CNS [Sorce and Krause 2009]. The ROS produced from NOX plays a crucial role in physiological signaling, but under pathological conditions, the uncontrolled production of ROS from NOX can have detrimental effects on neuronal homeostasis [Bedard and Krause 2007].

NOX1 was the first NADPH oxidase subunit to be cloned [Suh *et al.*1999]. It is highly expressed in the colon [Rokutan *et al.* 2008], but is also expressed in the uterus, the placenta [Cui *et al.* 2006], neurons, astrocytes, and microglia [Sorce and Krausse 2009]. NOX1 activation occurs when the cytosolic proteins $p47^{phox}$, $p67^{phox}$, and Rac1 translocate to the membrane and form a complex with the Nox1 subunit and $p22^{phox}$ [Kawahara *et al.* 2005; Banfi *et al.* 2003; Cheng *et al.* 2006]. NOX1 can also associate with NOXO1 and NOXA1, $p47^{phox}$ and $p67^{phox}$ analogs, respectively [Sumimoto *et al.* 2005]. In microglia lipopolysaccharide stimulation activates NOX1, which suggests a potential role in host defense [Cheret *et al.* 2008]. NOX1 has also been shown to negatively regulate neurite outgrowth in response to nerve growth factor [Ibi *et al.* 2006].

NOX2 is highly expressed in phagocytes, but also plays a critical role in the CNS [Sorce and Krause 2009]. NOX2 is regulated in a similar manner to NOX1. The activation of NOX2

occurs when the cytosolic proteins Rac1, $p47^{phox}$ and $p67^{phox}$ translocate to the membrane [Abo *et al.* 1994; Clark 1999; Clark *et al.* 1989] and complex with membrane proteins Nox2, also referred to as $gp91^{phox}$, and $p22^{phox}$ [El Benna *et al.* 1996; Brown and Griendling 2009].

NOX3, 4, and 5 were initially discovered in 2000 [Cheng *et al.* 2001]. NOX3 is highly expressed in the inner ear and its mechanism of activation has yet to be fully elucidated. Some studies demonstrate that $p22^{phox}$ is necessary for Nox3 activation [Ueno *et al.* 2005], while others suggest that full activation requires Rac1 and various combinations of the cytosolic subunits [Cheng *et al.* 2004; Ueyama *et al.* 2006; Miyano *et al.* 2009; Miyano and Sumimoto 2007]. However, most studies agree that NOXO1 and $p67^{phox}$ are sufficient to activate NOX3 [Kiss *et al.* 2006].

NOX4 is highly expressed in the kidney, but is also found in mesangial cells [Gorin *et al.* 2003], fibroblasts [Cucoranu *et al.* 2005], osteoclasts [Yang *et al.* 2001], and neurons [Vallet *et al.* 2005]. NOX4 is highly expressed compared to other NOX homologues [Krause 2004]. NOX4 is unique compared to other NOX subunits as it only requires $p22^{phox}$ to produce ROS and is constitutively active [Ellmark *et al.* 2005]. The superoxide produced from NOX4 is barely detectable as it is rapidly converted to hydrogen peroxide (H_2O_2) [Serrander *et al.* 2007]. NOX4 is expressed in the nucleus and the endoplasmic reticulum [Kuroda *et al.* 2005; Ambasta *et al.* 2004; Helmecke *et al.* 2008; Chen *et al.* 2008; Van Buul *et al.* 2005].

NOX5 is expressed in testis [Banfi *et al.* 2001], endothelial cells [BelAiba *et al.* 2007], uterus [Cheng *et al.* 2001], and lymphatic tissues [BelAiba *et al.* 2007], but has not been shown to be expressed in the CNS. NOX5 is activated by calcium, possibly through a calmodulin-dependent mechanism [Banfi *et al.* 2001; Cheng *et al.* 2001]. NOX5 is localized to plasma

membranes, and possibly internal cellular structures [Kawahara and Lambeth 2008; Serrander *et al.* 2008].

DUOX1/2 proteins were originally isolated in the thyroid [Dupuy *et al.* 1999], but are also expressed in the respiratory epithelium [Harper *et al.* 2006]. DUOX1/2 produce H_2O_2 by a two-electron reduction of oxygen [Dupuy *et al.* 1989]. DUOX1/2 proteins are localized to the plasma membrane [De Deken *et al.* 2000], and as of yet, these proteins have not been shown to be expressed in the CNS.

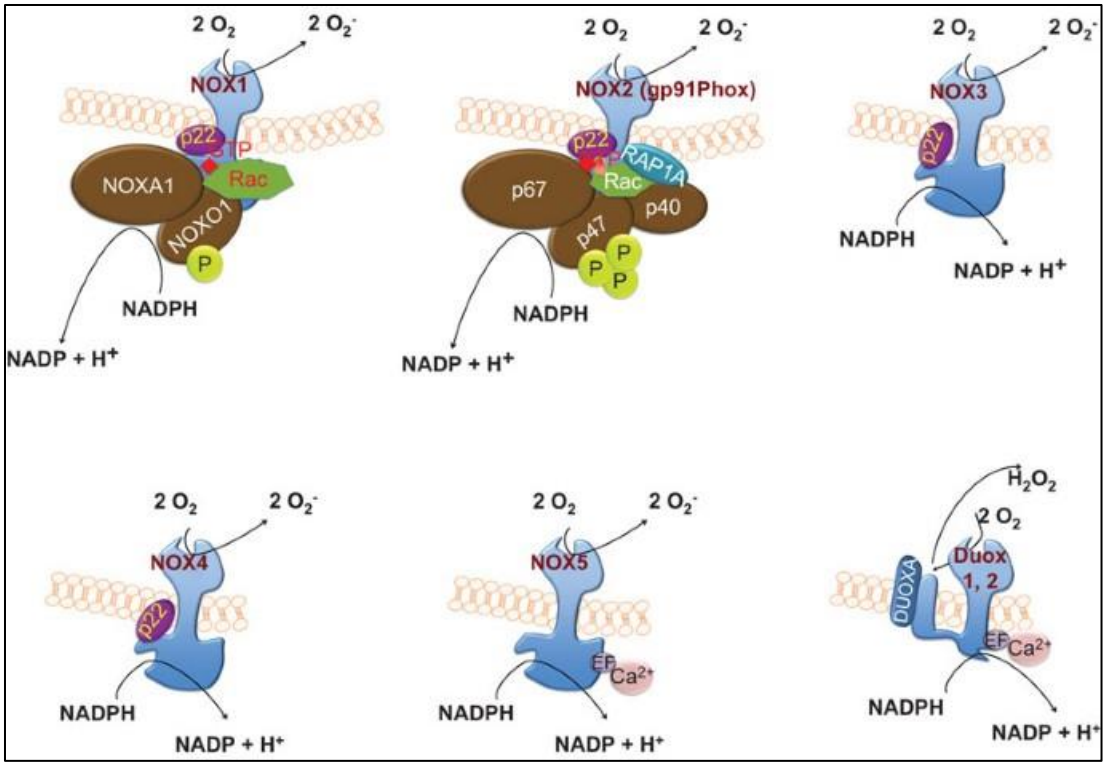

Diagram 1.9: Required proteins for activation of NADPH oxidases. Various NOX isoforms (NOX1-5, DUOX1-2) and the proteins required for their activation Figure from [Panday *et al.* 2014].

Within the CNS, Nox proteins are localized to dendrites, axons, as well as the soma [Tejada-Simon *et al.* 2005]. Superoxide plays a critical role in neuronal function, including LTP and intracellular signaling [Berry and Hare 2004; Di and Bernardi 2006; Di *et al.* 2007]. Treatment with superoxide scavengers prevents the induction of LTP, and overexpression of superoxide dismutase impairs LTP [Thiels *et al.* 2000]. Genetic ablation of regulatory proteins that comprise the NADPH oxidase complex, including $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$, lead to impaired cognitive function in mice [Kishida *et al.* 2006]. NADPH oxidase also plays a critical role in the regulation of NMDAR signaling. Oxidation of GluN1 and GluN2A NMDAR subunits can modify the kinetic properties of NMDARs [Lipton *et al.* 2002]. Indeed, inhibition of NADPH oxidase prevents NMDAR-dependent LTP [English and Sweatt, 1997; Kishida *et al.* 2005]. Calcium-entry from NMDARs can also regulate the cytosolic regulator proteins of NADPH oxidase, increasing activation of NADPH oxidase [Brennan *et al.* 2009].

It is clear now that neurons require a carefully regulated redox environment for normal physiologic function [Infanger *et al.* 2006], so NADPH oxidase must be carefully regulated as the overproduction, as well as underproduction, of ROS can have detrimental consequences. NOX-derived overproduction of ROS has been linked to stroke [Wang *et al.* 2006; Beske *et al.* 2014; Jackson *et al.* 2018; Achzet *et al.* 2021a], Parkinson's disease [Norris and Giasson, 2005], and traumatic brain injury [Dohi *et al.* 2010].

Oxidative Stress in Ischemia/Reperfusion

ROS is a large contributor to neuronal death following ischemic stroke. Following ischemia/reperfusion, the main source of ROS production is activation of NADPH oxidase, resulting in a large production of superoxide [Weston *et al.* 2013]. Mice with no $gp91^{phox}$, the

NOX2 membrane enzyme subunit, have less brain death following ischemia [Walder *et al.* 1997]. Diphenylene iodonium (DPI), which is a nonspecific flavoenzyme inhibitor, treatment decreases the amount of superoxide produced with ischemia [Miller *et al.* 2006], and pretreatment with apocynin, an inhibitor of NOX with scavenger properties (Diagram 1.10), is neuroprotective in the hippocampus following ischemia [Wang *et al.* 2006]. Knockdown of Tiam, a protein responsible for the activation of Rac1 (cytosolic GTPase required for NADPH oxidase activation) also reduces neuronal cell death in the hippocampus following ischemia/reperfusion [Blanco-Suarez *et al.* 2014].

There are also well-established differences in the redox profiles between males and females. For example, there is less oxidative damage in aged female brains compared to aged males due to greater antioxidant levels in females [Guevara *et al.* 2009; Guevara *et al.* 2011]. NOX1 and NOX3 protein levels in basilar arteries are higher in males compared to females. NADPH oxidase-stimulated superoxide production from males is also approximately two-fold greater than in females [Miller, *et al.* 2007; De Silva *et al.* 2009]. Age also plays a role in the oxidative profile of the brain. Hippocampal astrocytes cultured from aged animals have increased NOX activity, as well as decreased antioxidant levels, superoxide dismutase, catalase, and glutathione, compared to astrocytes cultured from young animals [Bellaver *et al.* 2017].

During ischemia/reperfusion, there is a triphasic burst of superoxide from three distinct sources. First during the ischemic phase, the dysfunctional electron transport chain produces superoxide from the mitochondria. Next during the ischemic phase, the cytosolic protein, xanthine oxidase, produces superoxide. Once oxygen and glucose are restored during reperfusion, NADPH oxidase produces a large amount of superoxide [Abramov *et al.* 2007].

There are also many other additional sources of ROS following ischemia/reperfusion including: monoamine oxidase, cyclooxygenase, and lipoxygenase [Allen and Bayraktutan, 2009]. There are also other reactive species produced during ischemia/reperfusion, including nitric oxide and reactive nitrogen species (RNS) [Allen and Bayraktutan, 2009]. Under certain conditions with ischemia, hydrogen peroxide can undergo a Fenton reaction, producing the highly reactive and harmful hydroxyl radical [Crack and Taylor, 2005]. Nitric oxide can also react with superoxide, forming peroxynitrite, a harmful oxidizing agent [Allen and Bayraktutan 2009].

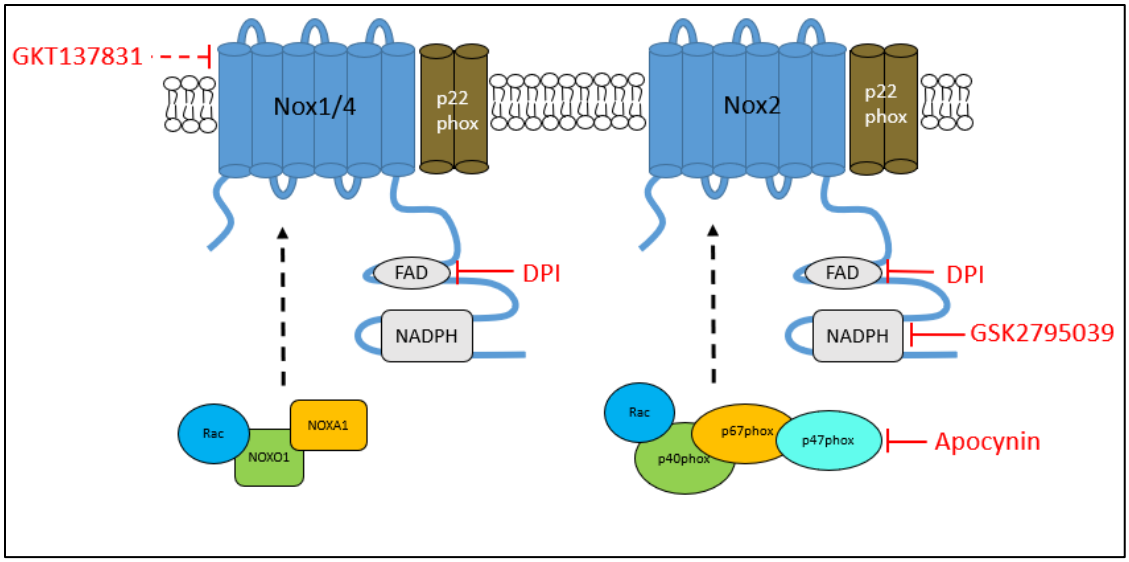

Diagram 1.10: Pharmacological Inhibitors of NOX1/4 and NOX2. Diphenyleneiodonium chloride (DPI) is a nonspecific inhibitor of flavoenzymes. GSK2795039 is a specific inhibitor of NOX2. GKT1378731 inhibits NOX1 and NOX4. Apocynin inhibits the translocation of cytosolic components of NOX, but also has scavenger properties.

Pathological levels of ROS and RNS can result in lipid peroxidation, a process where polyunsaturated fatty acids and ROS react to form a fatty acid radical. That fatty acid radical reacts with molecular oxygen, resulting in a peroxyl-fatty acid radical. This reaction propagates and the peroxyl-fatty acid radical reacts with another fatty acid to produce another fatty acid radical and a lipid peroxide. 4-hydroxynonenal (4-HNE) is a marker for lipid peroxidation and is toxic to neurons [McCracken *et al.* 2000]. High levels of ROS and RNS can also result in protein denaturation, DNA fragmentation, abnormal kinase signaling, and ultimately leads to cytochrome *c* release from the mitochondria, beginning the apoptosis cascade [Allen and Bayraktutan, 2009]. Inhibition of calcium-permeable AMPARs or pre-treatment with an ROS scavenger prevents the activation of the apoptosis signal-regulating kinase 1 (ASK1) pathway [Zhang *et al.* 2003]. Treatment with the free radical scavenger Resveratrol also prevents glutamate receptor-mediated cell death following ischemia [Zhang *et al.* 2008]. We hypothesize that oxidative stress plays a role in the ischemia/reperfusion-induced degradation of GluA2-containing AMPARs.

SPECIFIC AIMS

Specific Aim 1: To examine the effect of superoxide on the endocytic trafficking of AMPAR subunits, GluA1 and GluA2, following OGD/R in U251-MG astroglioma cells.

1.1) To determine whether treatment with a superoxide dismutase mimetic, MnTMPyP, affects the endocytic trafficking of GluA1 and GluA2 AMPAR subunits following OGD/R in U251-MG astroglioma cells.

1.2) To determine whether treatment with MnTMPyP affects the OGD/R-induced ubiquitination and subsequent degradation of GluA1 and GluA2 AMPAR subunits in U251-MG astroglioma cells.

Specific Aim 2: To identify the mechanism(s) responsible for the degradation of GluA2-containing AMPARs following OGD/R in aged (10-12 months of age) rat hippocampal slices.

2.1) To determine whether the OGD/R-induced degradation of GluA2-containing AMPARs occurs via a proteasomal or lysosomal-dependent mechanism.

2.2) To investigate whether a ubiquitination-dependent pathway is responsible for the OGD/R-induced degradation of GluA2-containing AMPARs.

2.3) To investigate the role of calcium and calcium channels in the OGD/R-induced degradation of GluA2-containing AMPARs.

Specific Aim 3: To examine the effect of NADPH oxidase (NOX) on the OGD/R-induced internalization and degradation of GluA2-containing AMPARs in aged (10-12 months of age) ♂/♀ rat hippocampal slices.

3.1) To determine whether superoxide is produced following OGD/R in aged ♂/♀ rat hippocampal slices.

3.2) To examine whether treatment with GKT137381, a NOX 1/4 inhibitor, prevents the ischemic-induced internalization and subsequent degradation of GluA2.

3.3) To examine whether treatment with GSK2795039, a NOX2 inhibitor prevents the OGD/R-induced internalization and subsequent degradation of GluA2-containing AMPARs.

CHAPTER TWO: OXIDATIVE STRESS UNDERLIES THE ISCHEMIC/REPERFUSION-INDUCED INTERNALIZATION AND DEGRADATION OF AMPA RECEPTORS IN U251-MG ASTROGLIOMA CELLS

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Abstract

Stroke is the fifth leading cause of death annually in the United States. Ischemic stroke occurs when a blood vessel supplying the brain is occluded. The hippocampus is particularly susceptible to AMPA receptor-mediated delayed neuronal death as a result of ischemic/reperfusion injury. AMPA receptors composed of a GluA2 subunit are impermeable to calcium due to a post-transcriptional modification in the channel pore of the GluA2 subunit. GluA2 undergoes internalization and is subsequently degraded following ischemia/reperfusion. The subsequent increase in the expression of GluA2-lacking, Ca^{2+}-permeable AMPARs results in excitotoxicity and eventually delayed neuronal death. Following ischemia/reperfusion, there is increased production of superoxide radicals. This chapter describes how the internalization and degradation of GluA1 and GluA2 AMPAR subunits following ischemia/reperfusion is mediated through an oxidative stress signaling cascade. U251-MG cells were transiently transfected with fluorescently tagged GluA1 and GluA2, and different Rab proteins to observe AMPAR endocytic trafficking following oxygen-glucose deprivation/reperfusion (OGD/R), an *in vitro* model for ischemia/reperfusion. Pre-treatment with MnTMPyP, a superoxide dismutase mimetic, ameliorates the OGD/R-induced, but not agonist-induced, internalization and degradation of GluA1 and GluA2 AMPAR subunits. Specifically, MnTMPyP prevents the increased

colocalization of GluA1 and GluA2 with Rab5, an early endosomal marker, and with Rab7, a late endosomal marker, but did not affect the colocalization of GluA1 with Rab11, a marker for recycling endosomes. These data indicate that an oxidative stress signaling cascade is involved in the OGD/R-induced internalization and degradation of GluA1 and GluA2 AMPAR subunits.

Introduction

Stroke is responsible for the death of nearly 140,000 people in the United States annually. Of the estimated 800,000 strokes that occur in the United States each year, approximately 87% are ischemic [Yang, *et al.* 2017]. Ischemic stroke occurs when a blood vessel in the brain is blocked, hindering the vessel's ability to provide oxygen and nutrients to brain tissue. While it is necessary to reintroduce blood flow to the infarcted area, this act also results in further damage by inflammation, oxidative stress, and delayed neuronal death (DND) within vulnerable neuronal populations, including CA1 hippocampal pyramidal neurons [Ford, *et al.* 1989; Mitani, *et al.* 1998]. During ischemia, the lack of energy available disrupts ATP-dependent processes that maintain ionic gradients, which are critical to cellular survival. Disrupting the ionic balance leads to excessive release of neurotransmitters, including glutamate, which are unable to be effectively cleared from the synapses [Camacho & Massieu, 2005]. Excessive stimulation of *N*-methyl-*D*-aspartate receptors (NMDARs) by glutamate is a contributing factor to DND [Faden, *et al.* 1989; Mcintosh, *et al.* 1989; Takagi, *et al.* 1997; Liu, *et al.* 2007], but multiple studies have reported that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) also contribute to DND [Liu, *et al.* 2004; Yin, *et al.* 2002; Anzai, *et al.* 2003; Calderone, *et al.* 2004; Noh, *et al.* 2005; Liu, *et al.* 2006].

AMPARs are ionotropic glutamate receptors composed of GluA1-4 subunits and can be either homomeric or heteromeric tetramers [Blanco-Suarez & Hanley, 2014]. A majority of AMPARs are impermeable to Ca^{2+} due to a post-transcriptional modification in the channel pore of the GluA2 AMPAR subunits [Pellegrini-Giampietry, *et al.* 1997]. Increases in intracellular calcium under physiological conditions regulates many cellular processes, including synaptic plasticity. The overactivation of post-synaptic receptors, such as NMDA receptors and Ca^{2+}-permeable AMPARs, leads to an overload of intracellular calcium. This is a key event responsible for a large amount of neuronal death associated with stroke [Arundine & Tymianski, 2003]. This process is tightly regulated under physiological conditions, and its dysregulation in pathophysiological conditions, such as ischemic stroke, is catastrophic.

Long-term potentiation (LTP), or strengthening of the synapse, is a tightly regulated process underlying how we form memories and learn. In the simplest form of LTP, AMPARs are inserted into the post-synaptic membrane. During long-term depression (LTD), or weakening of the synapse, AMPARs are removed from the post-synaptic membrane [Shepherd & Huganir, 2007]. The incorporation of GluA2-lacking Ca^{2+}-permeable AMPARs into the post-synaptic membrane is important in the induction of LTP [Terashima, *et al.* 2008]. However, when this GluA2 subunit composition switch from GluA2-containing, Ca^{2+}-permeable AMPARs to GluA2-lacking, Ca^{2+}-permeable becomes long-lasting, the results are detrimental to the cell and contribute to neuronal death in several central nervous system pathologies, including stroke [Noh, *et al.* 2005]. As a result of ischemia/reperfusion, AMPARs undergo a subunit composition switch from Ca^{2+}-impermeable, GluA2-containing AMPARs [Kuner, *et al.* 2001], to Ca^{2+}-

permeable GluA2-lacking AMPARs. This allows the AMPAR to conduct calcium which, in combination with excessive NMDAR stimulation, exacerbates cell death [Kwak & Weiss, 2006].

During ischemia/reperfusion there is triphasic burst of superoxide. Upon ischemic onset, superoxide is produced by the mitochondria as a by-product from the dysregulated electron transport chain. Next in the ischemic phase, the cytosolic enzyme xanthine oxidase produces reactive oxygen species, including hydrogen peroxide and superoxide. Once blood flow is restored during the reperfusion phase, NADPH oxidase, a membrane-bound superoxide generator, produces a large burst of superoxide [Abramov, *et al.* 2007]. Additional sources of reactive oxygen species following ischemic/reperfusion injury include monoamine oxidase, catecholamine metabolism, quinone formation, and oxidation of unsaturated fatty acids [Allen & Bayraktutan, 2009].

In this chapter, we show that internalization and degradation of GluA2-containing AMPARs following ischemia/reperfusion is mediated through an oxidative stress signaling cascade. U251-MG, a human astroglioma cell line, were utilized in this study due to their high transfection efficiency and consistent response to oxygen glucose-deprivation/reperfusion (OGD/R), an *in vitro* model for ischemia stroke. U251-MG cells have been used extensively to study various signaling pathways and protein trafficking [Raghu, *et al.* 2011; Cassoni, *et al.* 2007; Zhang, *et al.*, 2017; Xu, *et al.* 2017; Lee, *et al.* 2016; Johnson, *et al.* 2006; Overmeyer, *et* al. 2011]. U251-MG cells were transiently transfected with fluorescently tagged GluA1 and GluA2, as well as different Rab proteins to examine endocytic/intracellular trafficking of AMPARs following OGD/R. Rab proteins are GTPases that alternate between an inactive GDP-bound state, and an active GTP-bound state. The active GTP-bound state of Rab proteins allows

them to interact with downstream effectors [Stenmark & Olkkonen, 2001]. Previous studies have utilized pHluorin-tagged plasmids to observe AMPAR trafficking [Tin and Huganir, 2007; Thorsen, *et al.* 2010], but these tags have proven problematic [Rathie, *et al.* 2013]. To clarify the trafficking and intracellular localization of GluA1 and GluA2 AMPAR subunits following OGD/R, we utilized various Rab proteins as biomarkers. Rab5 is localized to early endosomes [Gorvel, *et al.* 1991]. Rab7 is localized to late endosomes destined for lysosomal degradation [Mukhopadhyay, *et al.* 1997]. Rab11 is localized to recycling endosomes that undergo exocytosis [Lock and Stow, 2005]. To examine whether superoxide plays a role in AMPAR trafficking following OGD/R, we pretreated the transfected U251-MG cells with Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), a cell-permeable superoxide dismutase mimetic, and examined the endocytic trafficking of GluA2 and GluA2 AMPAR subunits following OGD/R. Interestingly, we identified oxidative stress-dependent differential trafficking patterns between GluA1 and GluA2 AMPAR subunits following OGD/R.

Materials and Methods

Cell Culture

U251-MG cells, an immortalized human astrocytoma cell line, were maintained in DMEM, supplemented with 10% fetal bovine serum, 1X penicillin/streptomycin, 1X MEM non-essential amino acids, and 1X sodium pyruvate. All reagents for cell culture were purchased from Gibco (Gaithersburg, MD, USA). Once cells reached 75% confluency, they were passaged.

Transfection

U251-MG cells were transiently transfected with GluA1-tdTomato, GluA1-eGFP, GluA2-tdTomato, or GluA2eGFP, and either Rab5-cerulean, Rab7-RFP, or Rab11-RFP. Cells were transfected using Lipofectamine 2000 in Opti-MEM media (Fisher Scientific, Pittsburgh, PA, USA) for 4 hours before the transfection media was replaced with complete DMEM media. Experiments were performed 48 hours following transfection.

AMPA Stimulation

Transfected U251-MG cells (75,000 cells/well on 6-well plates) were exposed to saturating conditions of AMPA (100μM) [Chen, *et al.* 2014], cyclothiazide (100μM) [Fedorova, *et al.* 2009] to prevent AMPAR desensitization, and MnTMPyP (100μM) [MacKenzie and Martin, 1998; Nilkantan, *et al.* 2008] as previously indicated for 3 minutes before being removed and replaced with complete medium. U251-MG cells were fixed with 4% paraformaldehyde at time points: 5 and 10 minutes. Cells were washed twice with phosphate buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) and mounted on slides for microscopy analysis.

Oxygen Glucose-Deprivation/Reperfusion (OGD/R)

Transfected U251-MG cells were exposed to deoxygenated artificial cerebrospinal fluid (aCSF) without glucose (aCSF-glucose; NaCl [124mM], KCl [2.5mM], $NaHCO_3$ [26mM], $NaHPO_4$ [1.25mM], $CaCl_2$ [2.5mM], $MgCl_2$ [1.5mM], sucrose [10mM] or D-glucose [10mM] for reperfusion conditions (aCSF+glucose)) in a hypoxic chamber for 20 minutes. aCSF-glucose was deoxygenated with nitrogen for 18 hours prior to experiment to ensure hypoxic conditions. aCSF-gluocse was replaced with aCSF+glucose at various time-points: 0, 5, 15, 30, or 60

minutes. Experiments were conducted in the presence or absence of MnTMPyP [100μM], a superoxide dismutase mimetic. U251-MG cells were either prepared for western blotting analysis, or fluorescent microscopy. Normoxic controls were time-matched to the longest OGD/R time-point.

Microscopy

Following OGD/R or AMPA stimulation experiments, transfected U251-MG cells were imaged via confocal microscopy, Olympus FluoView1000/IX81 confocal microscope system (Olympus Corporations of the Americas Headquarter, Center Valley, PA, USA) and colocalization coefficients (Pearson's Correlation Coefficient) were obtained using FIJI software (freely available from National Institutes of Health, Bathesda, MD, USA). All microscopy and data analyses were conducted in a blind manner.

Western Blotting

Protein concentration was determined using a bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Waltham, MA, USA) and samples were heated at 100°C in LDS/reducing agent buffer (Thermo Fisher Scientific, Waltham, MA, USA) and resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were then transferred to a nitrocellulose membrane (Bio-Rad, Berkeley, CA, USA). Blots were blocked for one hour at room temperature with 5% (w/v) non-fat dry milk in tris buffered saline, 0.1% (v/v) tween-20, pH 7.5 (TBS-T). After blocking, blots were incubated with primary antibody overnight at 4°C at the concentration indicated below. Blots were incubated with secondary antibody for 1 hour at room temperature. The following antibodies were used: anti-RFP (1:500; Invitrogen, Waltham,

MA, USA), anti-β-Actin (1:10,000; Cell Signaling, Danvers, MA, USA), anti-mouse (1:2000; Cell Signaling, Dancers, MA, USA), and anti-rabbit (1:2000; Cell-Signaling, Dancers, MA, USA). Immunoreactive bands were visualized and captured with a Fuji imaging system using enhanced chemiluminescence. Bands were visualized using Fuji Image-Gauge software (FujiFilm North America Corporation, Valhalla, NY, USA).

Trypan Blue Exclusion

Following OGD/R, U251-MG cells were incubated in trypsin/EDTA (0.05%) and centrifuged at 1000 *x g* for 10 minutes. The cell pellet was resuspended in complete medium and trypan blue (1:1 dilution). Cell viability was determined using trypan blue exclusion on Countess II Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA).

NBT Assay

U251-MG cells were plated at $5x10^4$ in 6 well plates, and once they reached 70% confluency, were used for experiments. NBT assay was adapted from [Aukrust, *et al.* 1994; Choi, *et al.* 2006]. Briefly, after 4 hours of serum starvation, 0.5mg/mL NBT was added to cells and incubated for one hour. Excess NBT was washed away 3 times with warmed PBS. U251-MG cells underwent 20 minutes of OGD, and various reperfusion time points: 0, 15, 30, or 60 minutes with or without MnTMPyP treatment. Phorbol 12-myristate 13-acetate (PMA; 1μM; Sigma Aldrich, St. Louis, MO, USA) was used as a positive control. Following treatment, cells were fixed with absolute methanol, air dried, and formazan deposits were dissolved in 100% dimethyl sulfoxide and KOH [2M]. The absorbance was measured at 620nm with Spectra Max Gemini M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Data Analysis and Scientific Rigor

Either Student's t-test or One-way ANOVA with post-Hoc Tukey test were conducted using GraphPad Prism 8 software to determine statistical significance. All imaging and analyses were performed blindly. A p value below 0.05 was considered statistically significant.

Results

OGD/R does not alter cell viability in U251-MG cells.

We first sought to determine whether our model of OGD/R resulted in increased cell death compared to normoxic controls. There was no significant difference in cell viability by trypan blue exclusion in the OGD/R-exposed cells compared to the normoxic control (Figure 1.1), confirming that our *in vitro* model of ischemia/reperfusion is not lethal and any differences in protein levels is not a result of cell death.

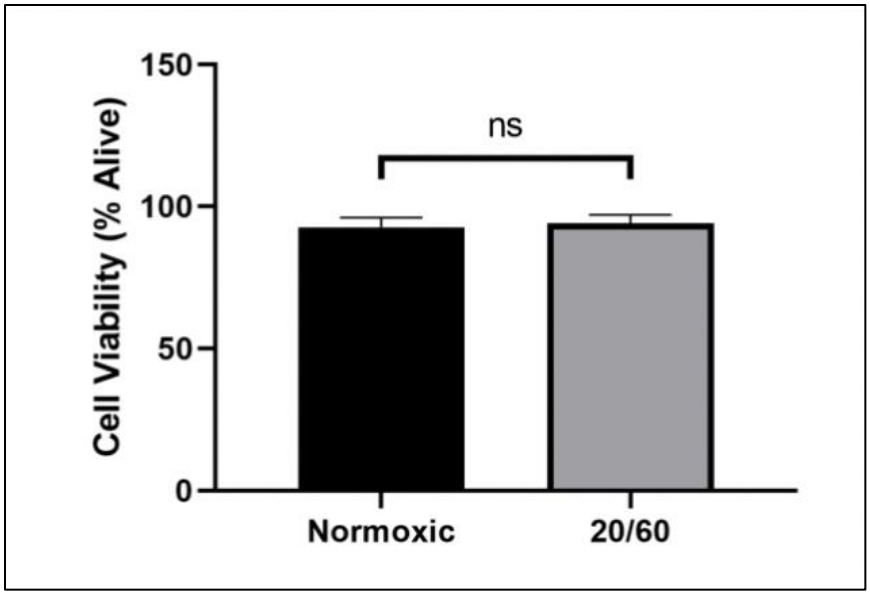
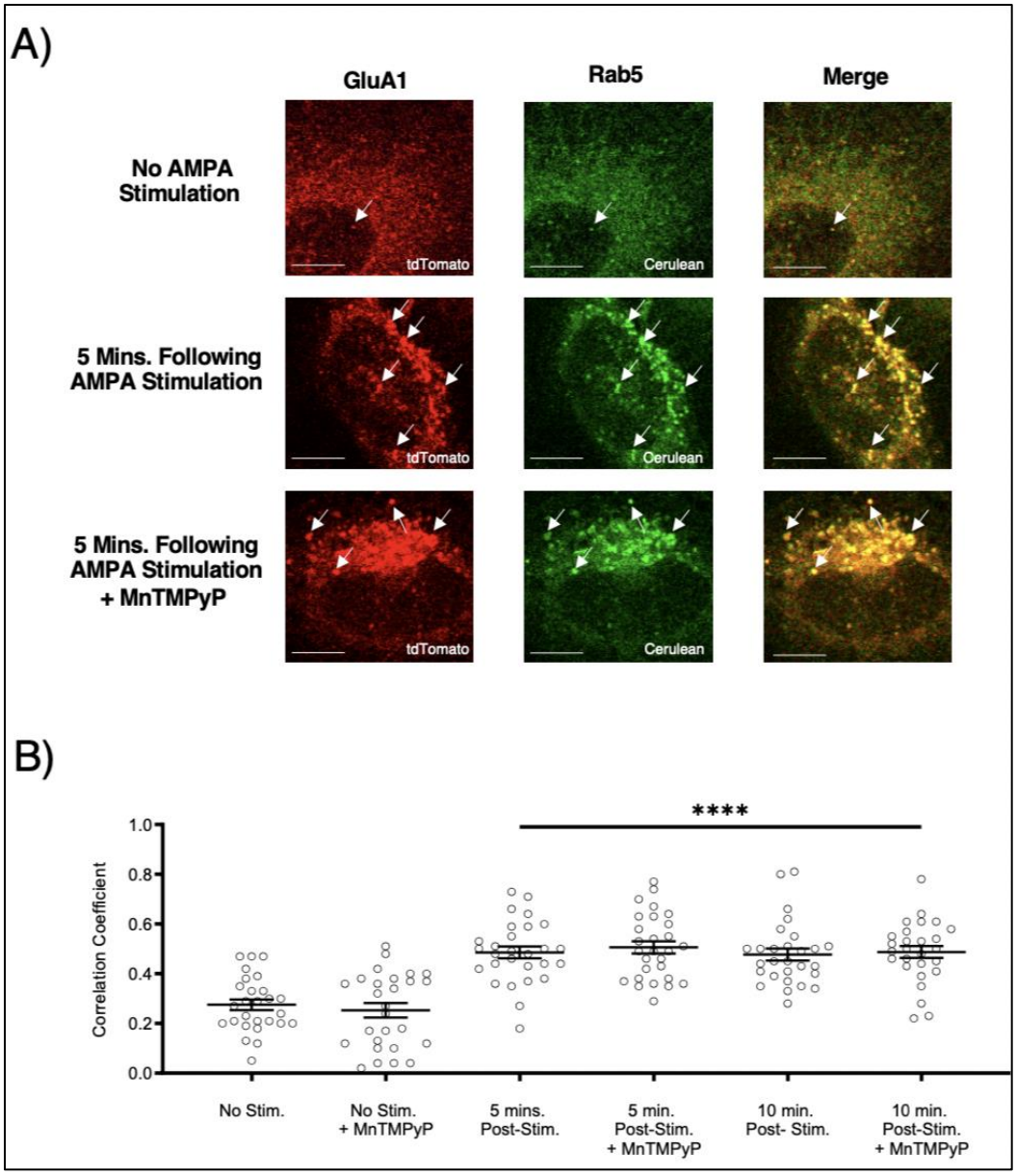


Figure 1.1: OGD/R does not alter viability of U251-MG cells. U251-MG cells underwent OGD for 20 minutes, followed by reperfusion for 60 minutes. There was no significant difference between the viability of normoxic control U251-MG cells and OGD/R-treated U251-MG cells (N=3; unpaired student t-test).

MnTMPyP pretreatment does not prevent agonist-induced internalization of AMPARs.

To examine whether our superoxide dismutase mimetic, MnTMPyP, prevents agonist-induced internalization, we first exposed U251-MG cells to 3 minutes of agonist stimulation (100μM AMPA, 100μM cyclothiazide) in the presence or absence of MnTMPyP (100μM). Following the 3 minutes of AMPA/cyclothiazide stimulation, cells were fixed at 5- or 10-minute time-points. There was an increase in colocalization between GluA1 and Rab5, and GluA2 and Rab5 following AMPA stimulation (Figure 1.2). This increased colocalization indicates that GluA1 and GluA2 AMPAR subunits are internalized and present within Rab5-positive early endosomes. Agonist-induced internalization of GluA1 and GluA2 in Rab5-positive endosomes is not antagonized by MnTMPyP.


A)
GluA1
Rab5
Merge
No AMPA Stimulation
5 Mins. Following AMPA Stimulation
5 Mins. Following AMPA Stimulation + MnTMPyP
tdTomato
Cerulean
B)
****
Correlation Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
No Stim.
No Stim. + MnTMPyP
5 mins. Post-Stim.
5 min. Post-Stim. + MnTMPyP
10 min. Post- Stim.
10 min. Post-Stim. + MnTMPyP

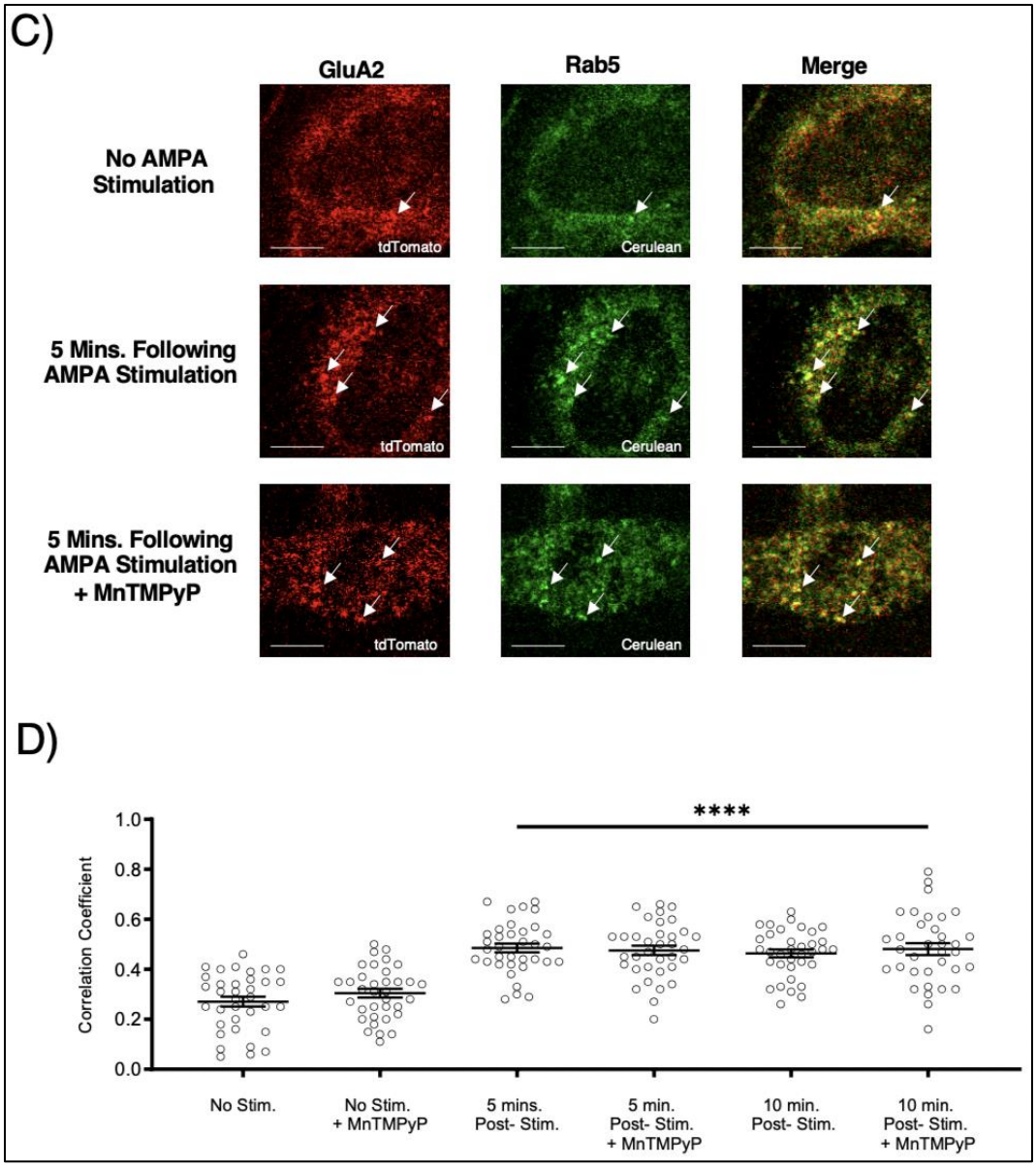


Figure 1.2. Pre-treatment with MnTMPyP does not prevent agonist-induced internalization of AMPARs. U251-MG cells transfected with either GluA1-tdTomato or GluA2-tdTomato and Rab5-cerulean, an early endosomal marker, and stimulated with AMPA agonist [100μM]. (A) and (B) Increased colocalization between GluA1 and Rab5, and GluA2 and Rab5, respectively, indicates internalization following agonist-stimulation. Pre-treatment with MnTMPyP, a superoxide scavenger does not prevent the agonist-induced internalization of GluA1 or GluA2 AMPAR subunits. (C) and (D) are quantifications of

(A) and (B), respectively (N=30). **** $p<0.0001$; ANOVA with Tukey *post hoc* test comparing agonist conditions to corresponding controls (No agonist stimulation ± MnTMPyP). Data are expressed as *mean* ± *SEM*.

OGD/R has been previously shown to produce reactive oxygen species (ROS) in hippocampal and cortical neurons [Abramov, *et al.* 2007], and we wanted to examine whether we observe a similar effect in our model system. Utilizing a nitroblue tetrazolium (NBT) assay, we determined that U251-MG cells produce ROS in a time-dependent manner with OGD/R exposure. Phorbol 12-myristate 13-acetate (PMA) activates NADPH oxidase resulting in increased ROS production [Kuwabara, *et al.* 2015] and was utilized as a positive control for the NBT assay. ROS production following OGD/R is first observed at reperfusion time-point 15 minutes, and maximally produced with 60 minutes of reperfusion. Pre-treatment with MnTMPyP ameliorates this OGD/R-induced increase in ROS (Figure 1.3). Based upon previous results [Beske, *et al.* 2015; Jackson, *et al.* 2018] within our lab, we sought to determine whether superoxide production had a direct effect on AMPAR subunit endocytic trafficking following OGD/R.

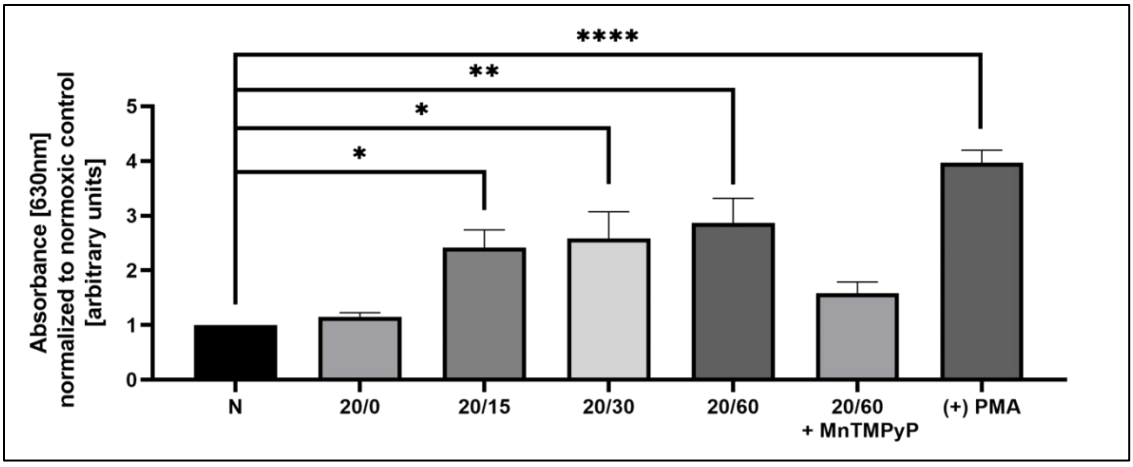
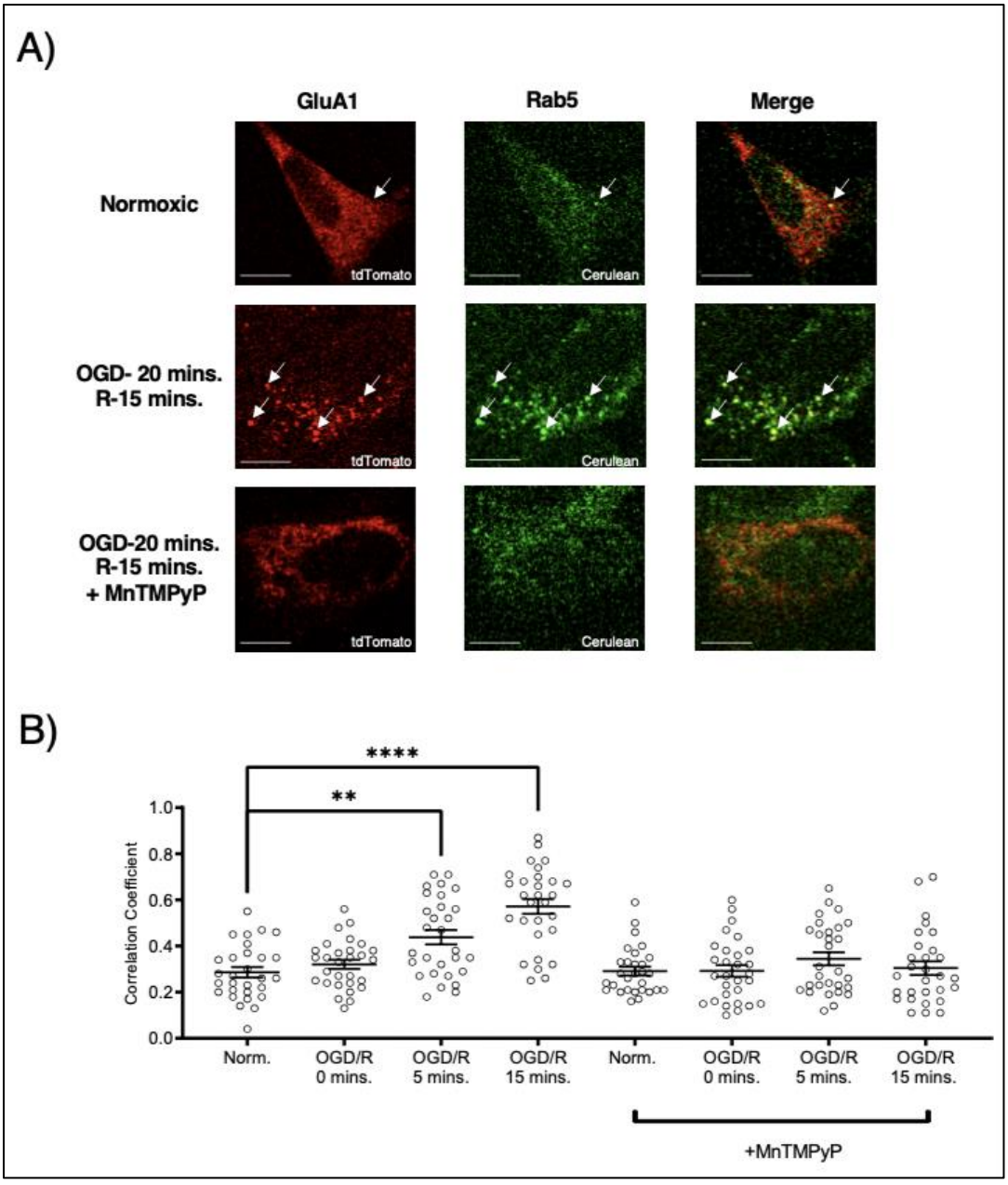


Figure 1.3: Pretreatment of U251-MG cells with MnTMPyP scavenged OGD/R-induced ROS. Spectrophotometric quantification of ROS following either OGD/R or normoxic conditions with or without pre-treatment of MnTMPyP (100µM) utilizing nitroblue tetrazolium chloride (NBT), to examine ROS production (N=3). Phorbol 12-myristate 13-acetate (PMA; 1µM for 15 minutes) was used as a positive control. * $p<0.05$; ** $p<0.01$; **** $p<0.0001$; ANOVA with Tukey *post hoc* test comparing OGD/R conditions to normoxic control ± MnTMPyP. Data are expressed as *mean* ± *SEM*.

Pretreatment with MnTMPyP attenuates the OGD/R-induced internalization of GluA1 and GluA2 AMPAR subunits.

Since our model system, U251-MG cells, produce ROS during OGD/R, we wanted to determine whether pre-treatment with a superoxide scavenger, MnTMPyP, affected the endocytic trafficking of AMPAR subunits, GluA1 and GluA2. Following OGD/R, GluA1 and GluA2 subunits both highly colocalized with Rab5-positive early endosomes. Interestingly, the time-points of GluA1 and GluA2 internalization differed with OGD/R. GluA1 was highly colocalized with Rab5 at OGD/R-5 and OGD/R-15-minute time-points. GluA2 colocalized with Rab5-positive early endosomes solely at the OGD/R-5-minute time-point, indicating that GluA2 moved quickly within the endocytic pathway from an early endosome to a sorting endosome, or a Rab7-positive late endosome. GluA1 and GluA2 transiently transfected in U251-MG cells internalized following OGD/R exposure. To determine whether oxidative stress played a role in this internalization, we pretreated the U251-MG cells with MnTMPyP. Pretreatment with MnTMPyP attenuated the OGD/R-induced colocalization of both GluA1 and GluA2 with Rab5-positive early endosomes (Figure 1.4).


A)
GluA1
Rab5
Merge
Normoxic
OGD- 20 mins.
R-15 mins.
OGD-20 mins.
R-15 mins.
+ MnTMPyP
tdTomato
Cerulean
B)
****
**
Correlation Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
Norm.
OGD/R 0 mins.
OGD/R 5 mins.
OGD/R 15 mins.
Norm.
OGD/R 0 mins.
OGD/R 5 mins.
OGD/R 15 mins.
+MnTMPyP

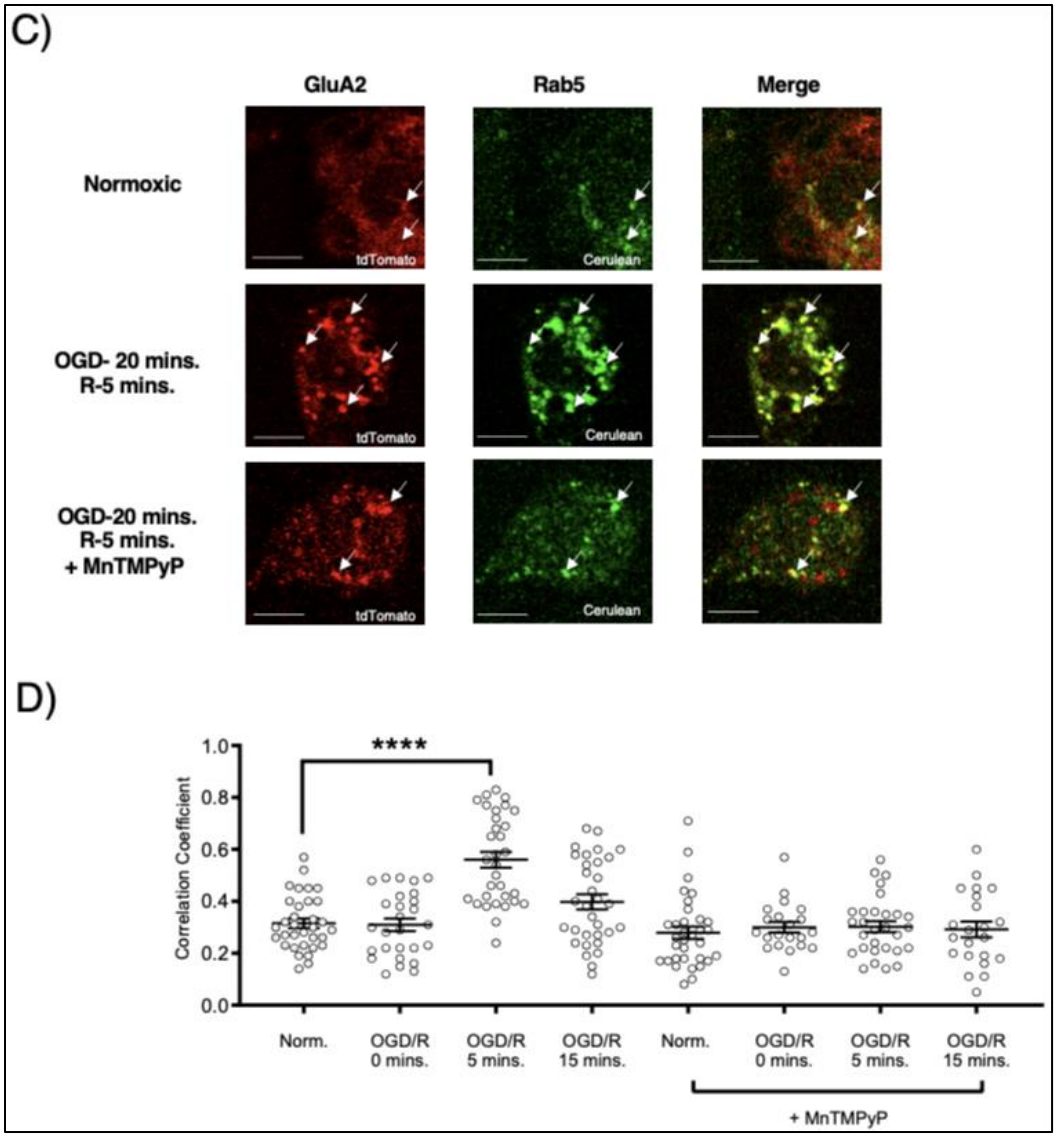
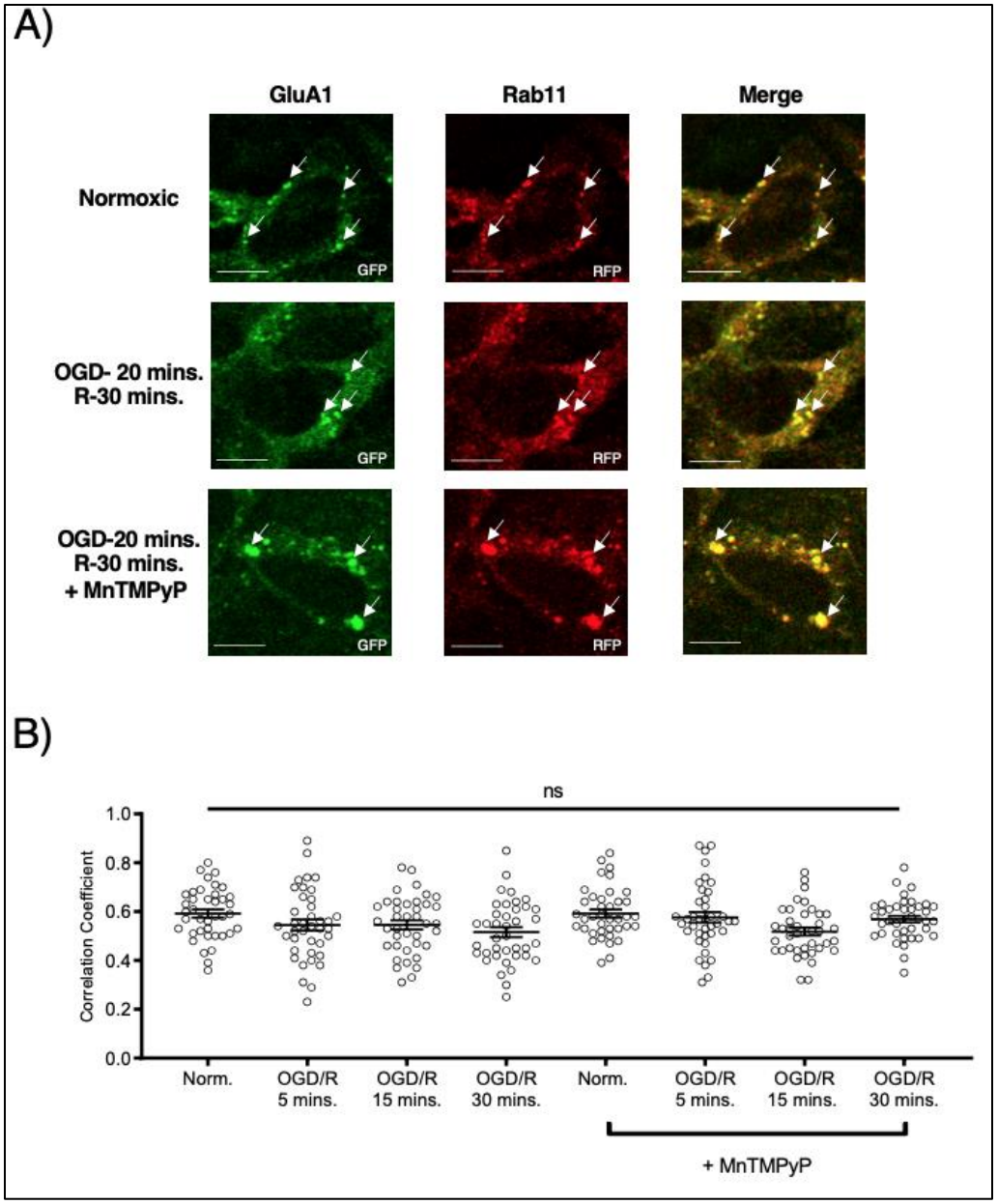


Figure 1.4: MnTMPyP prevents the OGD/R-induced internalization of GluA1 and GluA2 AMPAR subunits. U251-MG cells transfected with either GluA1-tdTomato or GluA2-tdTomato, and Rab5-cerulean, an early endosomal marker, and exposed to either normoxic or OGD/R conditions. (A) Increased colocalization between GluA1 and GluA2, indicating internalization, maximally at OGD/R-15 minutes; the effect is ameliorated with pre-treatment of MnTMPyP, a superoxide scavenger. (B) Quantification of (A) microscopy (N=30). (C) Increased colocalization between GluA1 and Rab5, indicating internalization, maximally at OGD/R-5 minutes; the effect is ameliorated with pre-treatment of MnTMPyP. (D)

Quantification of (C) microscopy results (N=30). *p<0.05; **p<0.01; ***p<0.001; ANOVA with Tukey *post hoc* test comparing OGD/R conditions to corresponding control (Normoxic ± MnTMPyP). Data are expressed as *mean* ± *SEM.*

GluA1 and GluA2 AMPAR subunits do not undergo OGD/R-induced degradation in the presence of MnTMPyP.

Following GluA1 and GluA2 AMPAR subunit internalization to Rab5-positive endosomes, experiments were performed to examine whether GluA1 or GluA2 AMPAR subunits are trafficking to Rab11-positive recycling endosomes in cells subjected to OGD/R. GluA1 remained highly colocalized with Rab11-positive recycling endosomes under both normoxic and OGD/R conditions. Treatment with MnTMPyP had no effect on this colocalization under normoxic nor OGD/R conditions (Figure 1.5A and 1.5C). Conversely, GluA2 was not highly colocalized with Rab11-positive recycling endosomes under any conditions, with or without MnTMPyP treatment (Figure 1.6A and 1.6C). It's likely that there is a recycling pool of GluA1 homomeric AMPARs under both basal and pathologic conditions, but not GluA2 containing AMPARs.


A)
GluA1
Rab11
Merge
Normoxic
OGD- 20 mins.
R-30 mins.
OGD-20 mins.
R-30 mins.
+ MnTMPyP
GFP
RFP
B)
ns
Correlation Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
Norm.
OGD/R 5 mins.
OGD/R 15 mins.
OGD/R 30 mins.
Norm.
OGD/R 5 mins.
OGD/R 15 mins.
OGD/R 30 mins.
+ MnTMPyP

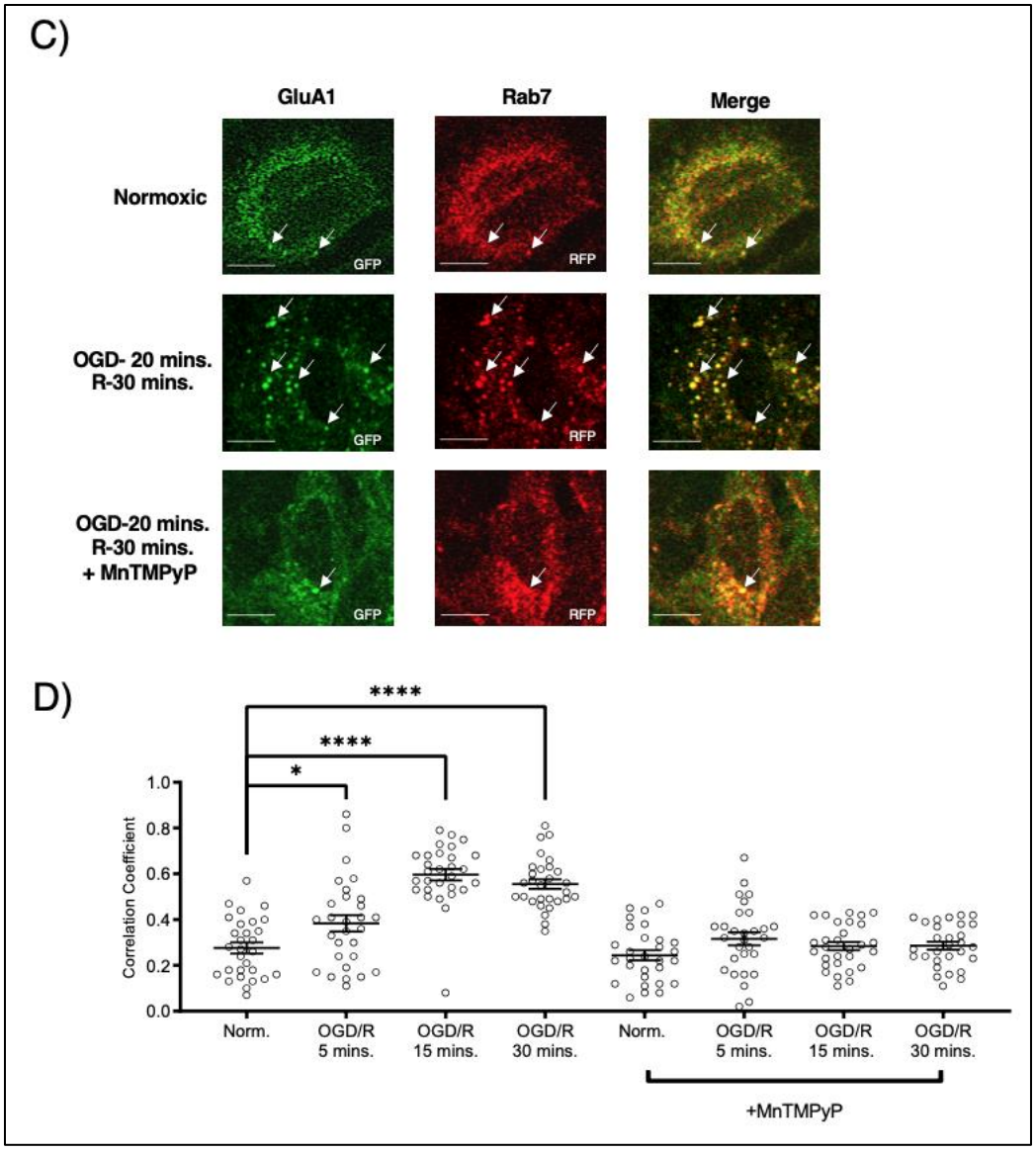
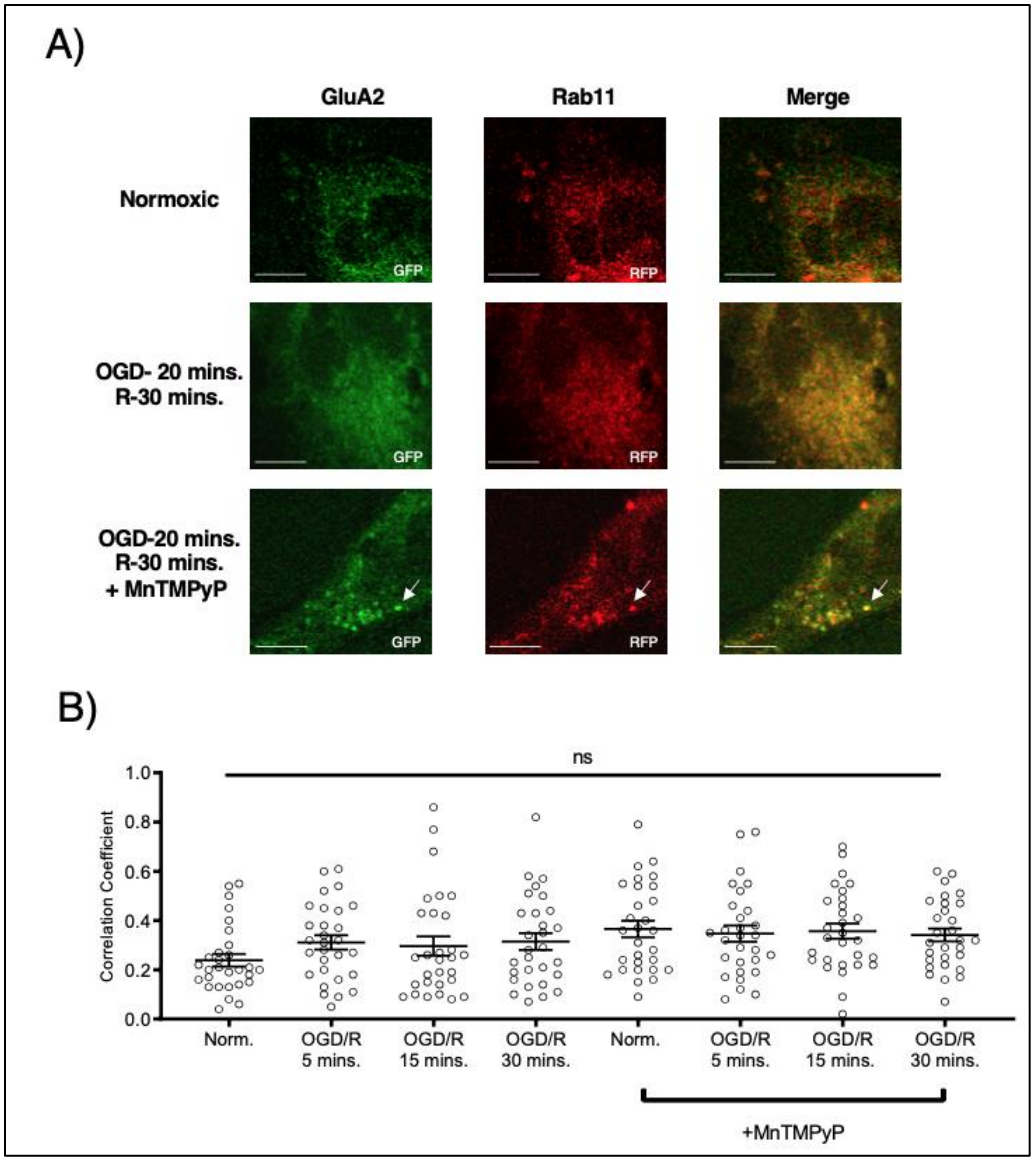


Figure 1.5: MnTMPyP prevents OGD/R-induced Rab-7, late endosomal sorting of GluA1 AMPAR subunits. GluA1 is present in Rab11-positive endosomes under all conditions. U-251MG cells transfected with GluA1-eGFP and either Rab11-RFP, a recycling endosome marker, or Rab7-RFP, a marker for late endosomes, and exposed to either normoxic or OGD/R conditions. (A) High colocalization between GluA1 and Rab11 under both normoxic and OGD/R conditions, unaffected by MnTMPyP treatment. (B) Quantification of (A) microscopy experiments (N=30). (C) Increased colocalization between GluA1 and Rab7, indicating lysosomal degradation, maximally at OGD/R-15 minutes; the effect is ameliorated with

pre-treatment of MnTMPyP. (D) Quantification of microscopy (N=30) results from (C). * $p<0.05$; **** $p<0.0001$; ns denotes no significance; ANOVA with Tukey *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic ± MnTMPyP). Data are expressed as *mean* ± *SEM*.

To examine whether the AMPAR subunits were being trafficked to the degradative pathway following OGD/R, we examined their colocalization with Rab7-positive late endosomes, destined for lysosomal degradation. Both GluA1 and GluA2 subunits were highly colocalized with Rab7-positive late endosomes following OGD/R, likely indicating they were fated for degradation (Figure 1.5B and 1.5D, Figure 1.6B and 1.6D). Pre-treatment with MnTMPyP, scavenging superoxide, prevented GluA1 and GluA2 AMPAR subunits being sorted to Rab7-positive late endosomes. This may occur because MnTMPyP treatment prevents the AMPAR subunits from internalizing with OGD/R or it prevents GluA1 and GluA2 subunits from trafficking to Rab7-positive endosomes with OGD/R exposure.


A)
GluA2
Rab11
Merge
Normoxic
OGD- 20 mins.
R-30 mins.
OGD-20 mins.
R-30 mins.
+ MnTMPyP
GFP
RFP
B)
ns
Correlation Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
Norm.
OGD/R 5 mins.
OGD/R 15 mins.
OGD/R 30 mins.
Norm.
OGD/R 5 mins.
OGD/R 15 mins.
OGD/R 30 mins.
+MnTMPyP

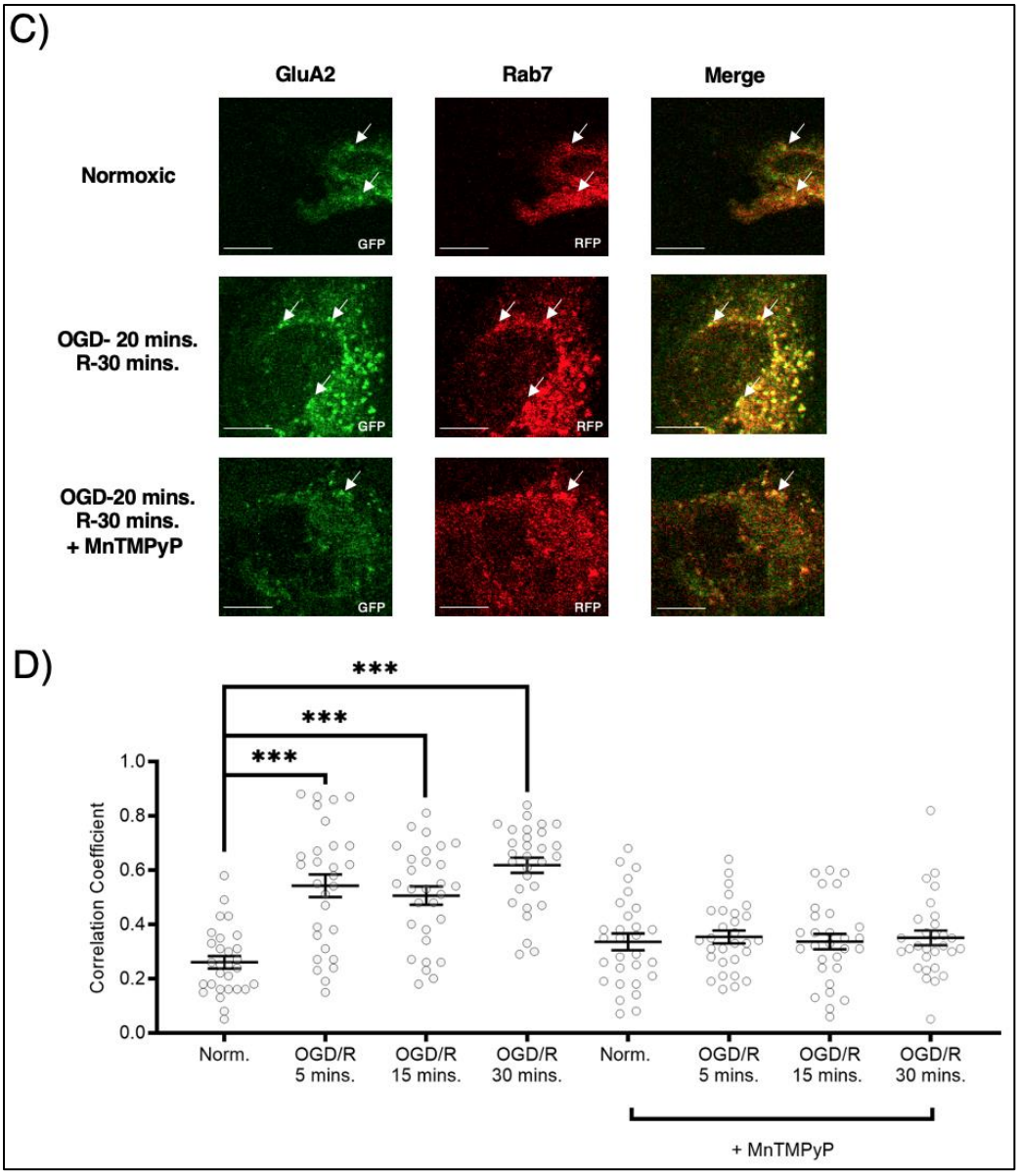


Figure 1.6: MnTMPyP prevents OGD/R-induced Rab-7, late endosomal sorting of GluA2 AMPAR subunits. GluA2 is not present in Rab11, recycling endosomes under any conditions. U-251 MG cells transfected with GluA2-eGFP, and either Rab11-RFP, a recycling endosome marker, or Rab7-RFP, a marker for late endosomes, and exposed to either normoxic or OGD/R conditions. (A) GluA2 does not colocalize with Rab11 under normoxic nor OGD/R conditions. (B) Quantification of microscopy (N=30) results from (A). (C) Increased colocalization between GluA2 and Rab7, indicating lysosomal degradation,

maximally at OGD/R-30-minutes; the effect is ameliorated with pre-treatment of MnTMPyP. (D) Quantification of microscopy (N=30) results from (C). ***p<0.0001; ANOVA with Tukey *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic ± MnTMPyP). Data are expressed as *mean* ± *SEM*.

To further confirm that GluA1 and GluA2 subunits are degraded with OGD/R, we performed western blot analysis to examine their respective protein levels. As expected, both GluA1 and GluA2 are degraded in a time-dependent manner with OGD/R. GluA1 protein levels decreased at OGD/R-30 minute and maximally at OGD/R-60-minute timepoints. GluA2 protein levels decreased at the OGD/R-60-minute timepoint. Pre-treatment with MnTMPyP prevented the OGD/R-induced degradation of GluA1 partially, and GluA2 completely (Figure 1.7).

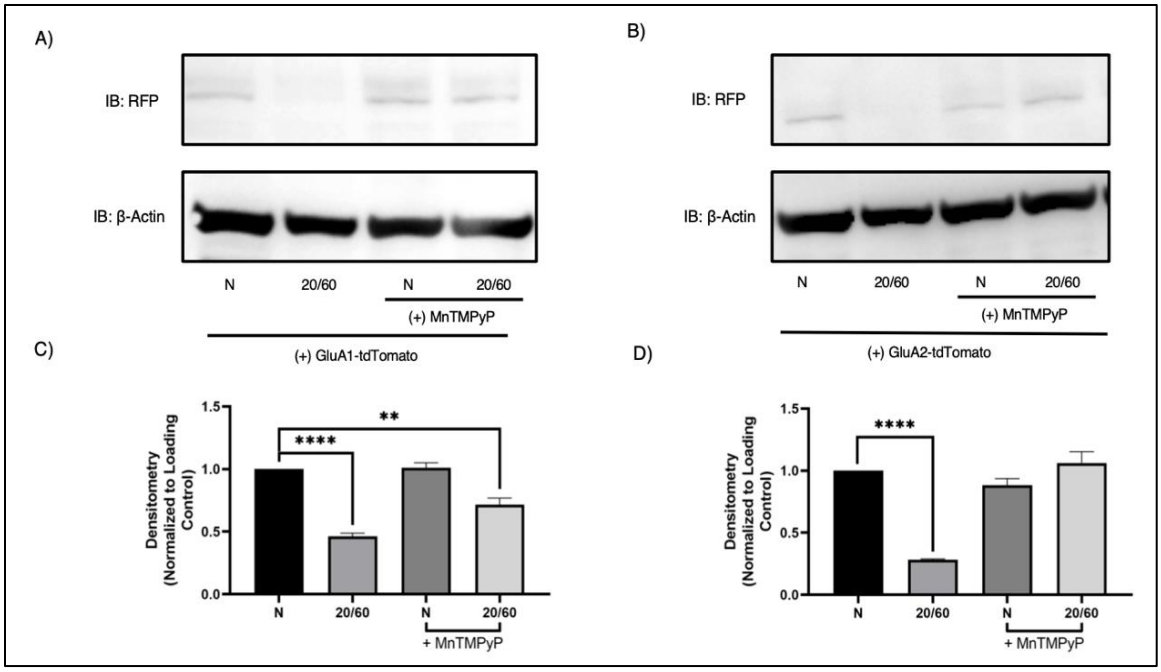


Figure 1.7: MnTMPyP prevents OGD/R-induced degradation of GluA1 and GluA2 AMPAR subunits. U-251 MG cells transfected with GluA1-tdTomato, or GluA2-tdTomato and exposed to either normoxic or OGD/R conditions. (A) Representative western blot demonstrating that pre-treatment with MnTMPyP ameliorates the OGD/R-induced decrease in total GluA1 protein -levels. (C) Quantification of (A) of total GluA1 protein levels normalized to β-Actin (N=3). (B) Representative western blot illustrating that pre-treatment with MnTMPyP ameliorates the OGD/R-induced decrease in total GluA2 protein levels. (D) Quantification of (B) of total GluA2 protein levels normalized to β-Actin (N=3). ****$p<0.0001$; ANOVA with Tukey *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic ± MnTMPyP). Data are expressed as *mean* ± *SEM*.

Discussion

In this study I have demonstrated that ischemic/reperfusion-induced internalization and subsequent endocytic trafficking of GluA1 and GluA2 AMPAR subunits to late endosomes is mediated in an oxidative stress signaling pathway. The loss of GluA2-containing AMPARs at the plasma membrane following ischemia/reperfusion occurs in vulnerable areas of the brain, such as the hippocampus, leading to delayed neuronal death [Liu, *et al.* 2004]. The subunit composition switch from GluA2-containing, Ca^{2+}-impermeable AMPARs to GluA2-lacking, Ca^{2+}-permeable AMPARs occurs via the internalization and degradation of the GluA2 subunit [Dixon, *et al.* 2009], transcriptional and translational downregulation of GluA2 levels [Pellegrini-Giampietry, *et al.* 1997], and the increase in GluA2-lacking AMPARs at the plasma membrane [Liu, *et al.* 2006]. In this study, we have examined the endocytic trafficking pathways of both GluA1 and GluA2 AMPAR subunits following OGD/R and the role of oxidative stress in mediating these processes.

It is well-studied that oxidative stress exacerbates cell death following ischemia/reperfusion [Beske, *et al.* 2015; Jackson, *et al.* 2018; Vornov, *et al.* 1997; Wang, *et al.* 2004; Zhang, *et al.* 2003; Beske, *et al.* 2012]. There are three distinct temporal oxidative stress mechanisms that contribute to neuronal injury following ischemia/reperfusion. With ischemia onset, mitochondria generate an initial burst of ROS followed closely by xanthine oxidase activation. Once reperfusion occurs, NADPH oxidase produces a large burst of ROS in a calcium-dependent manner [Abramov, *et al.* 2007]. In this study, we observed a robust increase in ROS production during the reperfusion phase of OGD/R in U251-MG cells. Astrocytes do express NADPH oxidase [Abramov, *et al.* 2005], so it is possible that the large increase in ROS

is due to activation of NADPH oxidase in U251-MG cells. Alternatively, there could be delayed ROS production from the mitochondria and/or xanthine oxidase. Further studies using pharmacological inhibitors and/or genetic tools are needed to identify the source of ROS in our U251-MG OGD/R model system. This study is the first to indicate that the endocytic trafficking of GluA1 and GluA2 subunits can be modulated with ROS following OGD/R. Utilizing MnTMPyP, a superoxide dismutase mimetic, we identified an ROS-mediated trafficking of GluA1 and GluA2 with OGD/R that is distinct from agonist-induced internalization. MnTMPyP treatment had no effect on agonist-induced internalization of GluA1 and GluA2 subunits, but attenuated both the internalization and degradation of GluA1 and GluA2 subunits with OGD/R.

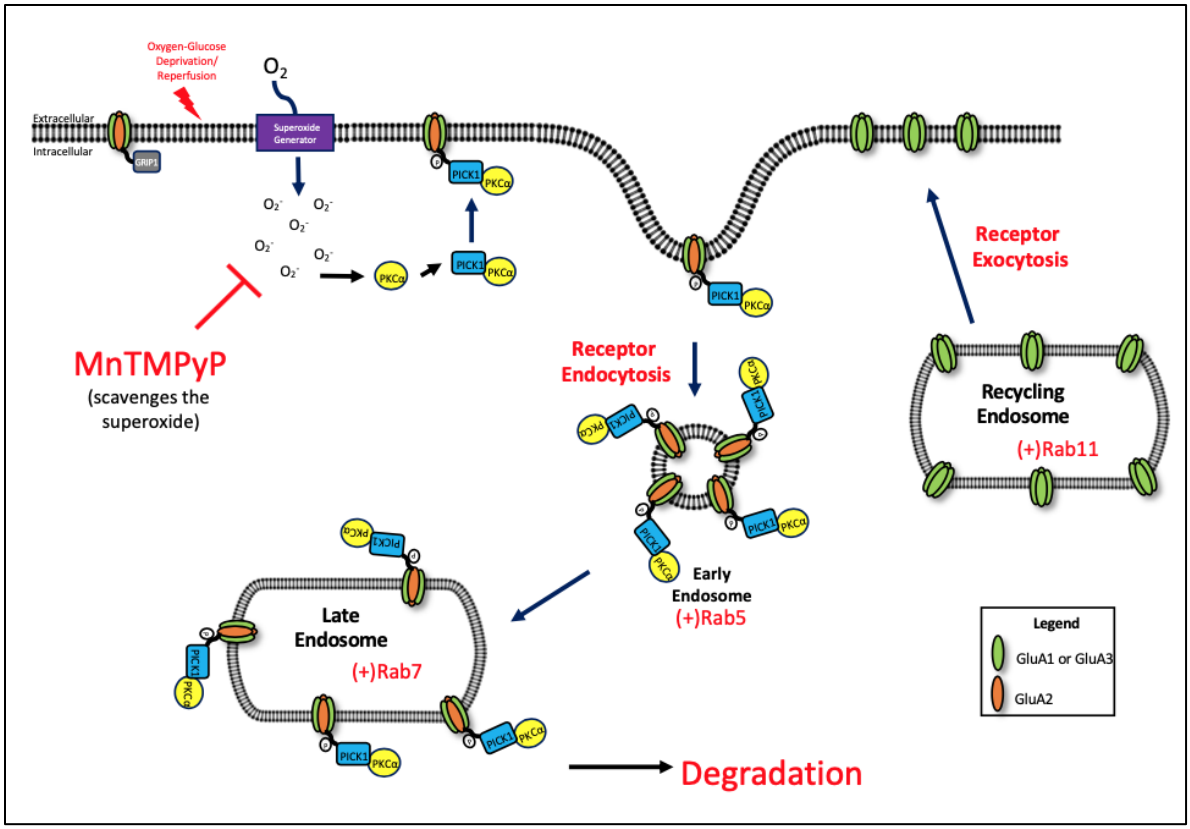


Figure 1.8: Potential mechanism of oxidative stress-mediated trafficking of GluA1 and GluA2 AMPAR subunits following OGD/R.

Under physiological conditions, the endocytosis of GluA2 is mediated by protein kinase C alpha (PKCα)-dependent phosphorylation of GluA2 Ser880 residue. PKCα is activated by increased intracellular calcium and is redox sensitive [Rimessi, *et al.* 2007; Ward, *et al.* 1998; Tuttle, *et al.* 2009]. Further studies are needed to examine the relationship between ROS and PKCα activation. Upon activation, PKCα translocates the plasma membrane by protein interacting with C kinase 1 (PICK1) [Perez, *et al.* 2001], where it phosphorylates GluA2 [Lu & Ziff, 2005]. The phosphorylation of GluA2 at the Ser880 residue, and increased association with PICK1, increases the internalization of GluA2-containing AMPARs [Terashima *et al.* 2008; Terashima, *et al.* 2004] thus reducing the surface population of AMPARs. This event allows for a transient increase in calcium permeable AMPARs at the plasma membrane, which is a critical component for LTP under physiologic conditions [Seidenman, *et al.* 2003], but is harmful when uncontrolled during pathophysiological conditions, like ischemia/reperfusion [Noh, *et al.* 2005] (Figure 1.8).

The present study examined the effect of superoxide on the internalization, degradation, and recycling of GluA1 and GluA2 AMPAR subunits with OGD/R in U251-MG cells. The superoxide scavenger, MnTMPyP, prevented both the internalization of both GluA1 and GluA2 receptor subunits following OGD/R. In the absence of superoxide, the degradation of GluA1 was partially rescued with OGD/R, whereas the degradation of GluA2 was completely prevented with MnTMPyP treatment. Interestingly, GluA1 was present within Rab11-positive recycling endosomes under both normoxic and OGD/R conditions, with MnTMPyP having no effect. This suggests that there is a pool of GluA1 homomeric AMPARs that is unique to GluA1, as GluA2 was not present in Rab11-positive endosomes under any conditions studied.

The recycling of GluA1 homomeric AMPARs to the plasma membrane, a critical component of LTP [Seidenman, *et al.* 2003] is a tightly regulated process. Activity-dependent endocytic sorting of GluA1 depends on phosphorylation [Ehlers, 2000] at the Ser845 residue by PKA, which promotes receptor insertion and decreases receptor endocytosis [Man, *et al.* 2007]. The fate of internalized GluA1-containing AMPARs to either Rab7-containing endosomes, fated for degradation, or Rab11-containing endosomes, fated for recycling, depends upon phosphorylation [Fernandez-Monreal *et al.* 2012]. It has also been reported that ubiquitination of GluA1 facilitates agonist-induced endocytosis [Lin, *et al.* 2011] and lysosomal targeting [Schwartz, *et al.* 2010]. GluA1-containing, GluA2-lacking, AMPARs are recruited to the plasma membrane during LTP by palmitoylation of A-kinase anchoring protein 150 (AKAP150), a scaffold that binds to and regulates GluA1 phosphorylation and trafficking [Purkey, *et al.* 2018]. Upon AMPA agonist stimulation, myosin Vb captures and mobilizes Rab11-positive, recycling endosomes for AMPAR insertion [Wang, *et al.* 2008]. The mechanisms for GluA1 trafficking during LTP are well understood, but whether all these mechanisms are conserved during pathologic ischemic/reperfusion injury remains to be determined. It is particularly interesting that GluA1 homomeric AMPAR subunits are presented within Rab11-positive endosomes, but GluA2 homomeric AMPAR subunits are not during OGD/R. This suggests that there is an unknown differential trafficking mechanism between GluA1 and GluA2 with regards to receptor recycling during OGD/R.

This study is the first to examine how oxidative stress mediates the endocytic trafficking of GluA1 and GluA2 AMPAR subunits following OGD/R, but further studies are needed to fully

characterize the role of superoxide in mediating the endocytic trafficking and degradation of the GluA1 and GluA2 AMPAR subunits with OGD/R.

CHAPTER THREE: MECHANISMS RESPONSIBLE FOR THE DEGRADATION OF GLUA2-CONTAINING AMPARS FOLLOWING OGD/R IN AGED RAT HIPPOCAMPAL SLICES.

Abstract:

In 2019 the Centers for Disease Control and Prevention identified stroke as the fifth leading cause of death. There is a great need for effective pharmacological therapies, however many therapies have failed in the clinical setting. Following ischemia/reperfusion, AMPA receptors (AMPARs) mediate delayed neuronal death through sustained expression of GluA2-lacking, calcium-permeable AMPARs, leading to dysregulation of intracellular calcium in a process referred to as excitotoxicity. Understanding the internalization and degradation of GluA2 AMPAR subunits, and preventing the surface removal of these GluA2-containing AMPARs, may yield new therapeutic targets for the treatment of ischemia/reperfusion. This study utilized acute organotypic hippocampal slices from aged male and female rats and subjected them to oxygen- glucose deprivation/reperfusion (OGD/R) to determine the mechanisms underlying the degradation of GluA2-containing AMPARs. Interestingly, hippocampal slices from male and female rats respond to OGD/R in a paradoxical manner with respect to AMPARs. GluA1 and GluA2 AMPAR subunits are degraded following OGD/R in males, but undergo an OGD/R-induced increase in total GluA1 and GluA2 protein levels in females. The mRNA expression of AMPAR subunits also differs in males compared to female following OGD/R. There is a rapid decrease in GRIA1 (GluA1) and GRIA2 (GluA2) mRNA levels in the male hippocampus following ischemic insult, but this is not observed in females. In agreement with studies conducted in other model systems, GluA2

undergoes lysosomal degradation following OGD/R in males, however this process is not ubiquitin-dependent. Chelation of all calcium, as well as inhibiting the intracellular influx of calcium through NMDA receptors and L-type voltage-gated calcium channels, prevents the OGD/R-induced degradation of GluA2 in males. The OGD/R-induced increase in GluA1 and GluA2 AMPAR subunits observed in females is likely due to an increase in synthesis of AMPARs. These data indicate a sex-dependent difference in how AMPARs in the hippocampus respond to ischemic insult, and may help explain, in part, why women have a lower incidence/severity of ischemic stroke compared to men.

Introduction:

Approximately 800,000 people in the United States suffer each year from a stroke [Yang *et al.* 2017]. Ischemic stroke, the most prevalent form of stroke, occurs when a vessel in the brain is occluded, resulting in decreased or absent blood flow. The current treatment for ischemic stroke is reperfusion to the infarcted area as quickly as possible, which can be accomplished in some patients with the thrombolytic tissue plasminogen activator (tPA) [Marier 1995]. While reperfusion is necessary, this also results in further tissue damage. During ischemia, there is a decrease in ATP-availability from the lack of glucose being delivered to the infarcted brain tissue. This lack of ATP results in disruption of ionic gradients that maintain neuron homeostasis, resulting in a massive release of neurotransmitters, including glutamate. Excess stimulation of ionotropic glutamate receptors contributes to delayed neuronal death in vulnerable CA1 pyramidal neurons of the hippocampus [Faden *et al.* 1989; Mcintosh *et al.* 1989; Takagi *et al.* 1997; Liu *et al.* 2007; Liu *et al.* 2004; Yin *et al.* 2002; Anzai *et al.* 2003; Calderone *et al.* 2004; Noh *et al.* 2005; Liu *et al.* 2006].

AMPA receptors (AMPARs) are glutamatergic ligand-gated cation channels composed of GluA1-4 subunits. The majority of AMPARs in the hippocampus are GluA1—2 or GluA2—3 AMPARs [Blanco-Suarez & Hanley, 2014]. The presence of the GluA2 subunit renders the AMPAR channel impermeable to divalent cations, such as calcium, due to a post-transcriptional modification by the RNA-editing enzyme adenosine deaminase enzyme 2 (ADAR2) [Pellegrini-Giampietry *et al.* 1997; Bass 2002]. An increase of GluA2-lacking, calcium-permeable, AMPARs to the plasma membrane plays a role in the induction of long-term potentiation (LTP), or synaptic strengthening [Terashima *et al.* 2008]. This is typically a short-lived change in AMPAR composition at the plasma membrane, but during ischemia/reperfusion, there is an increase in the amount of GluA2-lacking, calcium-permeable AMPARs [Noh *et al.* 2005].

This composition switch in AMPARs following ischemia/reperfusion from GluA2-containing AMPARs to GluA2-lacking AMPARs, leads to a cytotoxic accumulation of intracellular calcium. The increase in calcium triggers excitotoxicity, a process that is responsible for a large proportion of the neuronal death associated with ischemic stroke [Arudine and Tymianski 2003]. Blocking calcium-permeable, GluA2-lacking AMPARs has been shown to decrease cell death in the hippocampus following ischemia/reperfusion [Yin *et al.* 2002; Deng *et al.* 2003; Noh *et al.* 2005]. Targeting ionotropic glutamate receptors themselves can be difficult as these drugs can have severe side effects in patients, but targeting the trafficking and regulation of GluA2-containing AMPARs may be a viable drug target with translatability to the clinic.

During long-term depression (LTD), or weakening of synapses, AMPARs are removed from the plasma membrane and degraded by the lysosome [Lee *et al.* 2004; Fernandez-Monreal *et al.* 2012]. Palmitoylation, phosphorylation, and ubiquitination are post-translational

modifications that regulate AMPAR trafficking. In primary hippocampal neuronal cultures, agonist stimulation results in the ubiquitination and lysosomal degradation of AMPARs [Lin *et al.* 2011; Widagdo *et al.* 2015], however agonist stimulation of NMDA receptors (NMDARs) does not result in ubiquitination of AMPAR subunits [Lussier *et al.* 2011; Widagdo *et al.* 2015]. Following agonist stimulation, GluA2-containing AMPARs undergo endocytosis, a process mediated in part by protein kinase Cα (PKCα). An increase in intracellular calcium activates PKCα [Rimessi *et al.* 2007; Ward *et al.* 1998; Tuttle *et al.* 2009], and PKCα translocates the cell membrane where it binds to protein interacting with C kinase 1 (PICK1) [Perez *et al.* 2001]. This PICK1/PKCα complex binds to the C-terminus of the GluA2 AMPAR subunit, and PKCα phosphorylates GluA2 at the Ser880 residue [Lu and Ziff 2005], resulting in an increased internalization of GluA2-containing AMPARs [Terashima *et al.* 2004; Terashima *et al.* 2008]. By decreasing the amount of intracellular calcium from NMDARs and L-type voltage-gated calcium channels following ischemia/reperfusion, it may be possible to inhibit the internalization and degradation of GluA2-containing AMPARs. In primary hippocampal neuronal cultures, GluA2 AMPAR subunits are degraded by the lysosome following oxygen glucose deprivation/reperfusion (OGD/R), an *in vitro* model for ischemia/reperfusion injury in a PICK1-dependent manner [Koszegi *et al.* 2017].

In this study, we used acute organotypic hippocampal slices prepared from aged (10-12 months of age) male and female rats and exposed these slices to oxygen-glucose deprivation/reperfusion (OGD/R), our *ex vivo* model for ischemia/reperfusion injury. We determined that GluA2-containing AMPARs undergo lysosomal degradation following OGD/R, and that GluA2 AMPAR subunits are not ubiquitinated with OGD/R. Calcium plays a critical

role in the OGD/R-induced degradation of GluA2-containing AMPARs. Inhibition of NMDARs and L-type voltage-gated calcium channels, as well as chelation of all calcium (both intracellular and extracellular), prevents the OGD/R-induced degradation of GluA2 AMPAR subunits. Our work suggests that preventing the degradation of GluA2-containing AMPARs may be a target for therapeutic intervention for stroke patients. Redirecting GluA2-AMPARs to the surface by preventing their degradation can prevent the accumulation of calcium-permeable, GluA2-lacking AMPARs, and thereby reducing neuronal death in the hippocampus following ischemia/reperfusion injury.

Materials and Methods

Animals

Male and Female Sprague-Dawley rats (10-12 months of age; Envigo, Indianapolis, IN, USA) were used in this study. Animals were allowed free access to food and water for the duration of this study and housed in a temperature-controlled facility. All animals were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Washington State University Institutional Animal Care and Use Committee (IACUC).

Reagents

All antibodies used in this study are listed in the table below (Table 2.1). MG132 was purchased from UBPBio (Aurora, CO, USA). Chloroquine and Nitrendipine were purchased from Alfa Aesar (Tewksbury, MA, USA). Glutamate was purchased from Sigma (Greenacres, WA, USA).

Cycloheximide was purchased from Acros Organics (Carlsbad, CA, USA). AP5 and BAPTA-AM were purchased from Tocris Bioscience (Fisher Scientific; Waltham, MA, USA).

Preparation of acute rat hippocampal slices

The rats were anesthetized with isoflurane and decapitated. The brain was removed and placed in ice-cold Hank's Buffered Salt Solution (HBSS; Gibco, Amarillo, TX, USA) for no more than 30 seconds. Both hippocampi were rapidly dissected on ice and coronal 350µm-thick slices were prepared using a McIlwain tissue chopper (Stoelting Co., Wood Dale, IL, USA). Slices were equilibrated in oxygenated (95% O_2, 5%CO_2) artificial cerebrospinal fluid (aCSF; 124mM NaCl, 2.5mM KCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.5mM $MgCl_2$, 10mM D-glucose, pH7.4) at 37°C for 60 minutes prior to oxygen-glucose deprivation/reperfusion (OGD/R). Fresh aCSF was replaced every 15 minutes during the equilibration period and every 30 minutes during the reperfusion period.

Drug treatment of hippocampal slices

For MG132 [5µM; Zhao *et al.* 2016; Xie *et al.* 2014; Hou *et al.* 2006], chloroquine [60µM; Bendiske and Bahr 2003], cycloheximide [50µM or 100µM], nitrendipine [10µM; LoPachin *et al.* 2001; Santiago *et al.* 2008], AP5 [100µM; Widagdo *et al.* 2015], and BAPTA-AM [50µM; Wu *et al.* 2004] drug treatments, slices were pre-treated for 10 minutes prior to OGD/R conditions and drug was present for the duration of the experiment. Slices were treated with vehicle controls (VC), where appropriate, in 0.01% dimethyl sulfoxide (DMSO).

Agonist-stimulation of hippocampal slices

Following 60 minutes of equilibration, hippocampal slices were stimulated with glutamate [5mM; Frade *et al.* 2009] for 10 minutes. Following glutamate stimulation, slices were rinsed 3 times with ice-cold aCSF and lysates were prepared and subjected to immunoprecipitation/immunoblotting (IP/IB) as indicated below.

Triphenyltetrazolium chloride stain for cell viability

Following OGD/R, male and female hippocampal slices were incubated with triphenyltetrazolium chloride (TTC [1%]) for 10 minutes. Next, the slices were placed in 0.25M HCl and imaged utilizing a dissecting microscope (AmScope 3.5X Manufacturing 144-LED Zoom Stereo Microscope, Irvine, CA, USA). Following imaging, hippocampal slices were lysed in DMSO by sonication with 3 separate 5 second bursts at 25% power output. Absorbance was measured at 630nM for quantitative analysis and cell viability was determined by normalizing OGD/R conditions to Normoxic control. As a negative control, slices were incubated with 4% paraformaldehyde for 10 minutes to ensure low cell viability.

Ponceau stain of nitrocellulose membranes

Following immunoblotting, nitrocellulose membranes were stained with Ponceau (purchased from Acros Organics, Carlsbad, CA, USA) solution (0.1% Ponceau; 20% glacial acetic acid in distilled water) for total protein at room temperature for 10 minutes. Membranes were washed with distilled water at room temperature with agitation 3 times for 5 minutes each. Nitrocellulose membranes were imaged by dissecting microscope (AmScope 3.5X Manufacturing 144-LED

Zoom Stereo Microscope, Irvine, CA, USA) wide-field and quantified with ImageJ software (National Institutes of Health).

Oxygen-glucose deprivation/reperfusion of hippocampal slices

Following equilibration, hippocampal slices were placed in deoxygenated, glucose-free aCSF (aCSF with 10mM sucrose and no glucose, pH 7.4), and incubated for 40 minutes in a hypoxic glove box (Coy Laboratories, Grass Lake, MI, USA) containing 100% N_2. The glucose-free aCSF solution used for OGD was de-oxygenated using argon gas under vacuum to ensure completely anoxic aCSF. Following OGD, slices were transferred back to oxygenated glucose-containing aCSF for the time-periods indicated for each experiment (0, 30, 60, or 120 minutes). Normoxic controls remained in glucose-containing aCSF throughout the entirety of each experiment and time-matched to the last reperfusion time point of OGD/R-subjected slices.

Lysate preparation

Upon completion of OGD/R or normoxia, the hippocampal slices were lysed (50mM Tris, 140mM NaCl, 5mM EDTA, 1% Triton x-100, 1% Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Asheville, NC, USA), and 1mM phenylmethylsulfonyl fluoride) by dounce homogenization followed by sonication for 3 separate 5 second bursts at 25% power output (VirTis Ultrasonic Cell Disrupter; Gardiner, NY, USA). Samples were then centrifuged at 13,000 *x g* for 10 minutes (4°C). The supernatant was collected and a bicinchoninic acid assay (BCA) was performed to determine protein concentration. Samples were denatured in NuPAGE LDS (Fisher Scientific, Waltham, MA, USA) and heat (100°C) for 10 minutes and resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-

PAGE). Samples were transferred to a nitrocellulose membrane (Bio-Rad, Berkeley, CA, USA) for subsequent detection by immunoblotting.

Immunoblotting

Blots were blocked for 1 hour at room temperature with either SuperBlock (Thermo Fisher Scientific, Asheville, NC, USA) for phospho-antibody detection or 5% non-fat dry milk in tris buffered saline, 0.1% Tween 20, pH 7.5 (TBS-T) for non-phosphorylated antibodies. After blocking, blots were incubated with primary antibody overnight at 4°C at the concentration indicated in Table 2.1.

Antibody	Description	Host	Source	Dilution	Product #
GluA1	AMPA Receptor subunit 1	Rabbit	Cell Signaling	1:1000	13185S
GluA2	AMPA Receptor subunit 2	Rabbit	Cell Signaling	1:1000	13607S
GluA3	AMPA Receptor subunit 3	Rabbit	Cell Signaling	1:1000	5117S
GluA4	AMPA Receptor subunit 4	Rabbit	Cell Signaling	1:1000	8070S
Ubiquitin	Ubiquitin	Mouse	Santa Cruz	1:500	sc-8017
β-Actin	β-Actin	Rabbit	Cell Signaling	1:5000	8457S
PGK1	Phosphoglycerate kinase 1	Rabbit	Cell Signaling	1:1000	68540S
Na,K-ATPase β1	Na,K-ATPase subunit β1	Rabbit	Cell Signaling	1:1000	44759S

Table 2.1: Antibodies used in this study including source, product number, and dilution used.

Immunoreactive bands were visualized and captured utilizing a FujiFilm imaging system (Edison, NJ, USA) using enhanced chemiluminescence after addition of HRP-conjugated

secondary antibodies (Rabbit-HRP or Mouse-HRP, Cell Signaling, Danvers, MA, USA). Bands were analyzed using Fuji Image-Gauge software. Blots were stripped and reprobed up to 2 times with Restore Plus Western Blotting Stripping Buffer (Thermo Fisher Scientific, Asheville, NC, USA).

Immunoprecipitation

Rat hippocampal slices were lysed in a buffer containing 50mM Tris-HCl, 100mM NaCl, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1% Triton x-100, and 1% protease and phosphatase inhibitor cocktail. For all ubiquitination experiments, 10mM N-ethylmaleimide (Fisher Scientific, Waltham, MA, USA) was included in the lysis buffer to prevent de-ubiquitination from occurring. Protein concentration was then determined using a BCA assay, and lysates (500µg/sample in 500µL) were then pre-cleared using Surebeads Protein G magnetic beads (Bio-Rad, Berkeley, CA, USA) for 30 minutes at room temperature, followed by incubation with GluA2 antigen-bound protein G magnetic beads overnight at 4°C. The samples were washed 3 times with lysis buffer and eluted from the magnetic beads with Laemmli buffer (Bio-Rad, Berkeley, CA, USA) and heat (100°C) for 10 minutes. The samples were resolved by SDS-PAGE and immunoblotting occurred as already described.

Biotinylation of hippocampal slices

Acute aged rat hippocampal slices were prepared and treated to OGD/R or time-matched normoxia as previously described in this study. At the indicated time-point, slices were washed with ice-cold Buffer A (25mM HEPES, 119mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 30mM glucose, pH 7.4) and incubated at 4°C in Buffer A containing 0.5 mg/mL of sulfo-NHS-SS-biotin (Thermo Scientific, Waltham, MA, USA) for 30 minutes with gentle agitation. After

biotin labeling, slices were washed three times in ice cold Buffer A. Slices were then lysed via sonication with 3 separate 5 second bursts at 25% power output in Buffer A containing 1% protease and phosphatase inhibitor cocktail. The lysate was centrifuged at 100,000 *x g* in an ultracentrifuge. The biotin-labeled membrane fraction was then solubilized on ice for 30 minutes using Solubilization Buffer (10mM Tris, 150mM NaCl, 1% protease and phosphatase inhibitor cocktail, 1% Triton x-100, pH 8.0). The biotin-labeled membrane fraction was then sonicated with 3 separate 5 second bursts at 25% power output and a BCA assay was performed to determine protein content. Biotin-labeled membrane proteins (500µg lysate/500µL of Solubilization buffer) were then incubated overnight with gentle agitation at 4°C with 60µL of magnetic streptavidin beads (New England BioLabs, Ipswich, MA, USA). The streptavidin beads were then washed twice with High Salt Buffer (10mM Tris, 3M NaCl, 1% Triton x-100, pH 8.0), followed by two washes with Solubilization Buffer. The streptavidin-biotin bead complex was denatured with Laemmli and heat (80°C) for 10 minutes, subjected to SDS-PAGE, and transferred to nitrocellulose membranes for subsequent immunoblotting. Immunoreactive bands were analyzed using Fuji Image-Gauge software.

Reverse Transcription, Quantitative Polymerase Chain Reaction (RT-qPCR)

Acute aged rat hippocampal slices were prepared and treated to OGD/R or time-matched normoxia as previously described above. Total RNA was extracted utilizing RNeasy Plus Universal Mini Kit per manufacturer's instructions (QIAGEN, Germantown, MD, USA) following the manufacturer's protocol. Total RNA was reverse transcribed to cDNA with the Invitrogen High-Capacity Reverse Transcriptase Kit according to manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Comparative expression of genes of interest

was determined by quantitative polymerase chain reaction (qPCR). The conditions for qPCR were as follows: 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A melting curve was added to the end of the qPCR run to validate the primers. The PCR amplifications of each set of sample were performed in triplicates and averaged. β-Actin (ActB) was used as the internal control for normalization of our genes of interest. The relative fold changes in expression of genes of interest under OGD/R conditions were computed in comparison to the normoxia control group using the $2^{-\Delta\Delta Ct}$ algorithm [Schmittgen and Livak, 2008]. A one-way ANOVA was performed using the Sidak post hoc test to determine significance. The primers used are in Table 2.2 below.

#	Gene	Forward Primer	Reverse Primer	Product bps
1	*Gria1*	GGACAACRCAAGCGTCCAGA	CACAGTAGCCCTCATAGCGG	122
2	*Gria2*	TGGTTTTCCTTGGGTGCCTT	TCGATGGGAGACACCATCCT	170
3	*Gria3*	CCATGCTCTTGTCAGCTTCG	TGTGCTCCTGAACCGTGTTT	178
4	*Gria2 Q/R*	CTACGAGTGGCACACTGAGG	AACCACCACACACCTCCAAC	177
5	*Actb*	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC	74
6	*Adarb2*	GACGACACGCGGGAATATCT	GCCAGCAAGCACCTTCTCTA	131

Table 2.2: Primers used in this study [from Park *et al.* 2016].

Data analysis

One-way ANOVA with *post-Hoc* Sidak test were conducted using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) to determine statistical significance. A p-value less than 0.05 was considered statistically significant.

Results

No differences in basal protein level expression of AMPAR subunits in male and female aged rats.

First, we sought to determine whether basal protein levels of AMPAR subunits (GluA1-4) were different between male and females in different brain regions. Using western blot, we examined the protein levels of GluA1-4 in the hippocampus, cortex, cerebellum, and striatum of male and female aged (10-12 months old) Sprague Dawley rats. There were no significant differences in protein levels of AMPAR subunits in any brain region comparing male and female rats (Figure 2.1).

Acute organotypic hippocampal slice preparation, our *ex vivo* model system, utilizes viable brain tissue. It is important to determine whether there is any significant cell loss in the hippocampal slices following OGD/R. If there is significant cell loss, this could confound our results. Utilizing TTC stain, we examined the viability of our hippocampal slice model system comparing Normoxic and OGD/R conditions from aged male and female rats. The only significant difference in cell loss was in our control group, where slices were treated for 10 minutes with 4% paraformaldehyde to ensure very low cell viability. There was a decreasing trend in cell viability in male hippocampal slices following OGD/R treatment, but this was not significant. (Figure 2.2).

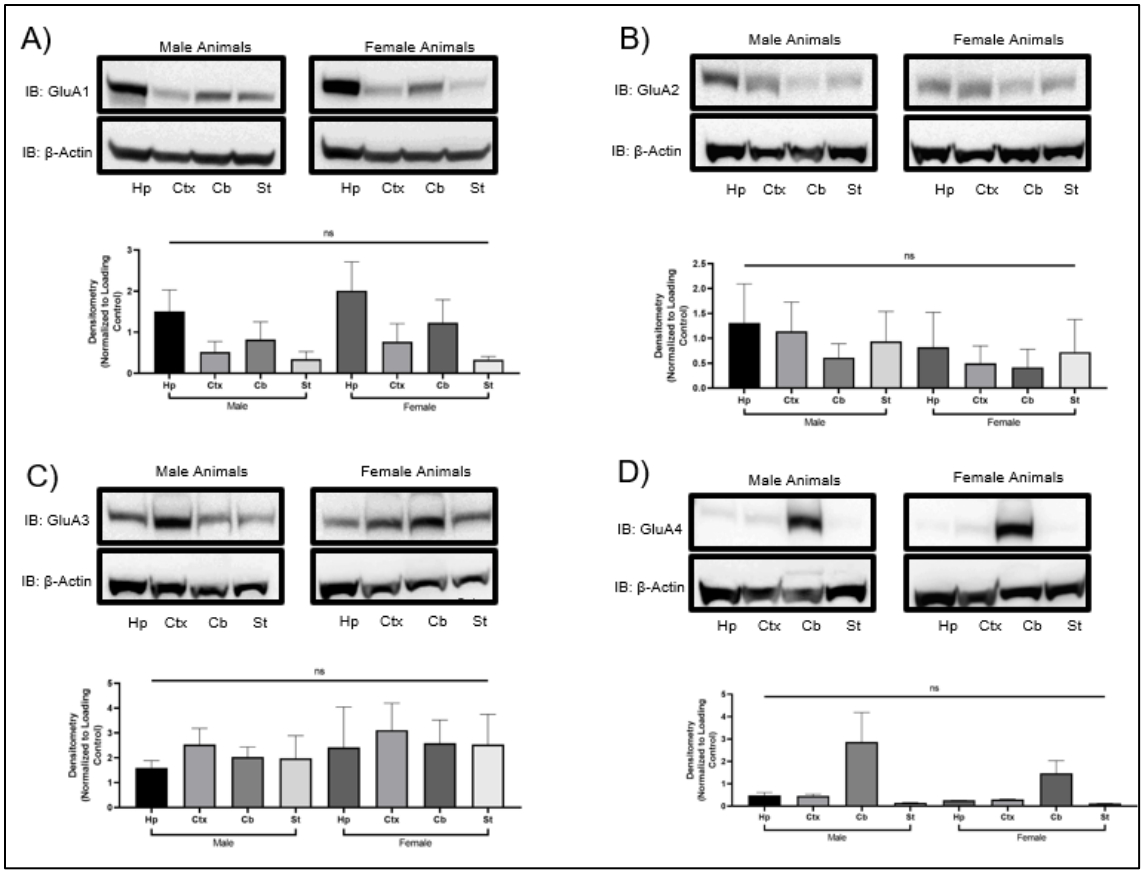


Figure 2.1: Basal protein levels of AMPAR subunits are similar in aged male and female rat brain regions. 10-12 month old male and female rat brain regions were examined by western blot to examine the total protein levels of GluA1, GluA2, GluA3, and GluA4 AMPA receptors subunit in the hippocampus (Hp), cortex (Ctx), cerebellum (Cb), and striatum (St). Representative western blots demonstrating that there are no significant differences in basal protein levels of AMPARs between male and female rats in the Hp, Ctx, Cb, or St. (A) GluA1 (B) GluA2 (C) GluA3 (D) GluA4 protein levels were normalized to β-Actin (N=3). ns denotes no significance; ANOVA with Sidak *post hoc* test comparing all brain regions. Data are expressed as *mean* ± *SEM*.

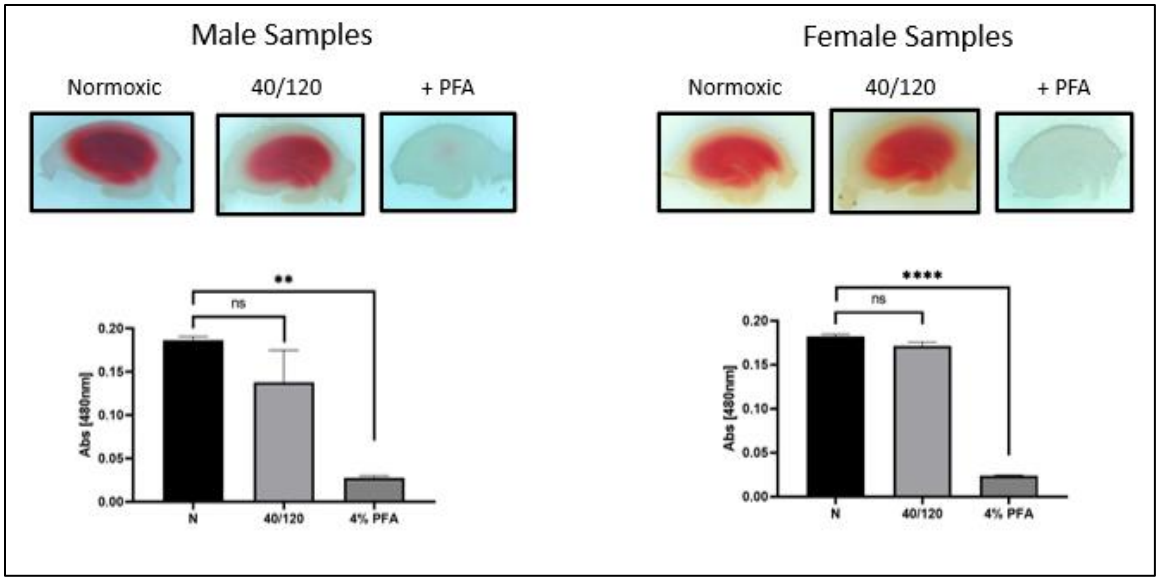


Figure 2.2: OGD/R does not significantly alter viability of hippocampal slices from male or female animals. Acute organotypic hippocampal slices from 10-12 month old male and female rats were exposed to 40 minutes of OGD, followed by 120 minutes of reperfusion, or normoxia. As a control to induce cell death, hippocampal slices were treated for 10 minutes with 4% paraformaldehyde (4% PFA). There was no significant difference between the viability of Normoxic controls and OGD/R-treated slices (N=3; ANOVA with Sidak *post hoc* test comparing OGD/R and 4% PFA conditions with normoxia). Data are expressed as *mean* ± *SEM*.

As a loading control to ensure equal transfer in our immunoblotting protocol, we performed a ponceau stain on the nitrocellulose membranes. There were no significant differences in total protein stain between normoxic and OGD/R treatment conditions in male or female hippocampal slice samples (Figure 2.3). This gave us high confidence that our immunoblotting conditions were ideal.

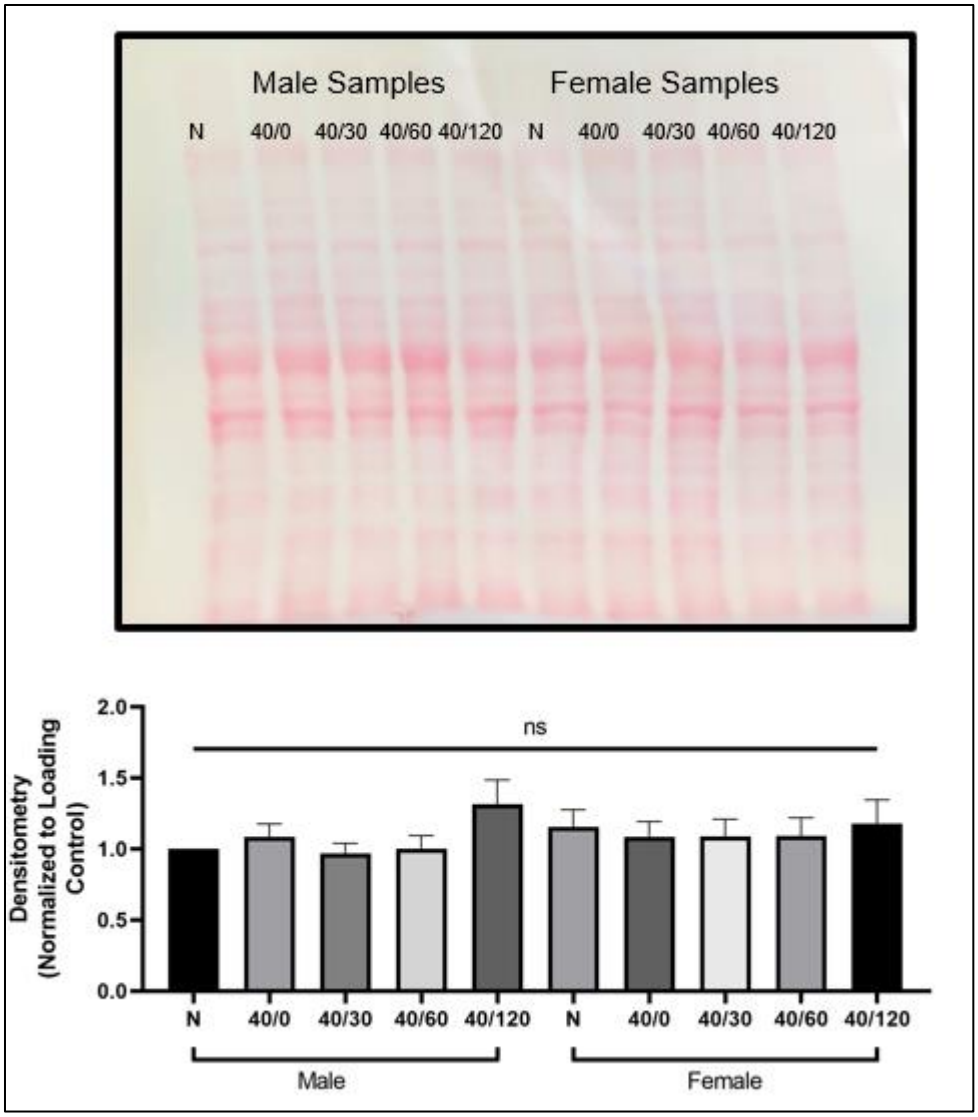


Figure 2.3: Determination of ideal transfer conditions in western blotting protocol. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Following immunoblotting, nitrocellulose membranes were incubated with Ponceau to stain total proteins. Blots were imaged and total proteins were quantified using ImageJ software (N=7; ANOVA with Tukey *post hoc* test comparing OGD/R conditions with normoxia). Data are expressed as *mean* ± *SEM*.

Sex-differences in GluA1 and GluA2 AMPARs protein levels with OGD/R.

Next, we performed 40 minutes of OGD on male and female aged rat hippocampal slices, and allowed 0, 30, 60, or 120 minutes of reperfusion. The Normoxic hippocampal slices were time-matched to the 120-minute reperfusion time-point. In male hippocampal slices GluA1 AMPAR subunit protein levels decreased with OGD/R treatment, maximally at the 120-minute end-point (Figure 2.4). This contrasts with female hippocampal slices, where GluA1 AMPAR protein levels increased with OGD/R treatment, maximally at 120 minutes of reperfusion (Figure 2.4).

It is well established that GluA2 AMPAR protein levels are decreased in CA1 hippocampal pyramidal neurons following ischemia/reperfusion (Beske 2015; Jackson *et al.* 2018; Koszegi *et al.* 2017). This is consistent with the results we observed in male hippocampal slices with OGD/R treatment. There was a significant decrease in GluA2 AMPAR protein levels with OGD/R treatment in male aged rat hippocampal slices, significantly with 120 minutes of reperfusion. However once again, in female animals, there is a significant increase in GluA2 AMPAR protein levels with OGD/R treatment, maximally at 120 minutes reperfusion (Figure 2.5).

Interestingly, GluA3 levels remain unchanged in either male or female hippocampal slices with OGD/R treatment (Fig. 2.6). This is in contrast to previous reports indicating GluA3 is also degraded in CA1 hippocampal pyramidal neurons following OGD/R (Koszegi *et al.* 2017). Since GluA3 protein levels did not change with OGD/R treatment, we only examined GluA1 and GluA2 for the remainder of these experiments. GluA4 protein levels were barely detectable in the hippocampus, so they were not examined in this study.

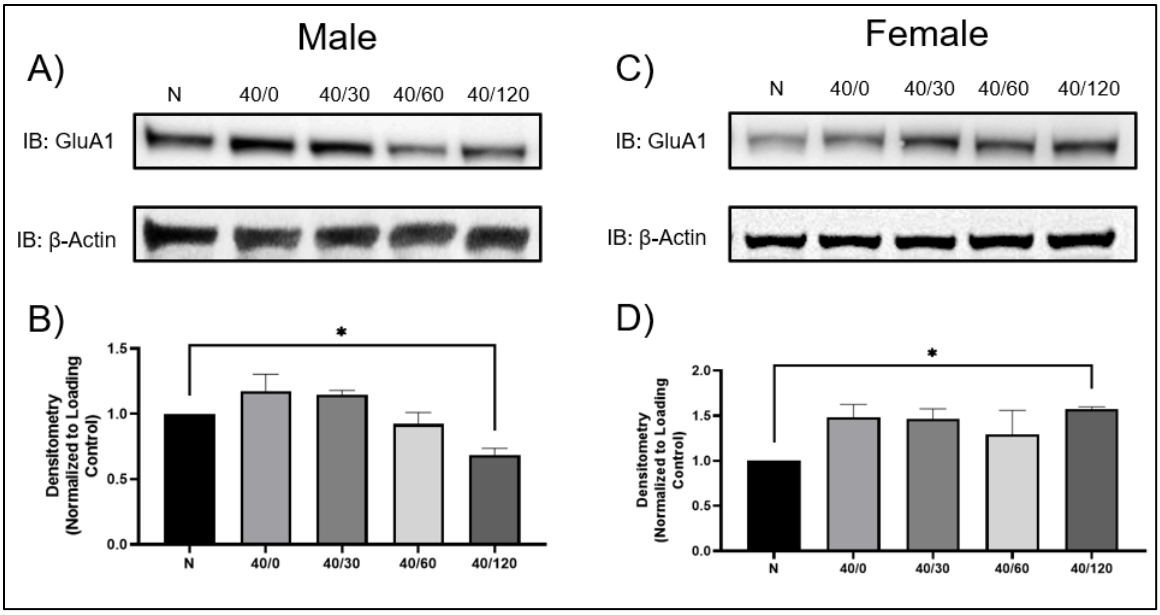


Figure 2.4: GluA1 decreases with OGD/R in male hippocampal slices, but GluA1 increases in female hippocampal slices following OGD/R. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. A) Representative western blot demonstrating that GluA1 decreases in male hippocampal slices following OGD/R, maximally at 120 minutes of reperfusion. (B) Quantification of (A) of total GluA1 protein levels normalized to β-Actin and Normoxic control group (N=4). (C) Representative western blot demonstrating that GluA1 increases in female hippocampal slices following OGD/R, maximally at 120 minutes of reperfusion. (D) Quantification of (C) of total GluA1 protein levels normalized to β-Actin and Normoxic control group (N=4). * $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

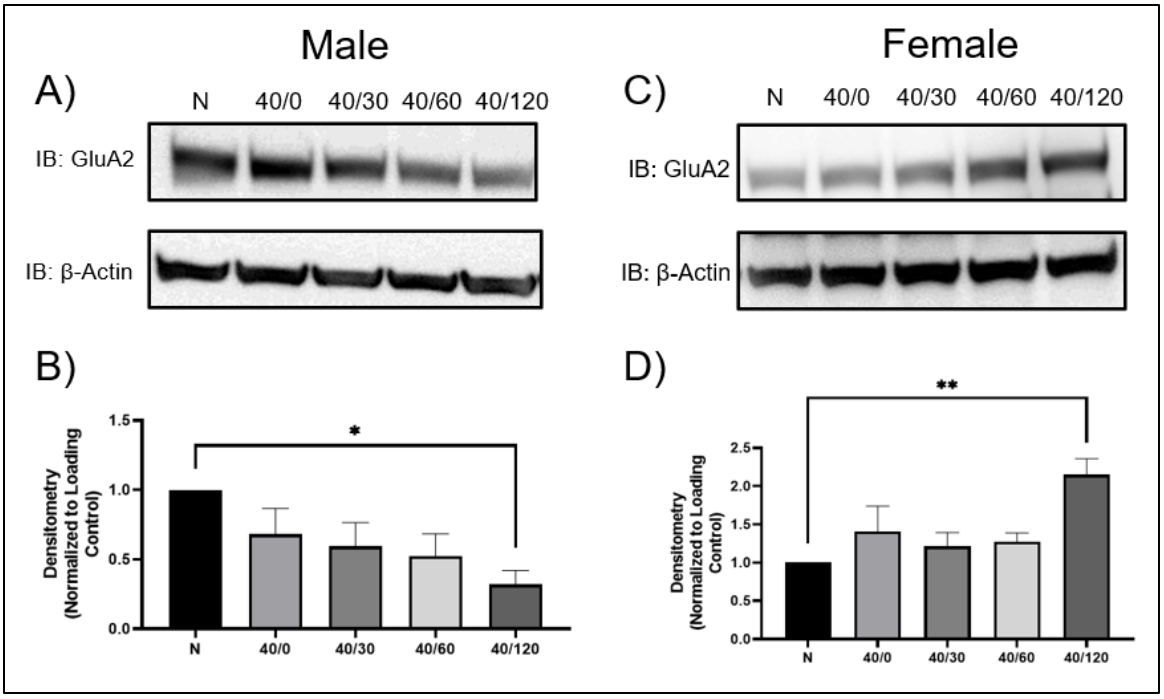


Figure 2.5: OGD/R induces degradation of GluA2 in male hippocampal slices and increases GluA2 protein levels in female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. A) Representative western blot demonstrating that GluA2 decreases in male hippocampal slices following OGD/R, maximally at 120 minutes of reperfusion. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=4). (C) Representative western blot demonstrating that GluA2 increases in female hippocampal slices following OGD/R, maximally at 120 minutes of reperfusion. (D) Quantification of (C) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=4). * $p<0.05$; **$p<0.01$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

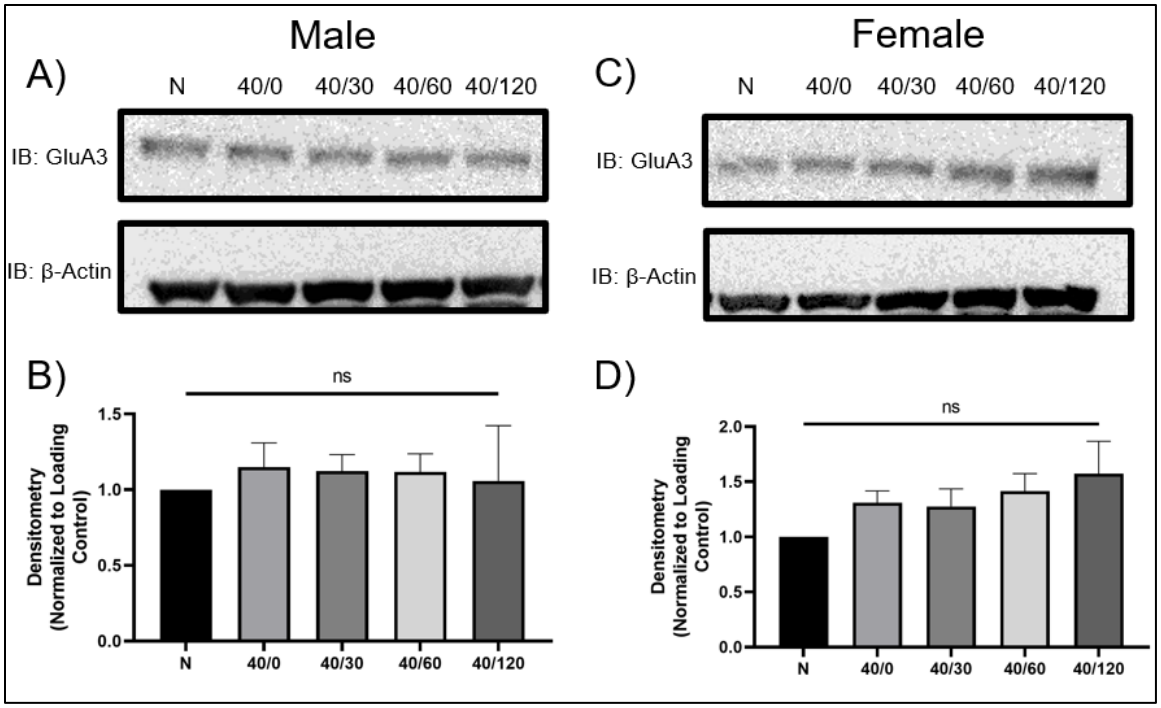


Figure 2.6: OGD/R does not alter GluA3 protein levels in male or female rat hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. A) Representative western blot demonstrating that GluA3 does not change in male hippocampal slices with OGD/R. (B) Quantification of (A) of total GluA3 protein levels normalized to β-Actin and Normoxic control group (N=3). (C) Representative western blot demonstrating that GluA3 does not change in female hippocampal slices following OGD/R. (D) Quantification of (C) of total GluA3 protein levels normalized to β-Actin and Normoxic control group (N=3). * $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

Increased OGD/R excitotoxicity in males compared to females

A popular method for determining the amount of AMPAR-mediated excitotoxicity is to determine the ratio of GluA1 to GluA2. An increase in the ratio of GluA1:GluA2 can be interpreted as more excitotoxic. Using immunoblots, we measured the total amount of GluA1 to the total amount of GluA2 in Normoxic and OGD/R conditions, in male and female aged rat hippocampal slices. The GluA1:GluA2 ratio was nearly 6-fold higher immediately following OGD/R and then returned to lower levels as reperfusion increased in male aged rat hippocampal slices (Fig. 2.7). There was also an increasing trend in the GluA1:GluA2 ratio in female aged rat hippocampal slices with OGD/R treatment, but it was not significant (Fig. 2.7). This supports the theory that even though we see a total decrease in GluA1 AMPAR protein levels with OGD/R in male hippocampal slices, the GluA1:GluA2 ratio increases, indicating more GluA1 with OGD/R, and potentially more excitotoxicity.

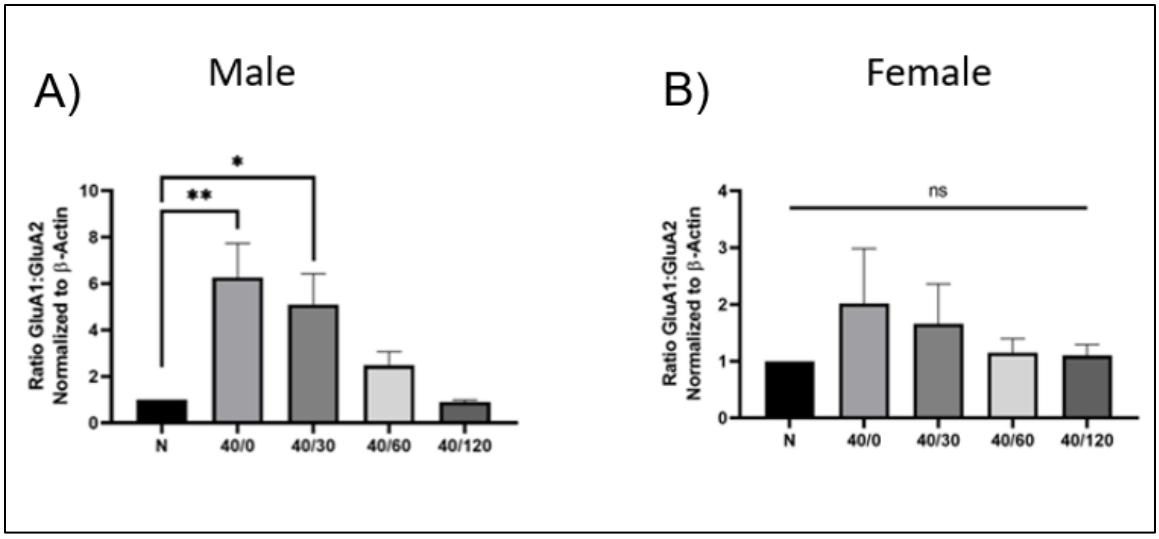


Figure 2.7: OGD increases the excitotoxic GluA1:GluA2 ratio in male hippocampal slices, but not significantly in female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. The ratio of GluA1:GluA2 was determined by immunoblotting. (A) Quantification of GluA1:GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3) in male hippocampal slices. The excitotoxic increase in GluA1:GluA2 ratio is maximal immediately following OGD. (B) Quantification of GluA1:GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3) in female hippocampal slices. There is no significant increase in GluA1:GluA2 ratio following OGD/R in female hippocampal slices. * $p<0.05$; **$p<0.01$; ns denotes no significance. ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

Sex differences in the OGD/R-induced internalization of GluA1 and GluA2 AMPAR subunits.

So far, we've examined total AMPAR subunit protein levels with OGD/R in hippocampal slices. It's been previously reported that GluA1 and GluA2 internalize with OGD/R in young male rat hippocampal slices [Beske *et al.* 2014]. We sought to determine whether GluA1 and GluA2 surface levels are changed with OGD/R in our aged rat model system. Since both GluA1 and GluA2 are degraded with OGD/R in male hippocampal slices, we wanted to determine whether these proteins are endocytosed prior to degradation. Utilizing a common technique, biotinylation (Diagram 2.1), we were able to measure the amount of GluA1 and GluA2 AMPAR subunits at the cell membrane surface. Surface GluA1 levels in male hippocampal slices decreased with OGD/R treatment, significantly at 60 and 120 minutes of reperfusion (Fig. 2.8). In male hippocampal slices there was an initial increase in GluA2 with OGD treatment, and then there was significantly less GluA2 at the membrane with 120 minutes of reperfusion (Fig. 2.9). GluA1 surface protein levels did not change with OGD/R in female hippocampal slices (Fig. 2.8). However, the amount of membrane GluA2 trends towards significantly decreasing with OGD/R in female hippocampal slices (Fig. 2.9; $p=0.0559$).

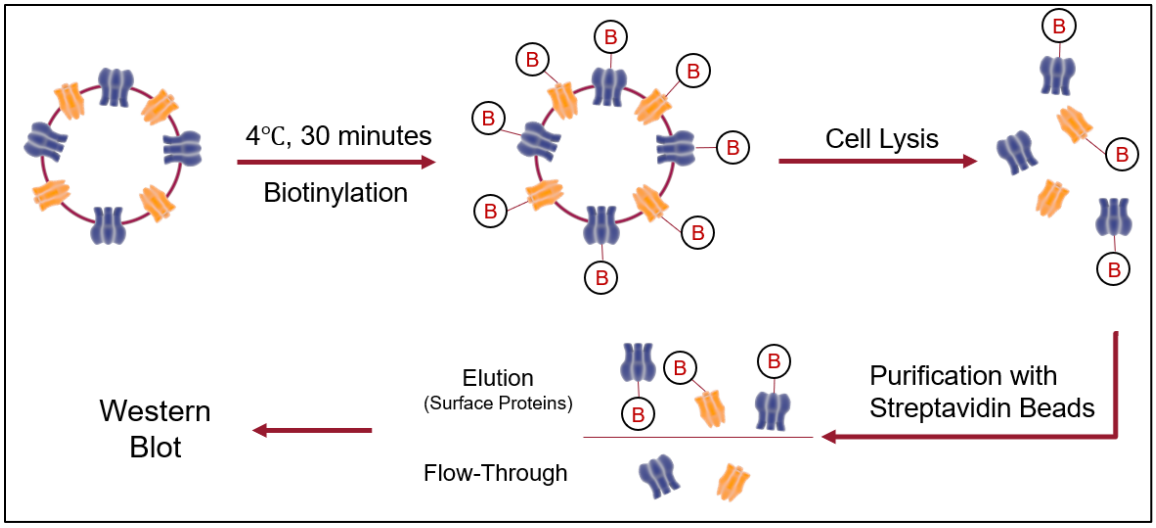


Diagram 2.1: Experimental methodology for biotinylation of surface proteins. Male and female hippocampal slices are subjected to OGD/R or normoxia, and biotinylated on ice for 30 minutes. Surface proteins are labeled with biotin, then the slices are lysed and incubated with streptavidin beads overnight at 4°C. Surface proteins were eluted with heat and resolved by SDS-PAGE.

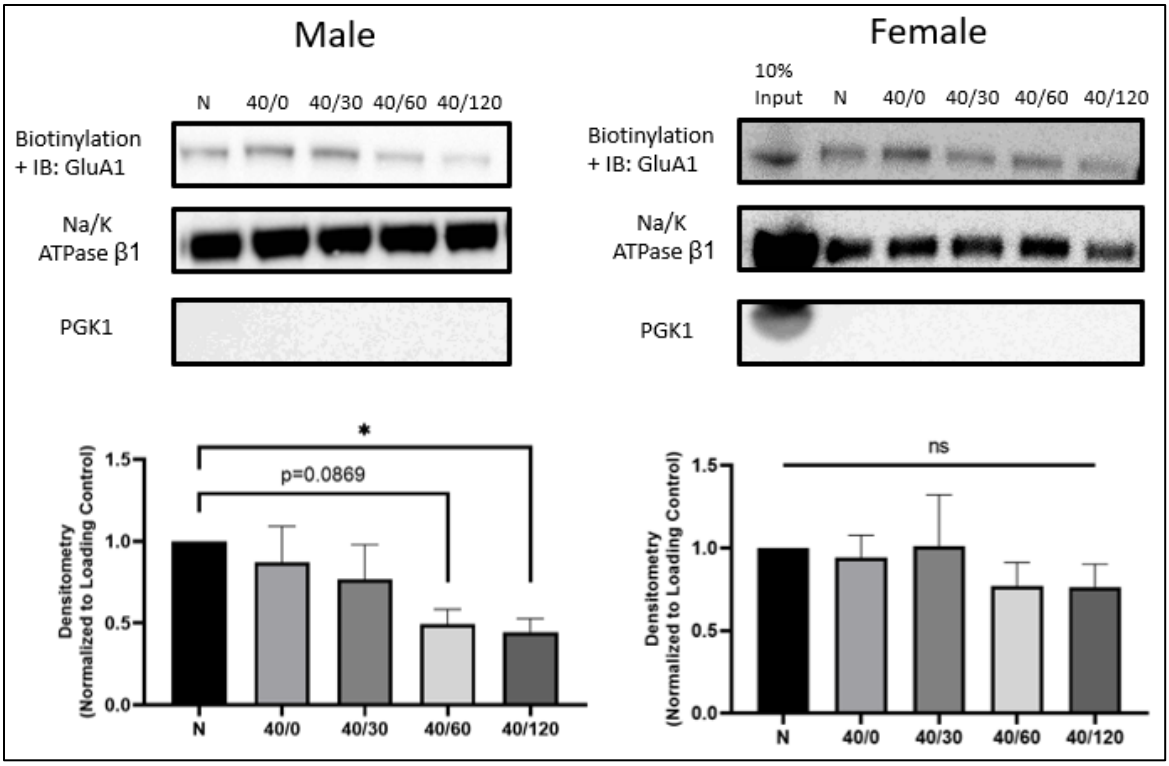


Figure 2.8: OGD/R reduces surface levels of GluA1 AMPAR subunits in male hippocampal slices, but does not alter surface levels of GluA1 in female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Surface proteins were biotinylated and resolved by immunoblotting to examine the amount of GluA1 AMPAR protein at the membrane surface. A) Representative western blot demonstrating that surface GluA1 decreases in male hippocampal slices with OGD/R. (B) Quantification of (A) of surface GluA1 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=4). (C) Representative western blot demonstrating that surface GluA1 does not change in female hippocampal slices following OGD/R. (D) Quantification of (C) of surface GluA1 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=4). * $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

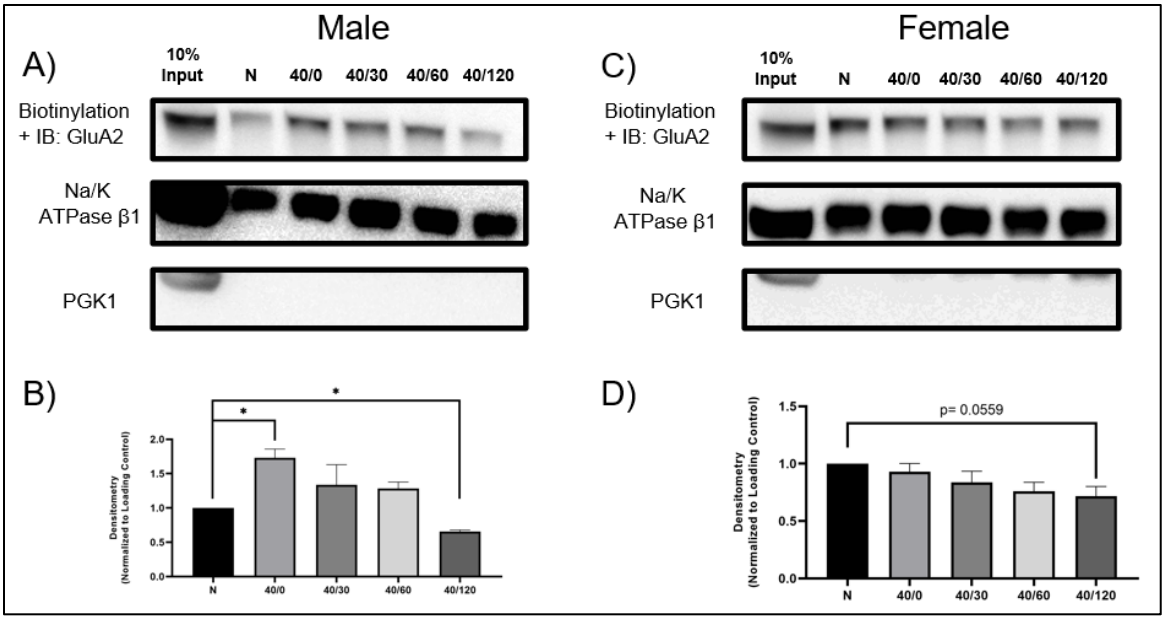


Figure 2.9: Sex-dependent differences in amount of surface GluA2 with OGD/R treatment in male and female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Surface proteins were biotinylated and resolved by immunoblotting to examine the amount of GluA2 AMPAR protein at the membrane surface. A) Representative western blot demonstrating an initial increase in surface GluA2 levels with OGD, and then a significant decrease in surface GluA2 with reperfusion at 120 minutes in male hippocampal slices. (B) Quantification of (A) of surface GluA2 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=4). (C) Representative western blot demonstrating that surface GluA2 trends toward decreasing in female hippocampal slices following OGD/R. (D) Quantification of (C) of surface GluA2 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=4). * $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

The OGD/R-induced degradation of GluA2 AMPAR subunits is mediated by the lysosome, not the proteasome.

Since AMPAR subunits are only degraded in male hippocampal slices, we utilized only male samples for the remainder of the experiments in this aim. We pre-treated male hippocampal slices with either chloroquine or MG132 to inhibit the lysosome or proteasome, respectively, and then performed normoxia or OGD/R experiments. Consistent with previous studies that examined OGD/R-induced degradation of AMPAR subunits [Koszegi *et al.* 2017], we found that the OGD/R-induced degradation of GluA2 is mediated by the lysosome, not the proteasome (Fig. 2.10).

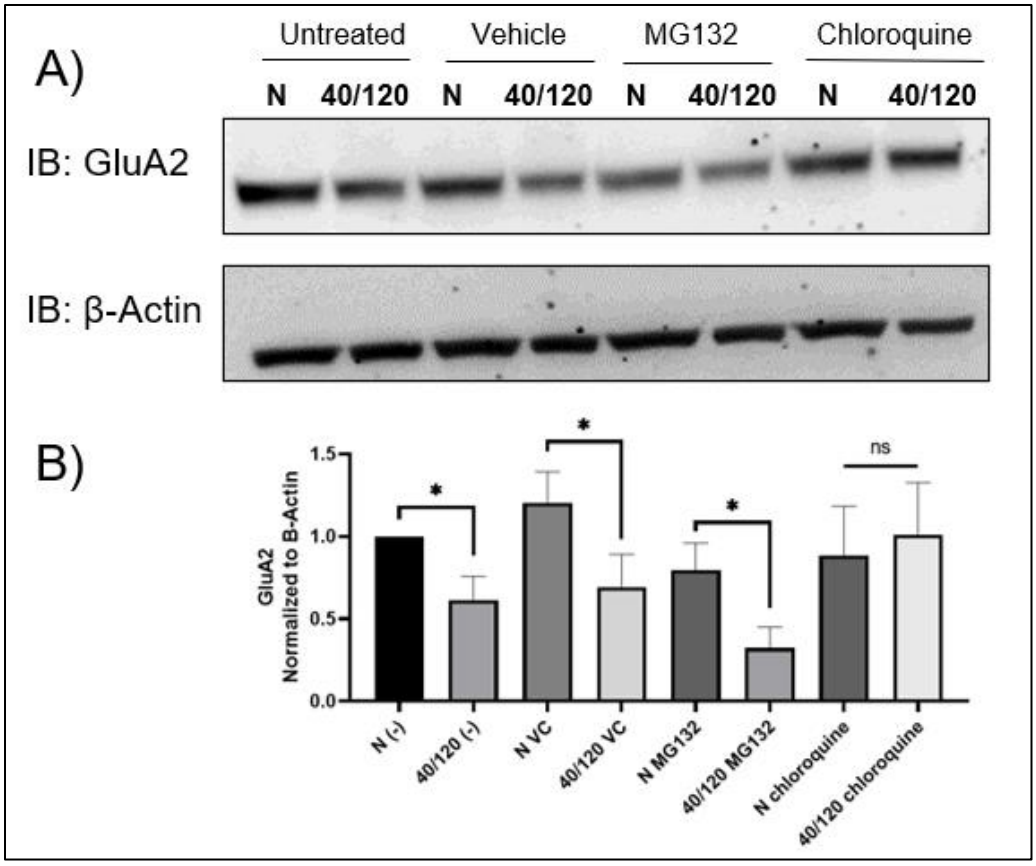


Figure 2.10: OGD/R induces lysosomal degradation of GluA2-containing AMPARs in male hippocampal slices. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of inhibitors of the proteasome or lysosomal, MG132 [5μM] or Chloroquine [60μM], respectively. (A) Representative western blot demonstrating that pre-treatment with chloroquine, a lysosomal inhibitor, prevents the OGD/R-induced decrease of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

GluA2 AMPAR subunit is not ubiquitinated with OGD/R treatment.

Previous reports [Widagdo *et al.* 2015] have indicated that agonist stimulation results in ubiquitination-dependent sorting of AMPAR subunits to the lysosome. We sought to determine whether GluA2-containing AMPARs in male hippocampal slices are ubiquitinated with OGD/R treatment. We first performed immunoprecipitation of GluA2 and immunoblotting (IP/IB) for ubiquitin in male hippocampal slices following OGD/R treatment and did not detect any ubiquitination (Fig. 2.11). To determine if the subunit was being degraded too quickly to detect levels of ubiquitination of the GluA2-containing AMPARs, we inhibited both proteasomal and lysosomal degradation as in Fig. 2.10, and performed IP/IB to examine ubiquitination of GluA2-containing AMPARs. Consistent with our previous experiment, we were unable to detect ubiquitination of GluA2 in male hippocampal slices with OGD/R treatment (Fig. 2.11).

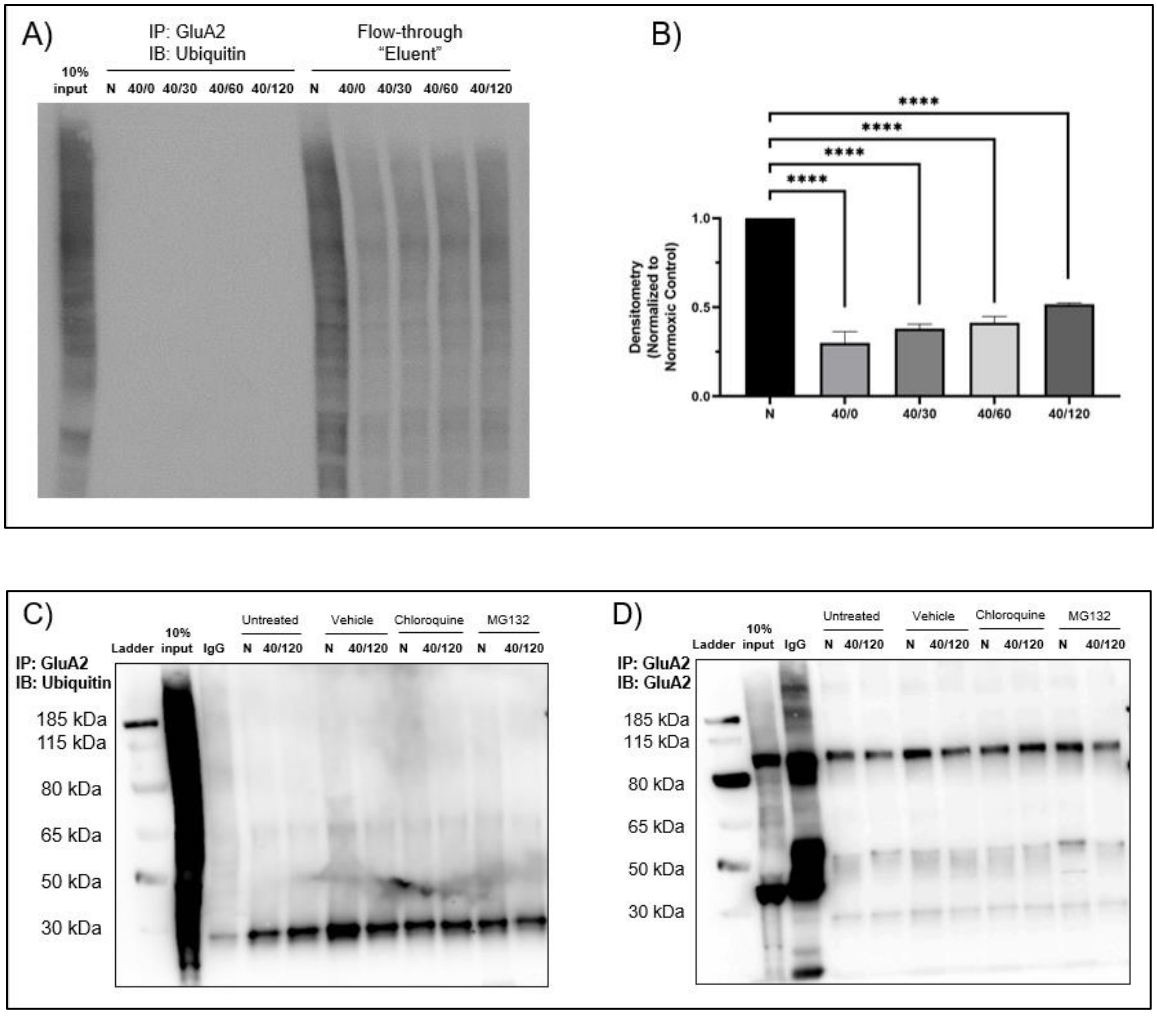


Figure 2.11: GluA2 AMPAR subunit is not ubiquitinated with OGD/R in male hippocampal slices. (A) Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. GluA2 was immunoprecipitated following OGD/R and was immunoblotted for ubiquitin. Representative western blot demonstrating that GluA2 is not ubiquitinated following OGD/R. (B) Quantification of (A) of flow-through "eluent", demonstrating the OGD/R-induced decrease in total protein ubiquitination in male hippocampal slices. Ubiquitin protein levels normalized to Normoxic control group (N=3). (C) Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes. Prior to OGD, and throughout reperfusion, slices were treated with either chloroquine or MG132. GluA2 was immunoprecipitated following OGD/R and was immunoblotted for ubiquitin.

Representative western blot demonstrating that GluA2 is not ubiquitinated following OGD/R, even in the presence of inhibitors for protein degradation. (D) Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes. Prior to OGD, and throughout reperfusion, slices were treated with either chloroquine or MG132. GluA2 was immunoprecipitated following OGD/R and was immunoblotted for GluA2 as a control experiment for (C). This demonstrates that GluA2 is not ubiquitinated with OGD/R and that the OGD/R-induced degradation of GluA2 is lysosomal-dependent. **** $p<0.0001$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

GluA2 undergoes agonist-induced ubiquitination in aged rat hippocampal slices.

To ensure that our methodology for detection of ubiquitination was adequate, we performed a positive control experiment. We stimulated male and female hippocampal slices with glutamate for 10 minutes and then immunoprecipitated GluA2 and performed immunoblotting for ubiquitin. We were able to detect agonist-induced ubiquitination of GluA2 (Fig. 2.12), but not OGD/R-induced ubiquitinated GluA2 (Fig. 2.11). This suggests that there is a differential mechanism in the late endosomal sorting of GluA2-containing AMPARs to the lysosome in hippocampal slices following OGD/R treatment.

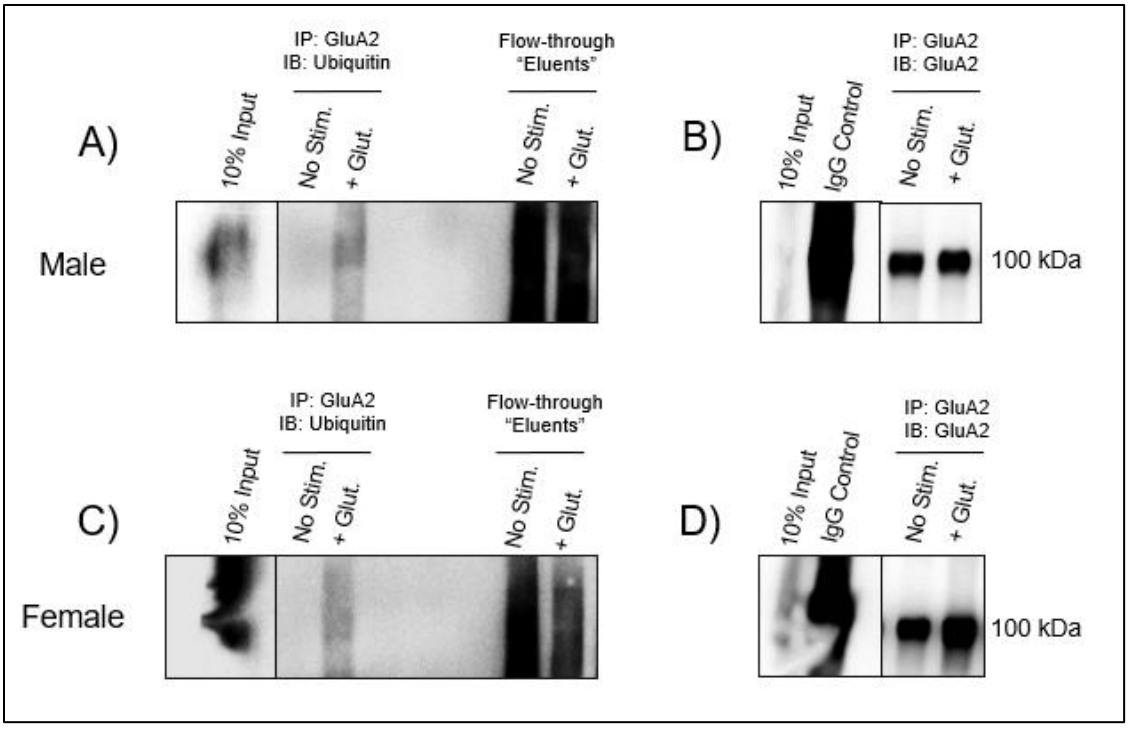


Figure 2.12: GluA2 AMPAR subunit is ubiquitinated with glutamate stimulation in male and female hippocampal slices. (A) Male hippocampal slices were stimulated with glutamate [5mM] for 10 minutes. GluA2 was immunoprecipitated following stimulation and was immunoblotted for ubiquitin. Representative western blot demonstrating that GluA2 is ubiquitinated following glutamate stimulation in male hippocampal slices. (B) GluA2 was immunoprecipitated following glutamate stimulation and GluA2 was immunoblotted to demonstrate successful IP/IB method in (A). (C) Female hippocampal slices were stimulated with glutamate [5mM] for 10 minutes. GluA2 was immunoprecipitated and was immunoblotted for ubiquitin following glutamate stimulation. Representative western blot demonstrating that GluA2 is ubiquitinated following glutamate stimulation in female hippocampal slices.(D) GluA2 was immunoprecipitated following glutamate stimulation and GluA2 was immunoblotted to demonstrate successful IP/IB method in (C). N=2 for both experiments.

The OGD/R-induced degradation of GluA2 is calcium-dependent.

Widagdo *et al.* [2015] determined that agonist-induced ubiquitination of AMPAR subunits is dependent upon calcium. We've eliminated ubiquitination as the sorting mechanism of GluA2-containing AMPARs to the lysosome following OGD/R, but we sought to examine if calcium plays a role in the degradation of GluA2-containing AMPARs with OGD/R. Chelation of all calcium utilizing the cell membrane-permeable calcium chelator, BAPTA-AM, blocked OGD/R-induced degradation of GluA2 in male hippocampal slices. Inhibition of calcium channels, NMDARs and L-type voltage-gated calcium channels, with AP5 and Nitrendipine, respectively, also prevented the OGD/R-induced degradation of GluA2-containing AMPARs (Fig. 2.13). These data indicate that calcium plays an important role in the degradation of GluA2-containing AMPARs with OGD/R.

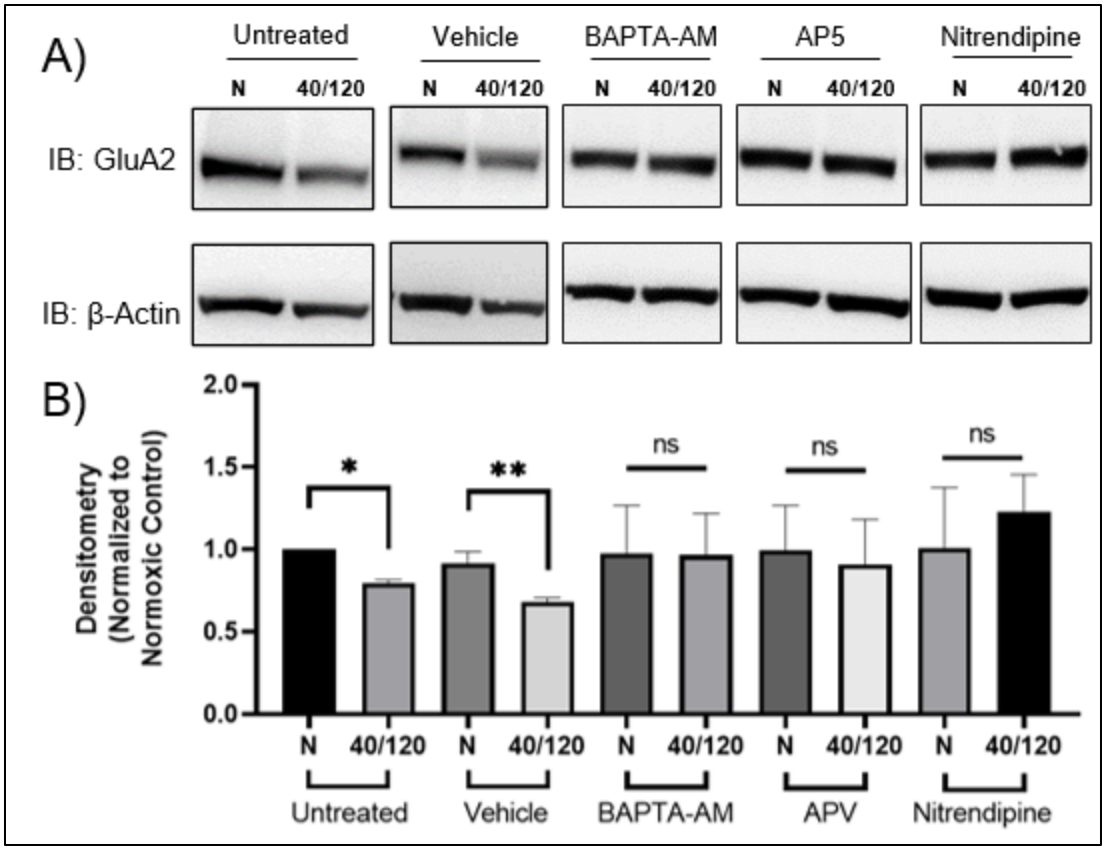


Figure 2.13: Calcium plays a critical role in the OGD/R-induced degradation of GluA2-containing AMPARs. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of inhibitors of NMDARs (APV [100µM]), L-type voltage-gated calcium channels (Nitrendipine [10µM]), or the membrane-permeable calcium chelator, BAPTA-AM [50µM]. (A) Representative western blot demonstrating that pre-treatment with BAPTA-AM, APV, or Nitrendipine prevents the OGD/R-induced decrease of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *p<0.05; **p<0.01; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

The OGD/R-induced increase in GluA1 and GluA2 in females occurs from newly synthesized AMPARs.

The most intriguing finding of this study is the opposite nature in which AMPARs subunits from male and female hippocampal slices respond with OGD/R treatment. Utilizing female hippocampal slices, we pre-treated with cycloheximide to determine whether inhibition of protein synthesis would block the OGD/R-induced increase in GluA1 and GluA2 AMPAR subunits. Indeed, the OGD/R-induced increase in both GluA1 and GluA2 AMPAR subunits was abolished with cycloheximide treatment (Figures 2.14 and 2.15). This did not lead to a decrease in AMPAR subunits following OGD/R though, indicating that there is a sex-dependent difference in AMPAR trafficking with OGD/R in the hippocampus.

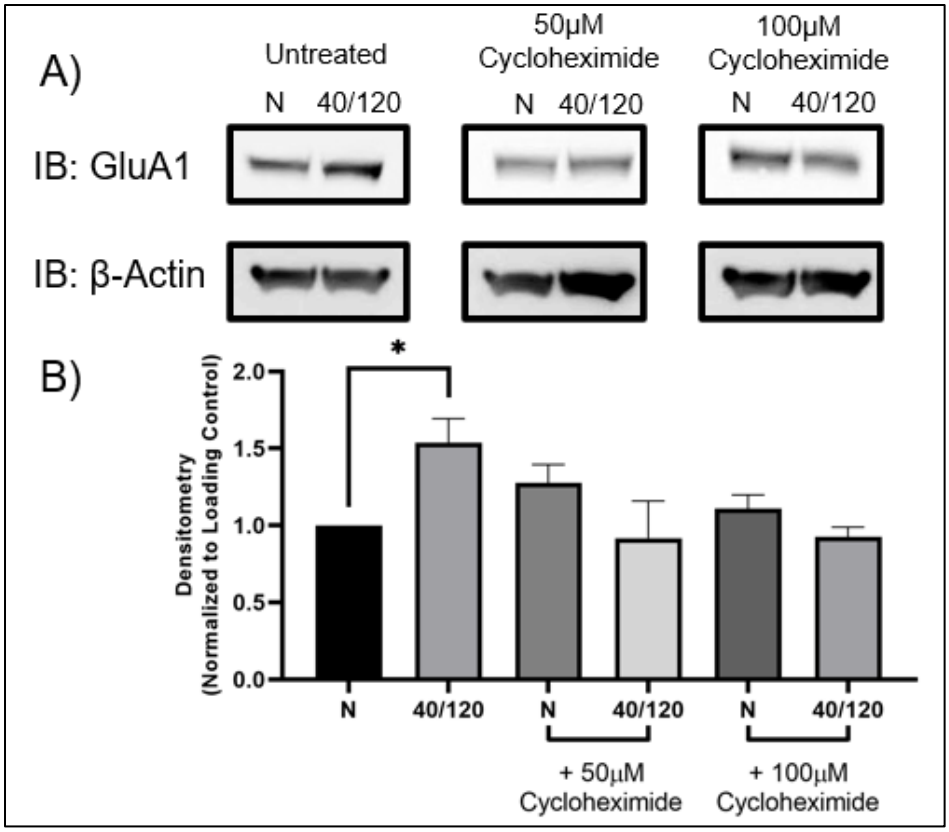


Figure 2.14: OGD/R-induced increase in GluA1 is due to increased protein translation in female hippocampal slices. Female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of cycloheximide [50μM] or [100μM], an inhibitor for protein synthesis. (A) Representative western blot demonstrating that pre-treatment with cycloheximide prevents the OGD/R-induced increase of GluA1 AMPAR subunits in female hippocampal slices. (B) Quantification of (A) of total GluA1 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

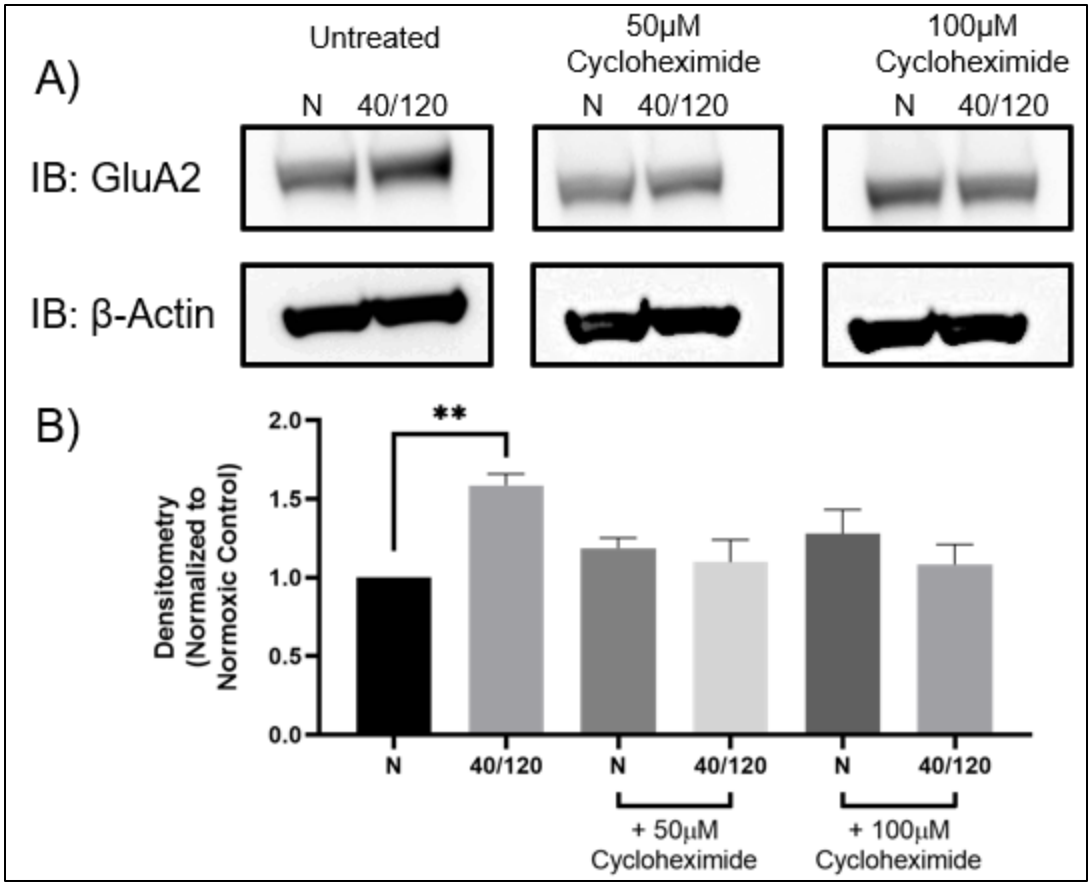


Figure 2.15: OGD/R-induced increase in GluA2 is due to increased protein translation in female hippocampal slices. Female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of cycloheximide [50μM] or [100μM], an inhibitor for protein synthesis. (A) Representative western blot demonstrating that pre-treatment with cycloheximide prevents the OGD/R-induced increase of GluA2 AMPAR subunits in female hippocampal slices. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

Sex differences in the mRNA expression of AMPARs with OGD/R.

Lastly, we examined whether the expression of AMPAR subunits were altered with OGD/R treatment in male and female hippocampal slices. Utilizing RT-qPCR we measured the expression of GRIA1 (GluA1), GRIA2 (GluA2), GRIA3 (GluA3), GRIA2Q/R (edited-GluA2), and ADARB2 (ADAR2) in male and female hippocampal slices following OGD/R treatment. Consistent with our previous work examining protein levels, the levels of GRIA1 and GRIA2 were decreased in male hippocampal slices with OGD/R, and GRIA3 levels did not change. Unexpectedly, the expression of GRIA1 and GRIA2 were unchanged with OGD/R treatment in female hippocampal slices (Fig. 2.16). Taken into consideration with the previous experiment (Fig. 2.14 and Fig. 2.15), this likely indicates that there is increased transcription and translation occurring with OGD/R treatment in female hippocampal slices to result in a total increase in GluA1 and GluA2 protein levels with reperfusion. Previous reports indicate there is a decrease in ADARB2 with OGD/R treatment [Peng *et al.* 2006], which we confirmed with our results in male hippocampal slices, but not in female hippocampal slices. Accordingly, the amount of edited GluA2 also decreased with OGD/R treatment in male hippocampal slices, but not female hippocampal slices (Fig. 2.17).

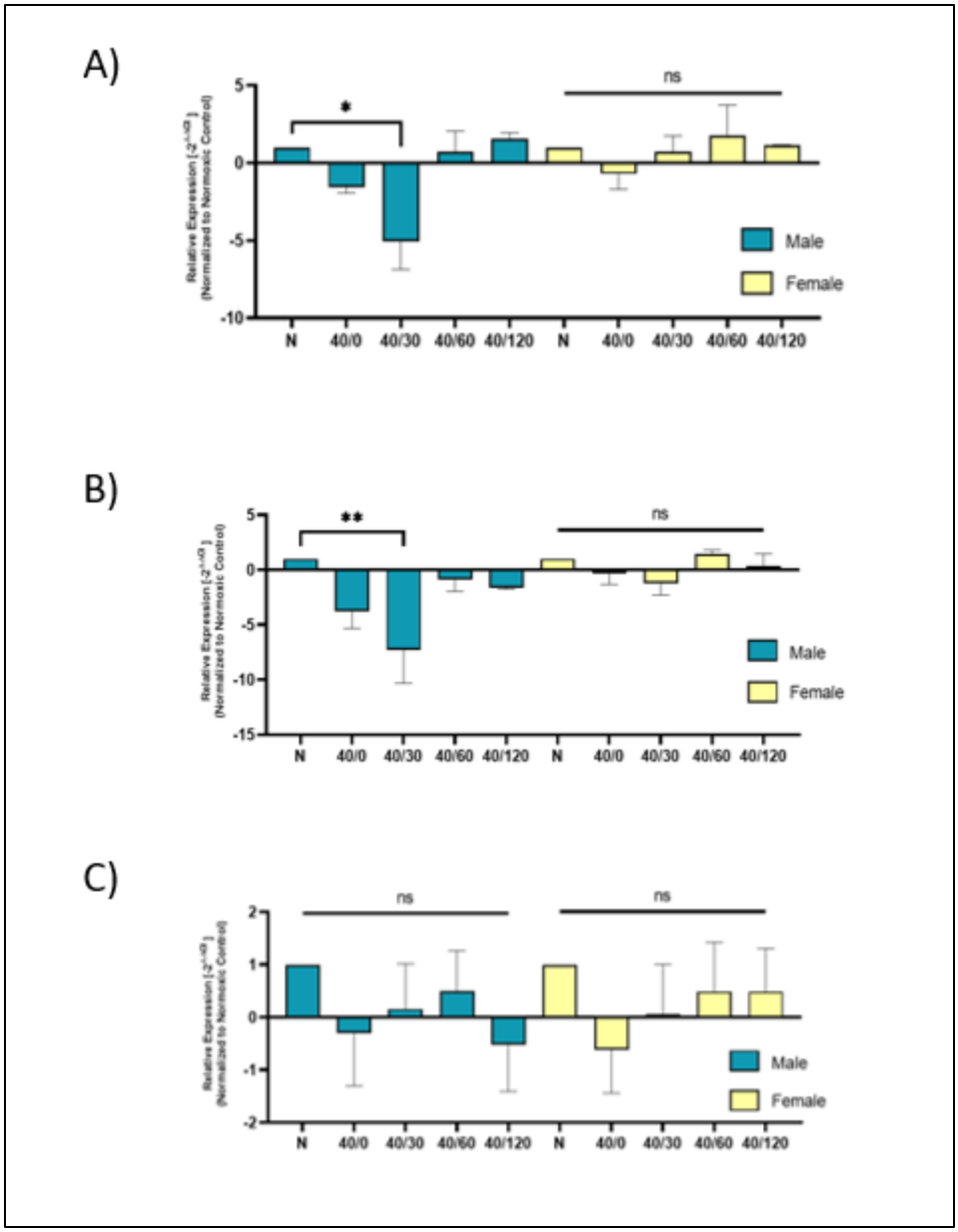


Figure 2.16: OGD/R decreases GRIA1 and GRIA2 expression in male hippocampal slices, but OGD/R does not significantly affect female GRIA expression in hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Utilizing rt-qPCR, we examined the expression of GRIA1 (GluA1), GRIA2 (GluA2), and GRIA3 (GluA3) with OGD/R treatment. (A) Expression of GRIA1 in male and female hippocampal slices with OGD/R treatment, normalized to Normoxic control group and ActB (β-Actin) (N=3). (B) Expression

of GRIA2 in male and female hippocampal slices with OGD/R treatment, normalized to Normoxic control group and ActB (β-Actin) (N=3). (C) Expression of GRIA3 in male and female hippocampal slices with OGD/R treatment, normalized to Normoxic control group and ActB (β-Actin) (N=3). *p<0.05; **p<0.01; ns denotes no significance. ANOVA with Sidak *post hoc* test comparing OGD/R values ($2^{-\Delta\Delta C_T}$) corresponding to Normoxic control. Data are expressed as *mean* ± *SEM*.

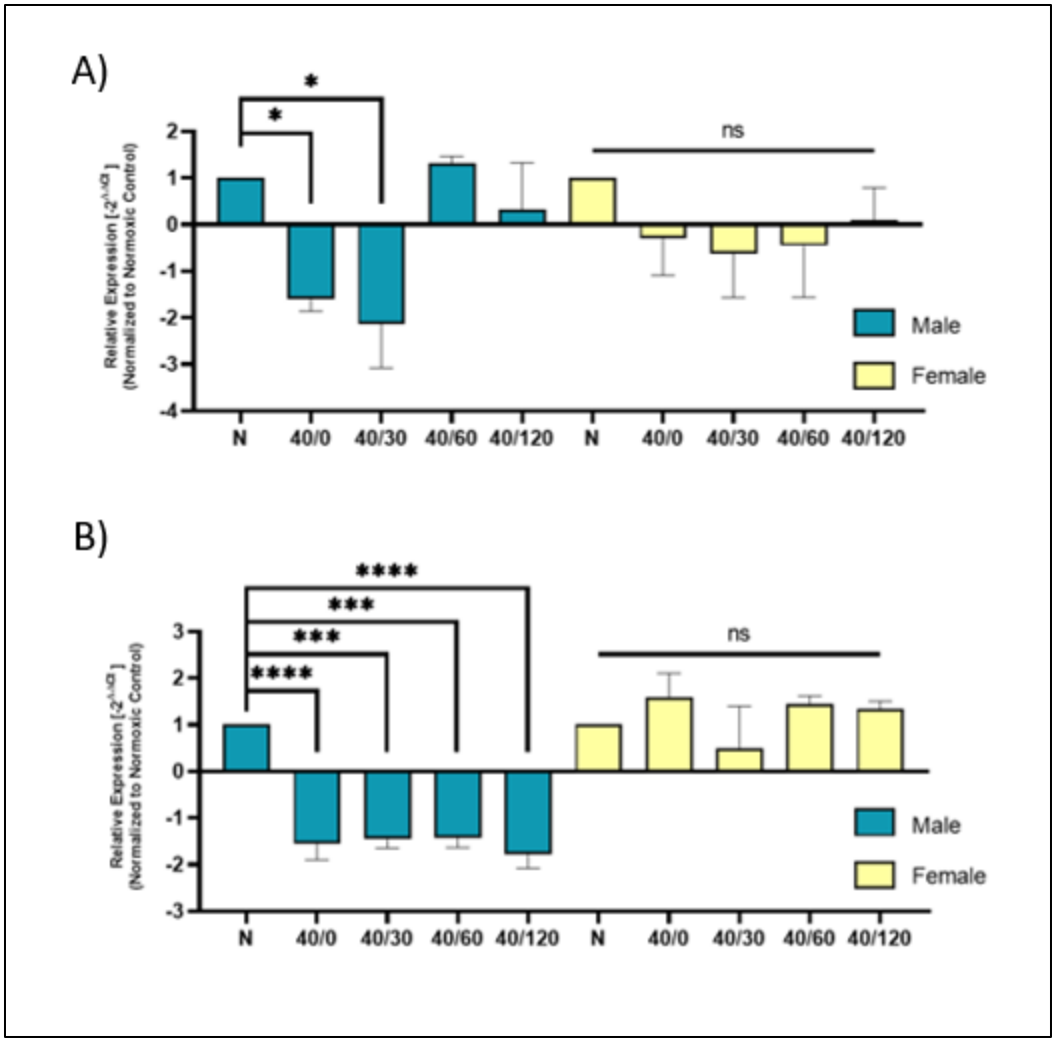


Figure 2.17: OGD/R decreases ADARB2 and GRIA2Q/R (Q/R-edited GluA2) expression in male hippocampal slices. ADARB2 and GRIA2Q/R expression levels are unaffected with OGD/R in female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Utilizing rt-qPCR, we examined the expression of ADARB2 (ADAR2) and GRIA2Q/R (Q/R-edited GluA2) with OGD/R treatment. (A) Expression of ADARB2 in male and female hippocampal slices with OGD/R treatment, normalized to Normoxic control group and ActB (β-Actin) (N=3). (B) Expression of GRIA2Q/R in male and female hippocampal slices with OGD/R treatment, normalized to Normoxic control group and ActB (β-Actin) (N=3). *$p<0.05$;

***p<0.001; **** p<0.0001; ns denotes no significance. ANOVA with Sidak *post hoc* test comparing OGD/R values ($2^{-\Delta\Delta C_T}$) corresponding to Normoxic control. Data are expressed as *mean* ± *SEM*.

Discussion

Our results indicate that the OGD/R-induced degradation of GluA2-containing AMPARs occurs via the lysosome and ubiquitination does not play a role in this process. We also provided evidence that increased calcium from both NMDARs and L-type voltage-gated calcium channels play a role in the degradation of GluA2 AMPAR subunits following OGD/R. We first established that the basal protein level expression of AMPAR subunits in the hippocampus is not significantly different in male versus female rats. There is also no difference in the amount of cell death following OGD/R in male hippocampal slices versus female hippocampal slices, although male hippocampal slices do trend slightly lower in cell viability than females as indicated in Figure 2.2.

Interestingly GluA1 and GluA2 AMPAR subunits respond in an opposite nature to OGD/R in male versus female aged rat hippocampal slices. In males, GluA1 and GluA2 AMPAR subunits decrease with OGD/R, maximally with 120 minutes of reperfusion. However, in females, GluA1 and GluA2 AMPAR subunits increase with OGD/R treatment, maximally at 120 minutes of reperfusion. We have previously demonstrated that GluA2, but not GluA1, decreases with OGD/R in 8-week-old male hippocampal slices [Beske *et al*. 2014; Jackson *et al.* 2018; Achzet *et al*. 2021b], and did not examine female hippocampal slices in those studies. There seems to be an age-dependent difference in the way GluA1 AMPAR subunits respond to ischemia/reperfusion stress.

Since GluA1 was also decreasing in male hippocampal slices, we wanted to determine whether there was a change in the ratio of GluA1:GluA2 AMPARs with OGD/R. We determined that there was an increase in the amount of GluA1 compared to GluA2 with OGD/R at 0 minutes

of reperfusion and 30 minutes of reperfusion. It has been well established that during LTP, there is a temporary increase in the amount of calcium-permeable AMPARs [Terashima *et al.* 2008], but more recently it's been demonstrated that hippocampal long-term depression also involves a transient increase in the amount of synaptic calcium-permeable AMPARs [Sanderson *et al.* 2016]. Indeed, we see an increase in the ratio of GluA1:GluA2 AMPAR subunits, potentially indicating increased expression of calcium-permeable AMPARs that may contribute to excitotoxicity with OGD/R in male hippocampal slices. In females, there is a trend towards an increase in this excitotoxic GluA1:GluA2 ratio with OGD/R, but this was not significant.

Next, we utilized biotinylation to quantify the amount of surface GluA1 and GluA2 AMPAR subunits. In male animals, we observed internalization of GluA1 with OGD/R, maximally at 120 minutes, but in females, surface GluA1 levels do not change. Interestingly, we see an increase in the amount of GluA2 AMPAR subunits with 40 minutes of OGD in male hippocampal slices, before we observe surface removal of GluA2-AMPAR subunits, maximally at 120 minutes of reperfusion. In females, we see a slight decrease in GluA2 surface levels, trending toward significance ($p=0.0559$). This is contradictory to what we expected to observe in male hippocampal slices. The increase in surface levels of GluA2 could be due to homomeric GluA2 AMPARs or GluA2/3 AMPARs in a typical LTP mechanism. However, we do not see a transient increase in the amount of GluA1 surface levels as we expected. This could be due to the slower recycling of GluA1 homomeric or GluA1/3 AMPARs that occurs during basal conditions compared to GluA2/GluA3 AMPARs [Passafaro *et al.* 2001; Shi *et al.* 2001]. We also may be missing the critical time-point during OGD where there could be a transient increase in the amount of GluA1 AMPAR subunits, as GluA1-containing (GluA2-lacking) AMPARs are rapidly

delivered to the synapse and then quickly internalized and substituted with GluA2-containing AMPARs [Hanley 2014]. This would explain why we see a transient increase in surface GluA2 AMPAR subunits in males, but not GluA1 AMPAR subunits with OGD treatment. This warrants further investigation examining different end-points during OGD to determine whether there is a change in the amount of GluA1 AMPAR subunits at the plasma membrane in male hippocampal slices.

We next determined that GluA2-containing AMPARs are degraded in a lysosomal-dependent manner with OGD/R in males, confirming what has been previously established in other models [Koszegi *et al.* 2017; Fernandez-Monreal *et al.* 2012]. However, the degradation of GluA2-containing AMPARs does not occur via ubiquitination, indicating a differential trafficking mechanism than agonist-induced ubiquitination [Lin *et al.* 2011; Widagdo *et al.* 2015]. During NMDAR-mediated chemical LTD, or weakening of the synapse, AMPARs are not ubiquitinated [Lussier *et al.* 2011; Widagdo *et al.* 2015]. This suggests there must be an alternative mechanism for the OGD/R-induced sorting of GluA2-containing AMPARs to degradation other than ubiquitination. The transmembrane auxiliary receptor protein (TARP) stargazin (γ2) binds to AMPARs and regulates the surface expression of AMPARs through its interaction with PSD-95. The adaptor protein (AP) complex 3 (AP-3) binds to TARPγ2, promoting AMPAR late endosomal sorting during long-term depression, in a process regulated by the phosphorylation of the C-terminus of TARPγ2 [Matsuda *et al.* 2013]. This is one potential mechanism for the OGD/R-induced sorting of GluA2-containing AMPARs to the lysosome, independent of ubiquitination and warrants further investigation. Cortactin has also recently been shown to play a role in facilitating the recycling of GluA2-containing AMPARs. Stimulation of

NMDARs disrupts the direct interaction between cortactin and AMPARs, which results in the lysosomal degradation of GluA2-containing AMPARs [Parkinson *et al.* 2018]. Perhaps one, or both, of these mechanisms play a role in the lysosomal sorting of GluA2-containing AMPARs with OGD/R.

Finally, we sought to determine whether the OGD/R-induced increase in GluA1 and GluA2 AMPAR subunits in female was due to an increase in protein translation, and indeed confirmed that inhibition of translation prevents the OGD/R-induced increase in AMPARs. This increase in AMPARs could be due to an increase in local translation, as neurons have the machinery required for protein synthesis in dendrites [Holt *et al.* 2019]. However, as we're not isolating a particular cellular location or cell type in our model, it's not possible to conclude where this increase in AMPARs is occurring. We do know that there is not an increase in GluA1 or GluA2 AMPARs at the synaptic membrane with OGD/R in females, given by our biotinylation results in this study. We also reported the mRNA expression levels of AMPAR subunits GluA1 [GRIA1], GluA2 [GRIA2], GluA3 [GRIA3], and edited GluA2Q/R [GRIA2Q/R], as well as the RNA-editing enzyme ADAR2 [ADARB2]. There is a rapid OGD/R-induced decrease of GRIA1, GRIA2, GRIA2Q/R and ADARB2 expression in males, but not in females. This further confirms that the OGD/R-induced increase in protein levels of GluA1 and GluA2 in females is due to an increase in protein translation, and not necessarily an increase in mRNA expression. The ischemia/reperfusion-induced decrease in mRNA expression of AMPAR subunits has been observed in other studies [Pelligrini-Giampietro *et al.* 1997; Noh *et al.* 2012], as well as the decreased expression of ADARB2 [Peng *et al.* 2006]. Both the transcriptional and

translational dysregulation of AMPAR subunits appear to have key roles in the trafficking of AMPARs with ischemia/reperfusion.

In summary, we have unraveled a key sexual dimorphism in the degradation of GluA2-containing AMPARs, which occurs via the lysosome in a calcium-dependent manner with OGD/R, independent of ubiquitination in males. There is an OGD/R-induced increase in GluA1 and GluA2 in females, which is not observed in males. This sex-dependent difference is peculiar and warrants further investigation to examine why females are protected against ischemia/reperfusion-induced AMPAR degradation. Ultimately, these data help elucidate the degradation pathway of AMPARs with ischemia/reperfusion in males and uncovered a sex-difference in AMPAR trafficking between males and females. This could help explain why females are more protected from ischemic stroke in their lifespan compared to males, and the precise regulation of AMPARs, with respect to sex, following ischemic stroke is an exciting new area for future investigation.

CHAPTER FOUR: THE EFFECT OF NADPH OXIDASE ON THE OGD/R-INDUCED INTERNALIZATION AND DEGRADATION OF GLUA2-CONTAINING AMPARS IN AGED RAT HIPPOCAMPAL SLICES

Abstract

Following ischemia/reperfusion, there is a pathological burst of superoxide from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). This uncontrolled production of reactive oxygen species (ROS) results in oxidative stress and neuronal damage following ischemic stroke. Previous studies from our laboratory have linked the overproduction of NADPH oxidase-derived ROS with the internalization and degradation of GluA2 AMPA receptor (AMPAR) subunits following ischemia/reperfusion. AMPARs are ionotropic glutamate receptors composed of GluA1-4 subunits. The presence of the GluA2 subunit renders the channel impermeable to calcium due to a post-transcriptional modification in the channel pore. Following ischemia/reperfusion, there is an increase in GluA2-lacking, calcium-permeable AMPARs at the synapse, which increased the excitability of neurons leading to cytotoxic accumulation of intracellular calcium and excitotoxicity. Preventing the ischemia/reperfusion-induced degradation of GluA2-containing AMPARs is a potential therapeutic target. However antagonists of AMPARs have not been successful in the clinic due to severe side-effects. In this study, we targeted NADPH oxidase 1 (NOX1), to prevent the ischemia/reperfusion-induced degradation of GluA2-containing AMPARs. Acute organotypic hippocampal slices from aged male rats were exposed to oxygen-glucose deprivation/reperfusion (OGD/R), an *in vitro* model for ischemia/reperfusion. Treatment with GKT137831, a NOX1/4 inhibitor, rescued the OGD/R-induced internalization and degradation of GluA2 AMPAR subunits. These data identify a key role for NOX1 in the oxidative

stress signaling cascade that mediates the ischemia/reperfusion-induced internalization and degradation of GluA2-containing AMPARs in males.

Introduction

One of the downstream consequences of ischemia/reperfusion is the pathological production of reactive oxygen species (ROS). Under physiological conditions superoxide is necessary for the induction of long-term potentiation (LTP) in the hippocampus [Klann *et al.* 1998]. Inhibition of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a superoxide producer, prevents LTP induction in the hippocampus [Kishida *et al.* 2006]. Stimulation of NMDA receptors (NMDARs) increases the activation of NADPH oxidase [Brennan *et al.* 2009; Girouard *et al.* 2009], resulting in a large burst of superoxide. NADPH oxidase 1 (NOX1) and NOX2 are activated upon translocation of cytosolic components $p47^{phox}$, $p67^{phox}$, and Rac1 to the membrane to form a complex with the Nox1, or Nox2, and $p22^{phox}$ subunits [Kawahara *et al.* 2005; Banfi *et al.* 2003; Cheng *et al.* 2006].

NADPH oxidase-derived ROS is important for LTP, but uncontrolled ROS production is pathologic and leads to oxidative stress. There is triphasic burst of superoxide following 40 minutes of oxygen-glucose deprivation/reperfusion (OGD/R), an *in vitro* model for ischemic stroke. The mitochondria and xanthine oxidase produce superoxide during the ischemic phase, and NADPH oxidase produces a large burst of superoxide upon the onset of reperfusion [Abramov *et al.* 2007]. This third superoxide burst that occurs during reperfusion results in cell death and inhibition of NADPH oxidase using apocynin is neuroprotective [Wang *et al.* 2006].

Another downstream consequence of ischemia is the excessive release of neurotransmitters, including the excitatory amino acid glutamate. The overactivation of ionotropic glutamate receptors, NMDARs and AMPARs results in a process referred to as excitotoxicity [Szydlowska and Tymianski 2010]. Intracellular calcium concentrations regulate many cellular processes, but too much calcium becomes pathologic. The activation of postsynaptic AMPARs and NMDARs is very tightly regulated during synaptic transmission. During LTP, AMPARs are mobilized to the postsynaptic membrane and during long-term depression (LTD), AMPARs are internalized and degraded. These processes work to strengthen or weaken a synapse, respectively [Shepherd and Huganir, 2007]. In the hippocampus, these processes are thought to underlie the basic molecular mechanisms of learning and memory.

A majority of AMPARs in the hippocampus are GluA1—GluA2 containing or GluA2—GluA3 AMPARs, with small pools of GluA2-lacking AMPARs [Wenthold *et al.* 1996; Reimers *et al.* 2011]. The presence of the GluA2 subunit renders the AMPAR impermeable to divalent cations, such as calcium [Traynelis *et al.* 2010]. Removal of GluA2-containing, calcium-impermeable AMPARs, at the synaptic membrane increases the excitability of neurons by increasing the neuron's calcium permeability through GluA2-lacking AMPARs [Lu and Ziff 2005] and can result in excitotoxicity during pathologies including: Huntington's disease, Parkinson's disease [Jayakar and Dikshit 2004], and ischemic stroke [Anzai *et al.* 2003; Noh *et al.* 2005].

We have previously linked the overproduction of ROS from NADPH oxidase following ischemia/reperfusion with the internalization and degradation of GluA2-containing AMPARs in the hippocampus [Beske *et al.* 2014; Jackson *et al.* 2018]. It's unclear however which isoform of

NADPH oxidase, NOX1 or NOX2, or both, contribute to the pathological trafficking of GluA2 AMPAR subunits with OGD/R. Utilizing pharmacological inhibitors, this study identified NOX1 as the isoform responsible for the OGD/R-induced internalization and degradation of GluA2-containing AMPARs in the hippocampus. Specifically, we found that treatment with GKT137831, a NOX1/4 inhibitor, rescued total and surface GluA2 AMPAR protein levels following OGD/R. NOX4 is constitutively active, producing hydrogen peroxide, and therefore is likely not the NOX isoform responsible for the OGD/R-induced production of ROS. The results in this study provide evidence that NOX1 plays a role in the oxidative stress-signaling cascade that mediates the internalization and degradation of GluA2-containing AMPARs following ischemia/reperfusion.

Materials and Methods

Animals

Male and Female Sprague-Dawley rats (10-12 months of age; Envigo, Indianapolis, IN, USA) were used in this study. Animals were allowed free access to food and water in an AALAC-accredited vivarium, and were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Washington State University Institutional Animal Care and Use Committee (IACUC).

Reagents

All antibodies used in this study are listed in the table below (Table 3.1). GKT137831 was purchased from (Selleckchem, Houston, TX, USA). GSK2795039 was purchased from (MedChemExpress, Monmouth Junction, NJ, USA).

Preparation of acute rat hippocampal slices

The rats were anesthetized with isoflurane and decapitated. The brain was removed and placed in ice-cold Hank's Buffered Salt Solution (HBSS; Gibco, Amarillo, TX, USA) for no more than 30 seconds. Both hippocampi were dissected on ice and coronal 350µm-thick slices were prepared using a McIlwain tissue chopper (Stoelting Co., Wood Dale, IL, USA). Slices were equilibrated in oxygenated (95% O_2, 5%CO_2) artificial cerebrospinal fluid (aCSF; 124mM NaCl, 2.5mM KCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.5mM $MgCl_2$, 10mM D-glucose, pH7.4) at 37°C for 60 minutes prior to oxygen-glucose deprivation/reperfusion (OGD/R). Fresh, oxygenated aCSF was replaced every 15-30 minutes during the equilibration period.

Drug treatment of hippocampal slices

Slices were treated with either GKT137831 or GSK2795039 following OGD, at the start of reperfusion, and drug was present throughout reperfusion. Slices were treated with vehicle controls where appropriate, in 0.01% dimethyl sulfoxide (DMSO).

Oxygen-glucose deprivation/reperfusion of hippocampal slices

Following equilibration, slices were placed in glucose-free aCSF (aCSF with 10mM sucrose and no glucose, pH 7.4), and incubated for 40 minutes in a hypoxic glove box (Coy Laboratories, Grass Lake, MI, USA) containing 100% N_2. The glucose-free aCSF media used for OGD was

de-oxygenated using argon gas under vacuum to ensure anoxic aCSF. Following OGD, slices were transferred back to oxygenated glucose-containing aCSF for the time-periods indicated for each experiment (0, 30, 60, or 120 minutes). Normoxic controls remained in glucose-containing aCSF throughout the entirety of each experiment and time-matched to the last reperfusion time point of OGD/R-subjected slices.

Lysate Preparation

Upon completion of OGD/R or normoxia, the hippocampal slices were lysed in lysis buffer containing (50mM Tris, 140mM NaCl, 5mM EDTA, 1% Triton x-100, 1% Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Asheville, NC, USA), and 1mM phenylmethylsulfonyl fluoride by dounce homogenization followed by sonication for 3 separate 5 second bursts at 25% power output (VirTis Ultrasonic Cell Disrupter; Gardiner, NY, USA). Samples were then centrifuged at 13,000 *x g* for 10 minutes (4°C). The supernatant was collected and a bicinchoninic acid assay (BCA) was performed to determine protein concentration. Samples were denatured in NuPAGE LDS (Fisher Scientific, Waltham, MA, USA) and heat (100°C) for 10 minutes and resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were transferred to a nitrocellulose membrane (Bio-Rad, Berkeley, CA, USA) for subsequent detection by immunoblotting.

Immunoblotting

Blots were blocked for 1 hour at room temperature with either SuperBlock (Thermo Fisher Scientific, Asheville, NC, USA) for phospho-antibody detection or 5% non-fat dry milk in tris buffered saline, 0.1% Tween 20, pH 7.5 (TBS-T) for non-phosphorylated antibodies. After

blocking, blots were incubated with primary antibody overnight at 4°C at the concentration indicated in Table 3.1.

#	Antibody	Source	Catalog #	Dilution
1	GluA1	Cell Signaling	13185S	1:1000
2	GluA2	Cell Signaling	13607S	1:1000
3	GluA1pSer845	Cell Signaling	8084S	1:1000
4	GluA2pY876	Cell Signaling	4027S	1:1000
5	p67phox	Santa Cruz	sc-374510	1:1000
6	gp91phox	BD Transduction	611414	1:500
7	SOD1	Cell Signaling	37385S	1:1000
8	SOD2	Cell Signaling	13141S	1:1000

Table 3.1: Antibodies used in this study including source, product number, and dilution used.

Immunoreactive bands were visualized and captured utilizing a FujiFilm imaging system (Edison, NJ, USA) using enhanced chemiluminescence after addition of HRP-conjugated secondary antibodies (Rabbit-HRP or Mouse-HRP, Cell Signaling, Danvers, MA, USA). Bands were analyzed using Fuji Image-Gauge software. Blots were stripped and reprobed up to 2 times with Restore Plus Western Blotting Stripping Buffer (Thermo Fisher Scientific, Asheville, NC, USA).

Biotinylation of hippocampal slices

Acute aged rat hippocampal slices were prepared and treated to OGD/R or time-matched normoxia as previously described in this study. At the indicated time-point, slices were washed with ice-cold Buffer A (25mM HEPES, 119mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 30mM glucose, pH 7.4) and incubated at 4°C in Buffer A containing 0.5 mg/mL of sulfo-NHS-SS-biotin (Thermo Scientific, Waltham, MA, USA) for 30 minutes with gentle agitation. After biotin labeling, slices were washed three times in ice cold Buffer A. Slices were then lysed via sonication with 3 separate 5 second bursts at 25% power output in Buffer A containing 1% protease and phosphatase inhibitor cocktail. The lysate was centrifuged at 100,000 *x g* in an ultracentrifuge. The biotin-labeled membrane fraction was then solubilized on ice for 30 minutes using Solubilization Buffer (10mM Tris, 150mM NaCl, 1% protease and phosphatase inhibitor cocktail, 1% Triton x-100, pH 8.0). The biotin-labeled membrane fraction was then sonicated with 3 separate 5 second bursts at 25% power output and a BCA assay was performed to determine protein content. Biotin-labeled membrane proteins (500µg lysate/500µL of Solubilization buffer) were then incubated overnight with gentle agitation at 4°C with 60µL of magnetic streptavidin beads (New England BioLabs, Ipswich, MA, USA). The streptavidin beads were then washed twice with High Salt Buffer (10mM Tris, 3M NaCl, 1% Triton x-100, pH 8.0), followed by two washes with Solubilization Buffer. The streptavidin-biotin bead complex was denatured with Laemmli and heat (80°C) for 10 minutes, subjected to SDS-PAGE, and transferred to nitrocellulose membranes for subsequent immunoblotting. Immunoreactive bands were analyzed using Fuji Image-Gauge software.

Nitro-blue tetrazolium assay

The nitro-blue tetrazolium (NBT) assay was adapted from Aukrust *et al.* (1994). In brief, slices were equilibrated for 50 minutes and incubated in oxygenated aCSF with NBT (0.5mg/mL; Sigma, Greenacres, WA, USA) for 10 minutes before OGD. Slices were rinsed with glucose-free aCSF and OGD/R treatment was performed as previously indicated. At the various reperfusion time-points, the NBT reaction was stopped with 0.25M HCl. Phorbol 12-myristate-13-acetate (PMA; 10µM, 10 min) was used as a positive control. Slices were visualized utilizing a dissecting microscope (AmScope 3.5X Manufacturing 144-LED Zoom Stereo Microscope, Irvine, CA, USA). Slices were then lysed by sonication in dimethylsulfoxide. 200µL of lysates were aliquoted into a 96-well plate and the absorbance was measured at 550nm using a spectrophotometric microplate reader (VersaMax plate reader, Molecular Devices, Sunnyvale, CA, USA). Normoxic controls were rinsed with glucose-containing aCSF after NBT incubation and left in glucose-containing aCSF time-matched to the last OGD/R time point.

Data analysis

One-way ANOVA with *post-Hoc* Sidak test were conducted using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) to determine statistical significance. A p-value less than 0.05 was considered statistically significant.

Results

Sex difference in the ROS production following OGD/R.

It's been well-established that acute organotypic hippocampal slices produce reactive oxygen species (ROS) following OGD/R (Beske *et al.* 2015), however this study was conducted using hippocampal slices from 8-week-old male rats. In the current study, we wanted to examine the amount of ROS produced in the hippocampus following OGD/R in aged male and female rats. Utilizing a nitroblue tetrazolium assay (NBT assay), we found that aged male hippocampal slices produce ROS in a time-dependent manner with OGD/R exposure, maximally at 120 minutes of reperfusion (Figure 3.1). In female slices, however, OGD does not significantly produce ROS compared to Normoxic control, although there is a trend of a slight increase with OGD/R treatment. Phorbol 12-myristate 13-acetate (PMA) was used as a positive control in this NBT assay because PMA activates NADPH oxidase, stimulating production of ROS [Kuwabara *et al.* 2015]. Based upon previous results within our laboratory [Beske *et al.* 2014; Jackson *et al.* 2018; Achzet *et al.* 2021], we sought to examine the role of ROS produced from NADPH oxidase on AMPAR subunit trafficking following OGD/R.

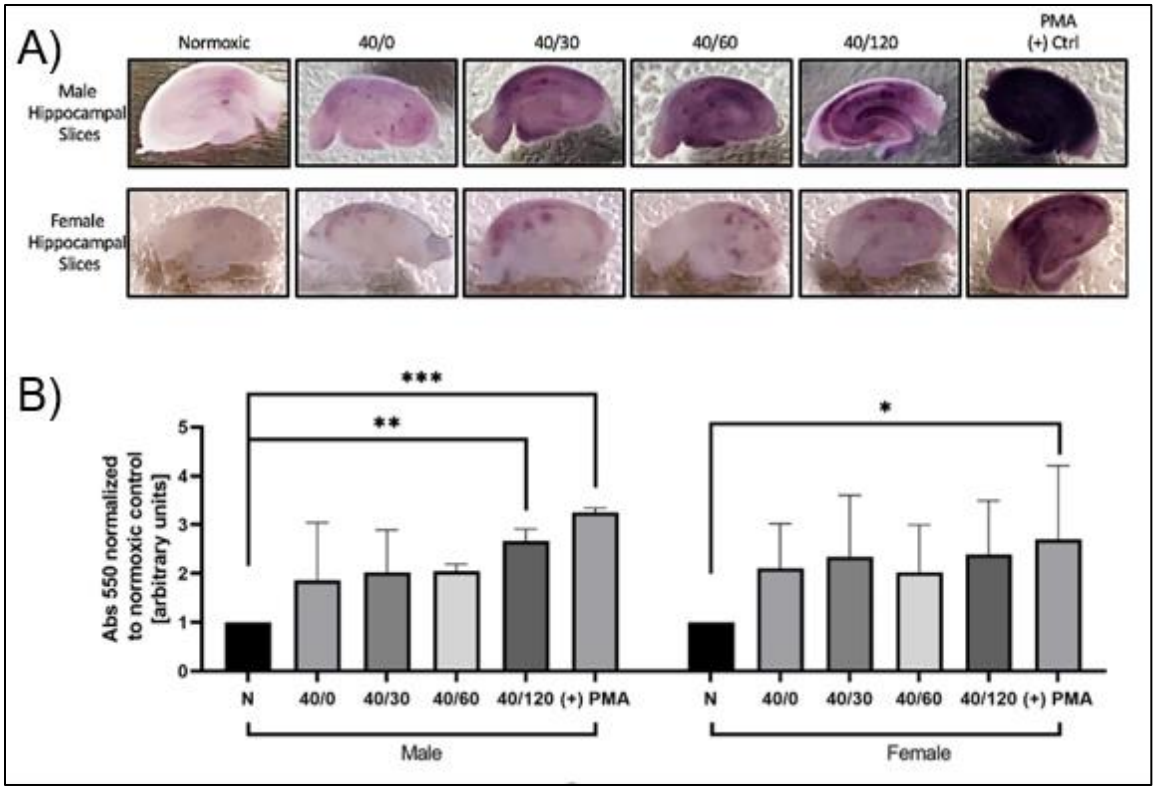


Figure 3.1: Sex differences in reactive oxygen species production in the aged rat hippocampal slices following ischemia/reperfusion. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. Hippocampal slices were incubated with Nitroblue Tetrazolium (NBT; 0.5mg/mL) 10 minutes prior to OGD and placed in 0.25M hydrochloric acid (HCl) following OGD/R or normoxia. Slices were incubated with Phorbol 12-myristate 13-acetate (PMA) following NBT incubation as a positive control. A) Representative images of male and female hippocampal slices following OGD/R demonstrating increased ROS production in male hippocampal slices following OGD/R, maximally at 120 minutes of reperfusion. (B) Following imaging of hippocampal slices in (A), slices were lysed in DMSO and the absorbance at 550nm was measured for a quantitative analysis of (A). Absorbances were normalized to Normoxic control group (N=4). *$p<0.05$, **$p<0.01$, ***$p<0.001$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

No differences in basal protein levels of NADPH oxidase subunits, $p67^{phox}$ nor $gp91^{phox}$, and antioxidants, SOD1 nor SOD2, in male or female aged rats.

Since aged male hippocampal slices produce more ROS following OGD/R compared to female hippocampal slices, we next sought to determine whether there was a misbalance between the oxidant and antioxidant proteins between sexes. Utilizing western blot analysis, we examined the total protein levels of NADPH oxidase 2 (NOX2) subunits, $p67^{phox}$ and $gp91^{phox}$, in the hippocampus, cortex, and cerebellum of aged male and female rats. It's already been established that NOX1 protein levels are higher in male compared to female in aged rats, so we did not examine the NOX1 protein levels in this study (Miller *et al.* 2007). There were no differences in the basal protein levels of $p67^{phox}$ nor $gp91^{phox}$ in any brain regions examined in male and female rats (Figure 3.2).

Next, we examined whether there were any differences in the protein expression of antioxidants, SOD1 and SOD2, that are responsible for the enzymatic reaction of superoxide to molecular oxygen and hydrogen peroxide. We hypothesized that since there were no differences in NOX2 protein levels, perhaps there were increased levels of antioxidants to prevent OGD/R-induced ROS production in female hippocampal slices. However, we also found no difference in the basal protein expression levels of SOD1 and SOD2 in the various brain regions examined comparing aged male and female rats (Figure 3.3).

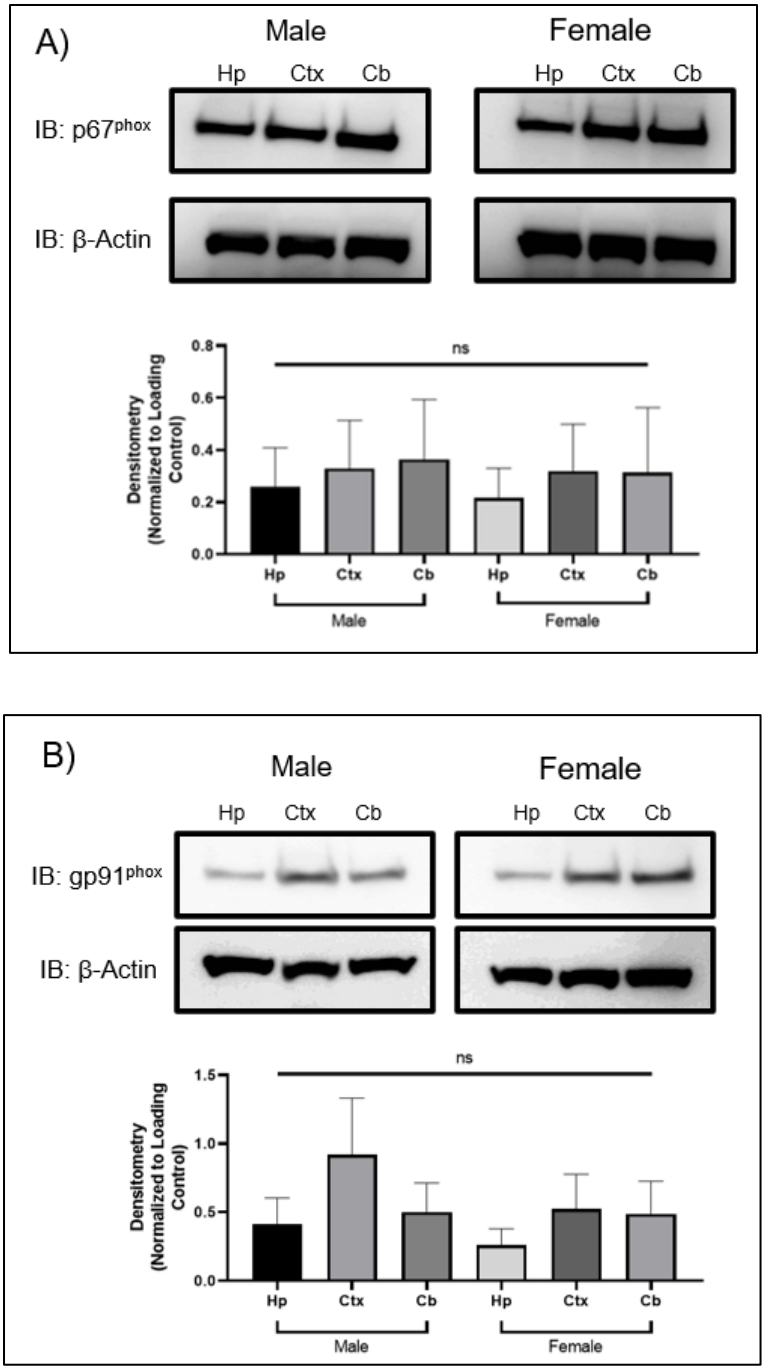
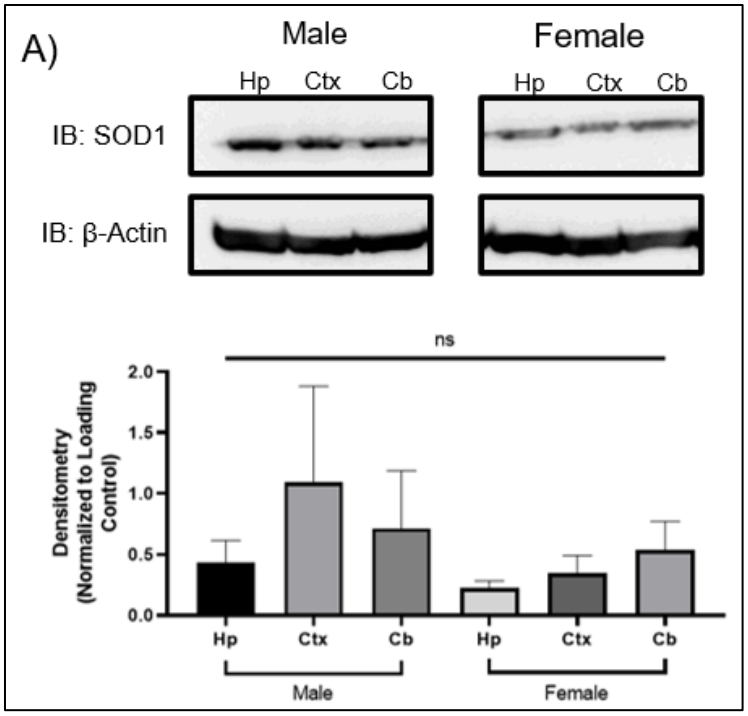


Figure 3.2: There are no differences in basal NADPH oxidase 2 (NOX2) subunit protein levels between aged male and female rats in various brain regions. 10-12 month old male and female rat brain regions were examined by western blot to examine the total protein levels of NOX2 subunits ($p67^{phox}$ and $gp91^{phox}$) in the hippocampus (Hp), cortex (Ctx), and cerebellum (Cb). (A) Representative western blots

demonstrating that there are no significant differences in basal protein levels of $p67^{phox}$ between male and female rats in the Hp, Ctx, or Cb. Total $p67^{phox}$ protein levels were normalized to β-Actin (N=3). (B) Representative western blots demonstrating that there are no significant differences in basal protein levels of $gp91^{phox}$ between male and female rats in the Hp, Ctx, or Cb. Total $gp91^{phox}$ protein levels were normalized to β-Actin (N=3). ns denotes no significance; ANOVA with Sidak *post hoc* test comparing all brain regions. Data are expressed as *mean* ± *SEM*.



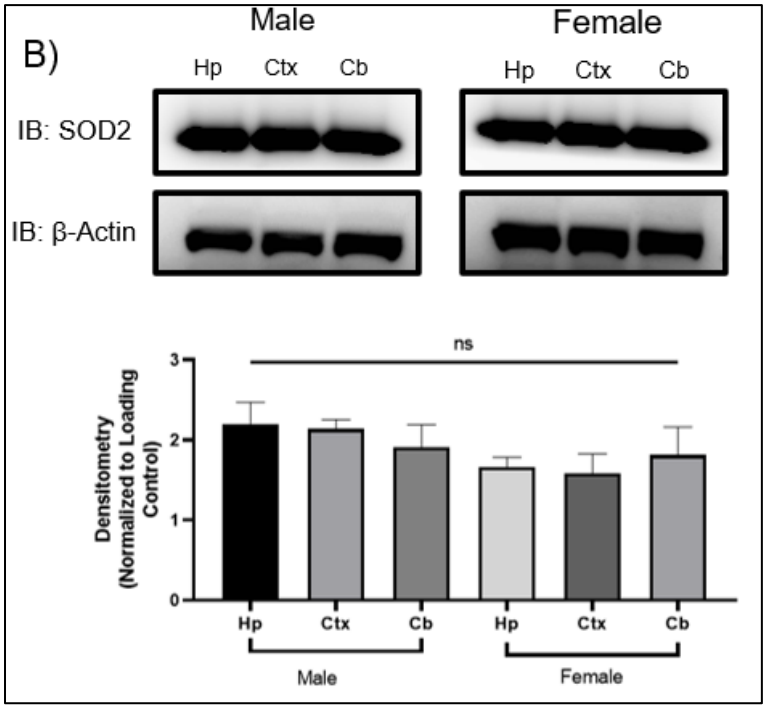


Figure 3.3: There are no differences in basal antioxidant protein levels, superoxidase dismutase 1 and 2 (SOD1 and SOD2) between aged male and female rats in various brain regions. 10-12 month old

male and female rat brain regions were examined by western blot to examine the total protein levels of SOD1 and SOD2 in the hippocampus (Hp), cortex (Ctx), and cerebellum (Cb). (A) Representative western blots demonstrating that there are no significant differences in basal protein levels of SOD1 between male and female rats in the Hp, Ctx, or Cb. Total SOD1 protein levels were normalized to β-Actin (N=3). (B) Representative western blots demonstrating that there are no significant differences in basal protein levels of SOD2 between male and female rats in the Hp, Ctx, or Cb. Total SOD2 protein levels were normalized to β-Actin (N=3). ns denotes no significance; ANOVA with Sidak *post hoc* test comparing all brain regions. Data are expressed as *mean* ± *SEM*.

Inhibition of NADPH Oxidase 1/4 prevents the OGD/R-induced degradation of AMPAR subunits in male hippocampal slices.

In the previous chapter of this work, we established that GluA1 and GluA2 AMPAR subunits are degraded in male hippocampal slices with OGD/R, but not in female hippocampal slices. For the remainder work in this chapter, we utilized only male rats to examine whether NADPH oxidase plays a role in the OGD/R-induced degradation of AMPAR subunits. First, we utilized a pharmacological agent, GKT137831, that selectively inhibits NADPH oxidase 1 and 4 isoforms [Aoyama *et al.* 2012]. Since it's nearly impossible and impractical to pre-treat stroke patients in the clinic with drug treatments, we chose to treat the hippocampal slices with GKT137831 at the onset of reperfusion, to increase the translatability of this work.

GKT137831 has not been used in hippocampal slices prior to this work, so we used three different concentrations 250nM, 500nM, and 5µM, ensuring that at least one concentration was ten times more concentrated than the K_i (100nM) [Aoyama *et al.* 2012]. Inhibition of NOX1/4 with GKT137831 prevented the OGD/R-induced decrease of GluA1 (Figure 3.4) and GluA2 (Figure 3.5) AMPAR subunits in male hippocampal slices.

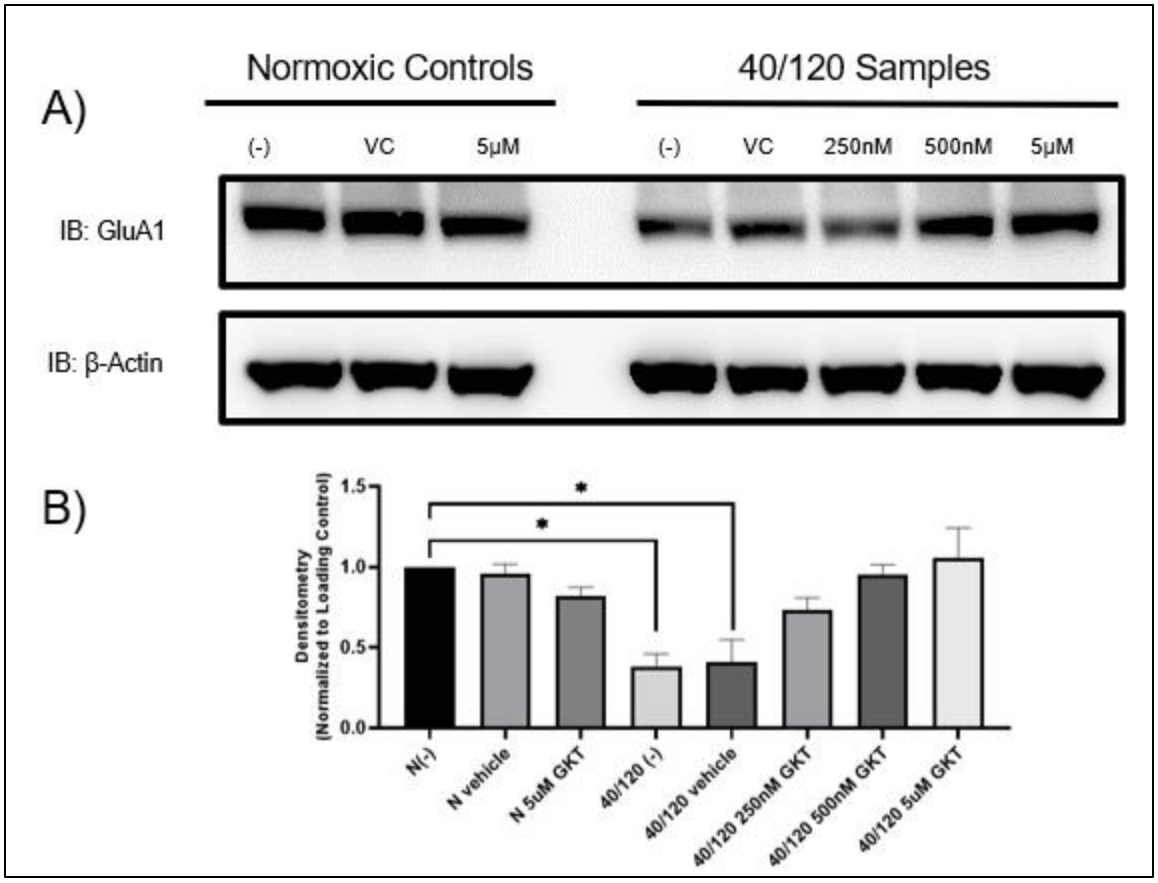


Figure 3.4: Inhibition of NADPH oxidase 1/4 (NOX1/4) prevents the OGD/R-induced degradation of GluA1 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX1/4 inhibitor, GKT137831 [250nM; 500nM; 5μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GKT137831, a NOX1/4 inhibitor, prevents the OGD/R-induced decrease of GluA1-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA1 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

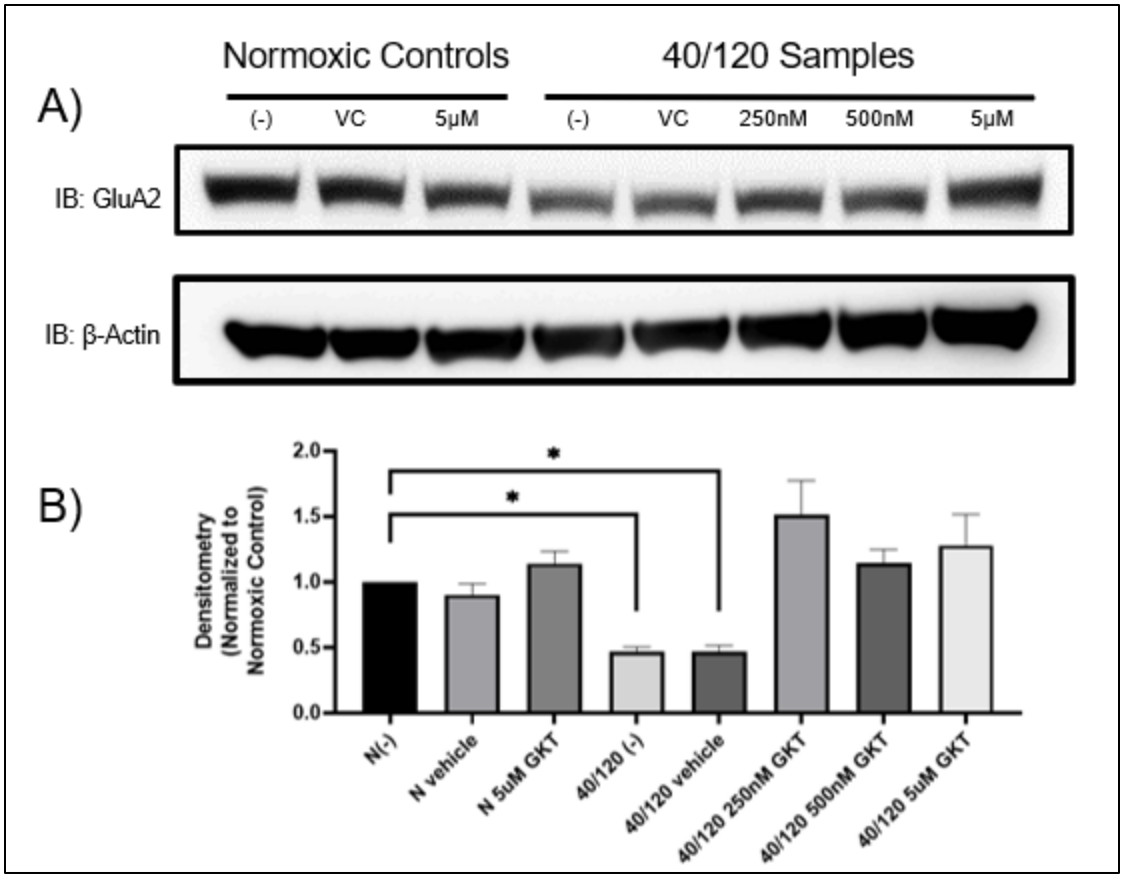


Figure 3.5: Inhibition of NADPH oxidase 1/4 (NOX1/4) prevents the OGD/R-induced degradation of GluA2 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX1/4 inhibitor, GKT137831 [250nM; 500nM; 5μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GKT137831, a NOX1/4 inhibitor, prevents the OGD/R-induced decrease of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

Sex differences in phosphorylation states of GluA1 and GluA2 AMPAR subunits following OGD/R

It's been well-established that phosphorylation of GluA1 at the Ser845 residue correlates with the induction of long-term depression, or a decrease in the amount of AMPARs at the synaptic membrane [Lee *et al.* 2000]. In the previous chapter, we demonstrated that GluA1 surface levels are decreased with OGD/R in male hippocampal slices, but not in female hippocampal slices. We wanted to examine whether GluA1 is dephosphorylated with OGD/R treatment in male or female hippocampal slices. Indeed, there is a time-dependent decrease of phosphorylated GluA1 at the Ser845 residue with OGD/R treatment in both male and female hippocampal slices (Figure 3.6).

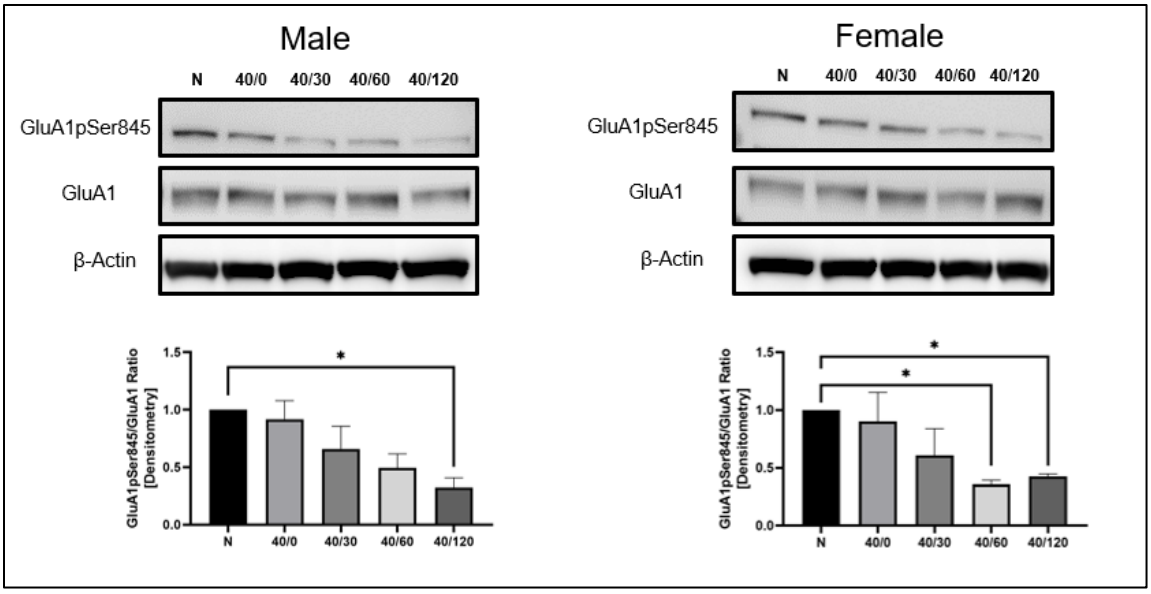


Figure 3.6: OGD/R induces dephosphorylation of Ser845 residue of GluA1 in male and female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. A) Representative western blot demonstrating the dephosphorylation of GluA1 Ser845 residue in male hippocampal slices following OGD/R, maximally at 60 and 120 minutes of reperfusion. (B) Quantification of (A) of GluA1 Ser845 residue protein levels normalized to β-Actin and total GluA1. OGD/R conditions normalized to Normoxic control group (N=3). (C) Representative western blot demonstrating the dephosphorylation of GluA1 Ser845 residue in female hippocampal slices following OGD/R, maximally at 60 and 120 minutes of reperfusion. (D) Quantification of (C) of GluA1 Ser845 residue protein levels normalized to β-Actin and total GluA1. OGD/R conditions normalized to Normoxic control group (N=3).* $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

The Tyr876 residue of GluA2 is also important for mediating synaptic plasticity, specifically mGluR-mediated LTD is associated with dephosphorylation of Tyr876 residue of GluA2 [Moult *et al.* 2006; Gladding *et al.* 2009], however it may not be crucial for the induction of LTD or LTP [Yong *et al.* 2020]. Even though the exact role that Tyr876 phosphorylation of GluA2 has yet to be elucidated with regards to LTP or LTD, we still determined whether the phosphorylation state of this residue is differentially altered with OGD/R in male and female hippocampal slices. OGD/R does not affect the phosphorylation of the Tyr876 residue of GluA2 in female slices, however OGD/R does result in dephosphorylation of this residue in male hippocampal slices (Figure 3.7). It is unclear whether this decrease in phosphorylation of the Tyr876 residue of GluA2 in male hippocampal slices is due to a decrease in total GluA2 protein, or whether the dephosphorylation of Tyr876 is involved in the degradation of GluA2 AMPAR subunits. We've established that treatment of male hippocampal slices with GKT137831 prevents the OGD/R-induced decrease in GluA2 AMPAR subunits (Figure 3.5). To examine whether the tyrosine dephosphorylation of GluA2 is due to a decrease in total GluA2, we treated male hippocampal slices with GKT137831 and quantified the phosphorylation state of Tyr876 residue by western blot. Interestingly, inhibition of Nox1/4 with GKT137831 did not prevent the OGD/R-induced dephosphorylation of the Tyr876 residue of GluA2 (Figure 3.8). This suggests that the OGD/R-induced dephosphorylation of GluA2 at the Tyr876 residue is not a result of the decrease in total GluA2 in male hippocampal slices. This also suggests that regulation of tyrosine phosphorylation of GluA2 AMPAR subunit with OGD/R may not be dependent on ROS production from NOX1/4.

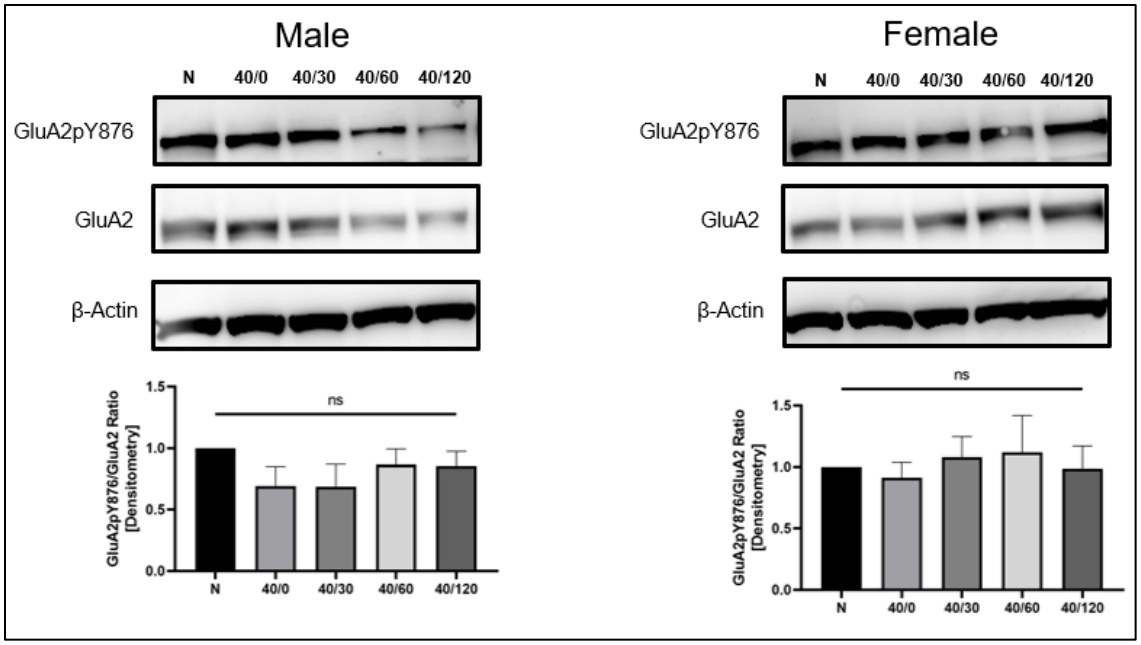


Figure 3.7: OGD/R induces dephosphorylation of Tyrosine876 residue of GluA2 in male hippocampal slices, but not in female hippocampal slices. Male and female hippocampal slices were exposed to 40 minutes of OGD and then reperfused for various time-points up to 120 minutes. A) Representative western blot demonstrating the dephosphorylation of GluA2 Tyr876 residue in male hippocampal slices following OGD/R, maximally at 60 and 120 minutes of reperfusion. (B) Quantification of (A) of GluA2 Tyr876 residue protein levels normalized to β-Actin and total GluA2. OGD/R conditions normalized to Normoxic control group (N=3). (C) Representative western blot demonstrating no change in phosphorylation state of GluA2 Tyr876 residue in female hippocampal slices following OGD/R. (D) Quantification of (C) of GluA2 Tyr876 residue protein levels normalized to β-Actin and total GluA2. OGD/R conditions normalized to Normoxic control group (N=3). ns denotes no significance; ANOVA with Sidak *post hoc* test comparing OGD/R conditions corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

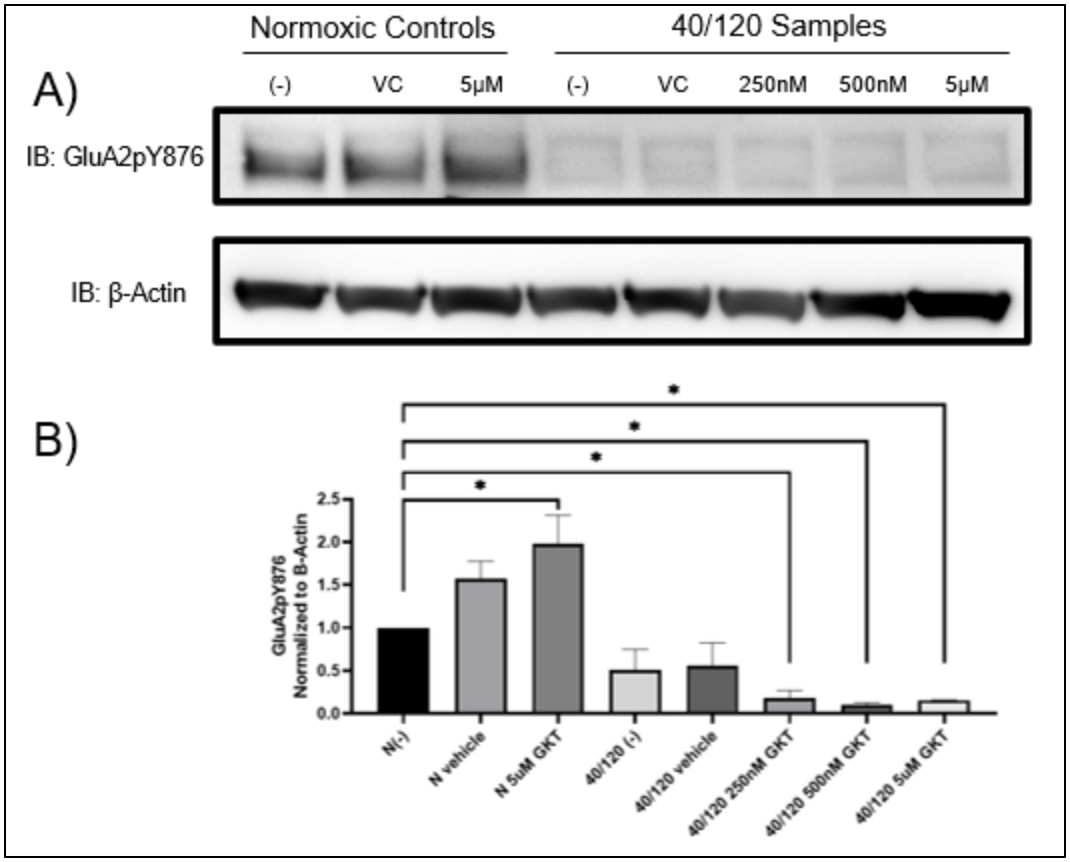


Figure 3.8: Inhibition of NADPH oxidase 1/4 (NOX1/4) does not prevent the OGD/R-induced dephosphorylation of Tyr876 residue of GluA2 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX1/4 inhibitor, GKT137831 [250nM; 500nM; 5μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GKT137831, a NOX1/4 inhibitor, does not prevent the OGD/R-induced decrease in Tyr876 phosphorylation of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of phosphorylated Tyr876 of GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

NADPH oxidase 2 does not play a role in the OGD/R-mediated degradation of AMPAR subunits in male hippocampal slices.

Previous studies from our lab have indicated that NOX2 plays a role in the OGD/R-induced degradation of GluA2-containing AMPARs [Beske *et al.* 2014; Jackson *et al.* 2018]. These studies utilized apocynin as an inhibitor of NADPH oxidase, but apocynin may act as a superoxide scavenger [Heumuller *et al.* 2008]. To confirm whether NOX2 plays a role in the OGD/R-induced degradation of GluA1 or GluA2 AMPAR subunits, we utilized the compound GSK2795039. GSK2795039 is a selective inhibitor for NOX2, with 30-fold selectivity over the other NOX isoforms [Hirano *et al.* 2015]. This compound, GSK2795039, has not yet been used in hippocampal slices, so we selected three different concentrations, with two concentrations being greater than ten times the K_i (~0.15μM) [Hirano *et al.* 2015] to ensure maximal inhibition of NOX2.

Unexpectedly, inhibition of NOX2 with GSK2795039 does not prevent the OGD/R-induced degradation of GluA1 [Figure 3.9] or GluA2 [Figure 3.10] in male hippocampal slices. Treatment with GSK2795039 also did not prevent the OGD/R-induced dephosphorylation of GluA2 at the Tyr876 residue in male hippocampal slices (Figure 3.11). Taken together, these data indicate that NOX2 does not play a role in the OGD/R-induced degradation of GluA1 or GluA2 in male hippocampal slices.

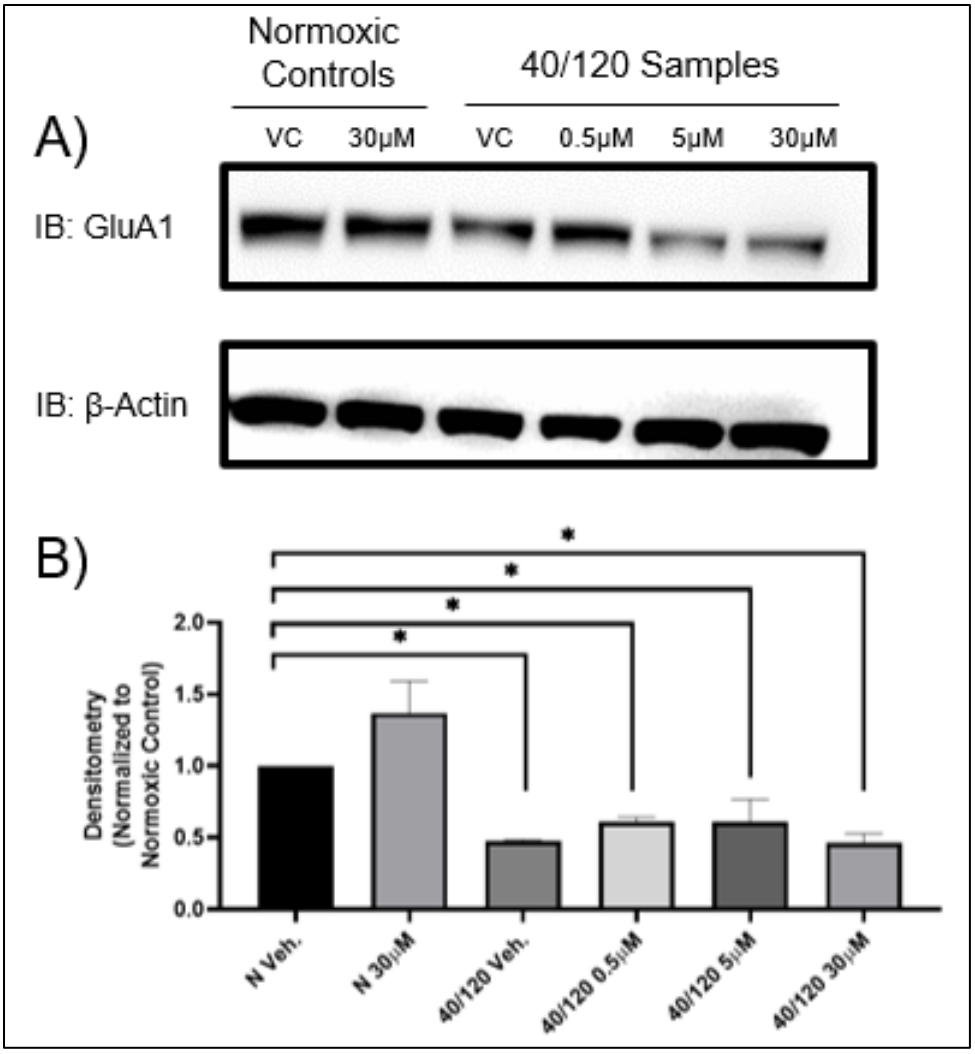


Figure 3.9: Inhibition of NADPH oxidase 2 (NOX2) does not prevent the OGD/R-induced degradation of GluA1 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX2 inhibitor, GSK2795039 [0.5μM; 5μM; 30μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GSK2795039, a NOX2 inhibitor, does not prevent the OGD/R-induced decrease of GluA1-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA1 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

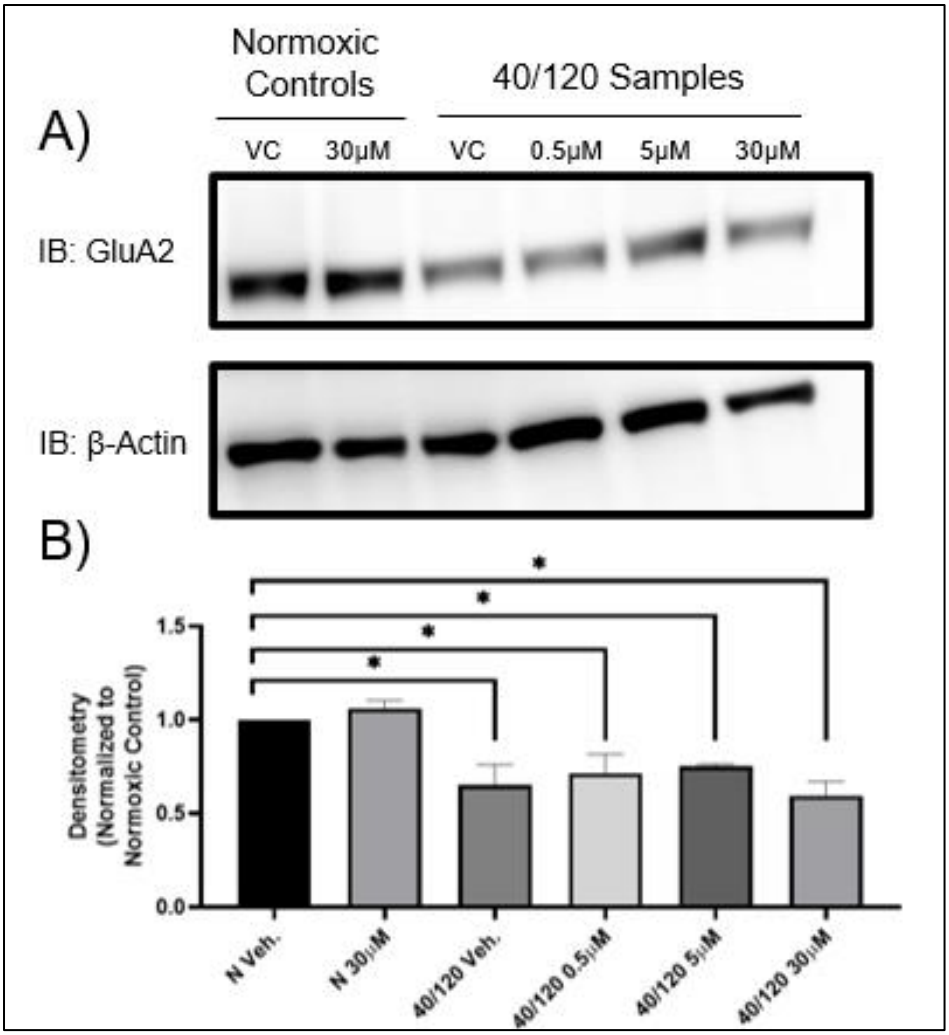


Figure 3.10: Inhibition of NOX2 does not prevent the OGD/R-induced degradation of GluA2 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX2 inhibitor, GSK2795039 [0.5μM; 5μM; 30μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GSK2795039, a NOX2 inhibitor, does not prevent the OGD/R-induced decrease of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of total GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

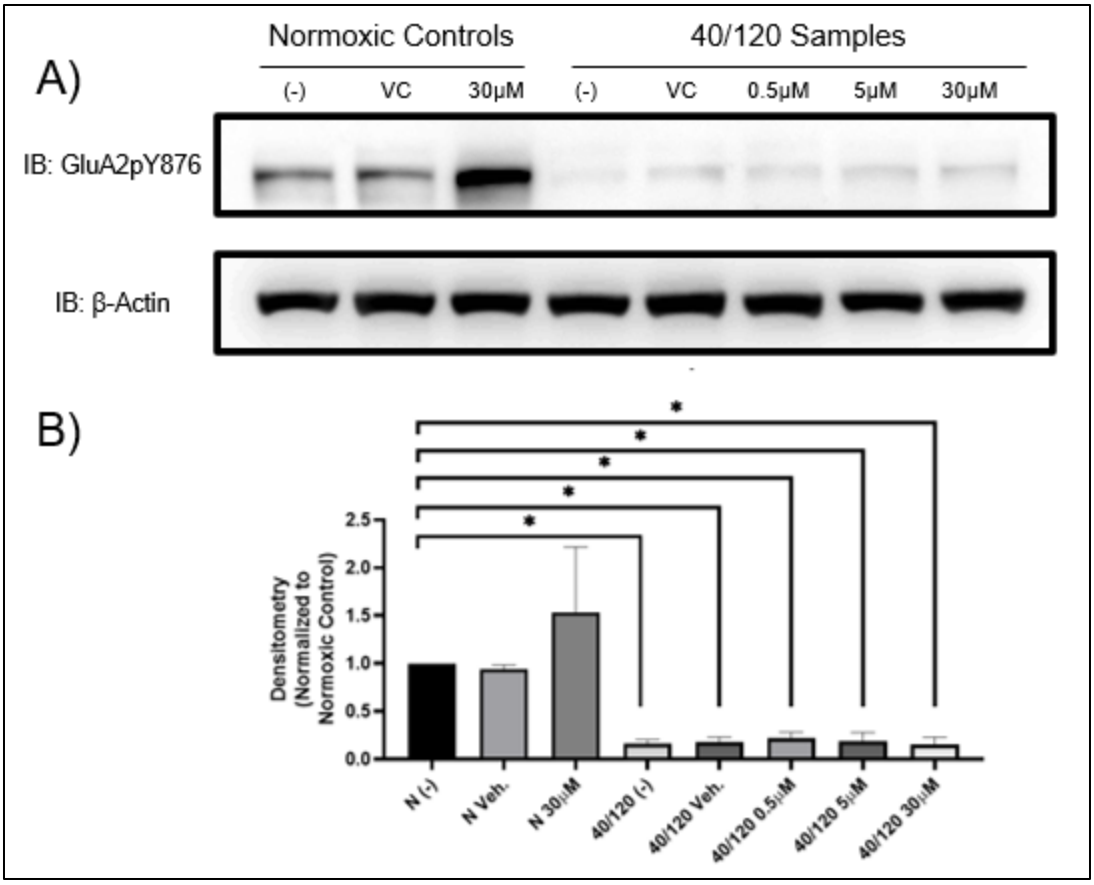


Figure 3.11: Inhibition of NOX2 does not prevent the OGD/R-induced dephosphorylation of Tyr876 residue of GluA2 AMPAR subunits. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes in the presence or absence of the NOX2 inhibitor, GSK2795039 [0.5μM; 5μM; 30μM]. (A) Representative western blot demonstrating that treatment during reperfusion with GSK2795039, a NOX2 inhibitor, does not prevent the OGD/R-induced decrease in Tyr876 phosphorylation of GluA2-containing AMPARs in male hippocampal slices. (B) Quantification of (A) of phosphorylated Tyr876 of GluA2 protein levels normalized to β-Actin and Normoxic control group (N=3). *$p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

NOX1/4 plays a role in the OGD/R-induced decrease in surface levels of GluA2 AMPAR subunits, but not GluA1 AMPAR subunits.

Since inhibition of NOX1/4 prevented the OGD/R-induced degradation of GluA1 and GluA2, we determined whether GKT137831 could also prevent the internalization of GluA1 and GluA2 with OGD/R treatment. Utilizing biotinylation to label surface proteins, we measured the amount of GluA1 and GluA2 at the cell surface following OGD/R with or without treatment of GKT137831 or GSK2795039. Treatment with GSK2795039 [5μM] during reperfusion was unable to rescue GluA1 (Figure 3.12) or GluA2 (Figure 3.13) surface protein levels following OGD/R in male hippocampal slices. Inhibition of NOX1/4 with GKT137831 prevented the OGD/R-induced loss of surface GluA2 AMPAR subunits in male hippocampal slices (Figure 3.13), but was unable to prevent the loss of GluA1 AMPAR subunits with OGD/R (Figure 3.12). These data indicate that NOX1/4 plays a critical role in the OGD/R-induced internalization and degradation of GluA2-containing AMPARs.

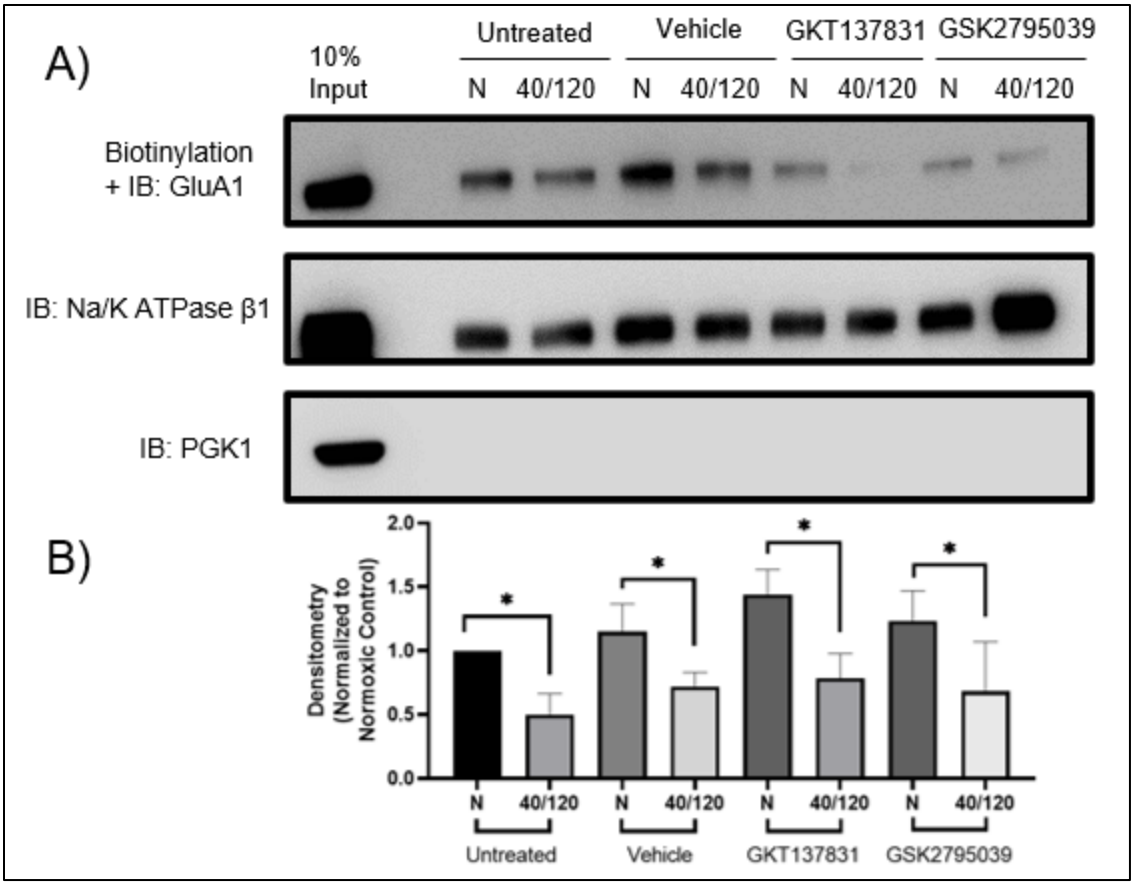


Figure 3.12: Inhibition of NADPH Oxidase isoforms 1, 2, and 4, (NOX1/4, NOX2) does not prevent the OGD/R-induced decrease in surface levels of GluA1 AMPAR subunit in male hippocampal slices. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes. Surface proteins were biotinylated and resolved by immunoblotting to examine the amount of GluA1 AMPAR protein at the membrane surface. A) Representative western blot demonstrating that surface GluA1 decreases in male hippocampal slices with OGD/R., and treatment with GKT137831 (NOX1/4 inhibitor; [500nM]) nor GSK2795039 (NOX2 inhibitor; [5μM]) does not prevent the OGD/R-induced decrease in GluA1 AMPAR subunits. (B) Quantification of (A) of surface GluA1 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=3). * $p<0.05$; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

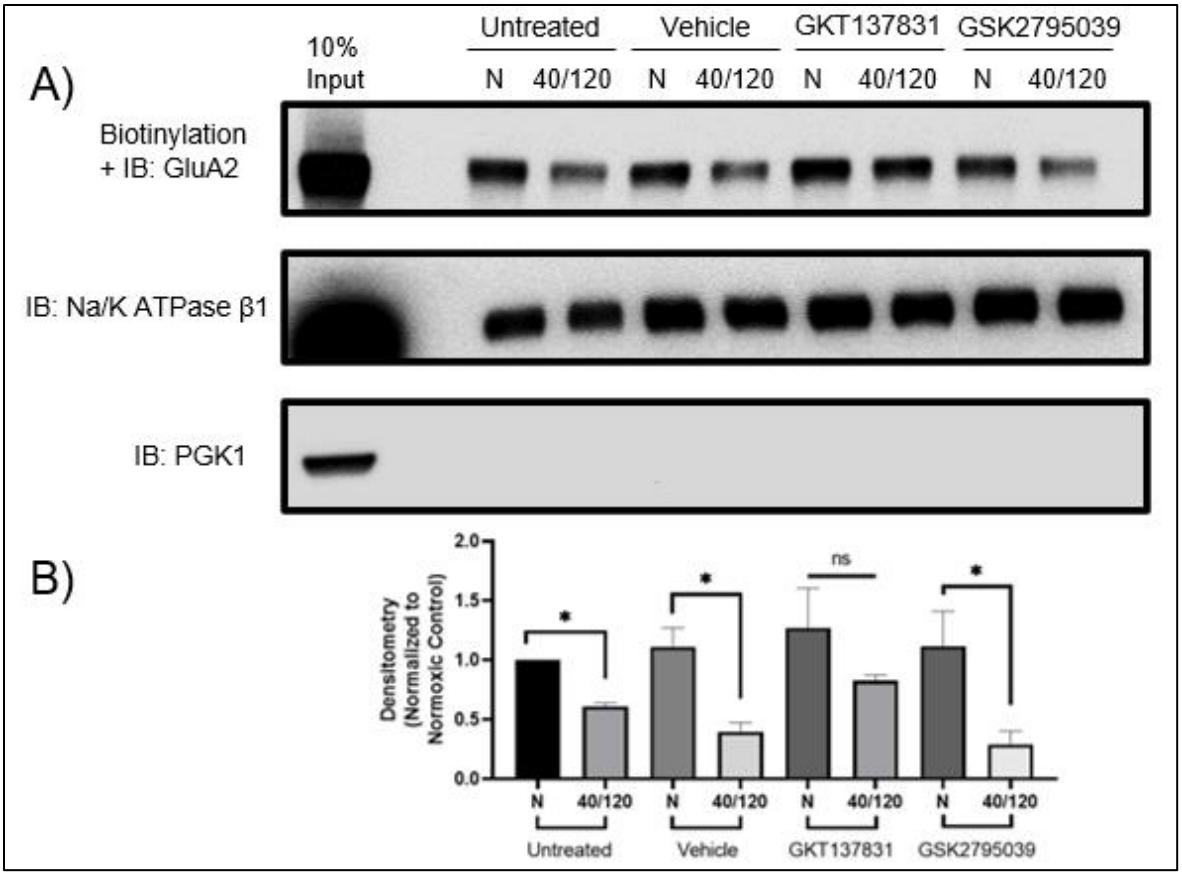


Figure 3.13: Inhibition of NADPH Oxidase 1/4 (NOX1/4), but not NADPH Oxidase 2 (NOX2), prevents the OGD/R-induced decrease in surface GluA2 AMPAR subunits in male hippocampal slices. Male hippocampal slices were exposed to 40 minutes of OGD and then reperfused for 120 minutes. Surface proteins were biotinylated and resolved by immunoblotting to examine the amount of GluA2 AMPAR protein at the membrane surface. A) Representative western blot demonstrating that surface GluA2 decreases in male hippocampal slices with OGD/R., and treatment with GKT137831 (NOX1/4 inhibitor; [500nM]), but not GSK2795039 (NOX2 inhibitor; [5μM]), prevents the OGD/R-induced surface decrease in GluA2 AMPAR subunits. (B) Quantification of (A) of surface GluA2 protein levels normalized to Na/K ATPase β1 and Normoxic control group (N=3). * $p<0.05$; ns denotes no significance; ANOVA with Sidak *post hoc* test comparing OGD/R and drug treatments corresponding to control (Normoxic). Data are expressed as *mean* ± *SEM*.

Discussion

The loss of synaptic GluA2-containing AMPARs following ischemia/reperfusion plays a critical role in delayed neuronal death in the hippocampus [Lieu *et al.* 2004]. This excitotoxic switch from GluA2-containing, calcium-impermeable AMPARs, to GluA2-lacking, calcium-permeable AMPARs disrupts neuronal calcium homeostasis. The internalization and degradation of GluA2 AMPAR subunits [Dixon *et al.* 2009] and the increase in synaptic GluA2-lacking AMPARs [Liu *et al.* 2006] contributes to the excitotoxic calcium overload following ischemia/reperfusion injury, ultimately resulting in neuronal death.

In this chapter we've identified NOX1 as the NADPH oxidase isoform responsible for the internalization and degradation of GluA2-containing AMPARs. In the previous chapter, we demonstrated that there is a sex-dependent difference in the internalization and degradation of GluA1 and GluA2 AMPAR subunits with OGD/R. Herein, we also demonstrated that there is a sex-dependent difference in the amount of ROS production following OGD/R, with males producing significantly more ROS than females. This difference in ROS production was not due to a difference in the amount of NADPH oxidase proteins or in the antioxidants, SOD1 nor SOD2. Previous studies have demonstrated that there is less oxidative damage in aged female brains compared to males due to greater levels of antioxidants in females [Guevara *et al.* 2009; Guevara *et al.* 2011]. There is also increased production of NADPH oxidase-derived superoxide in males compared to females [Miller *et al.* 2007; De Silva *et al.* 2009]. This may explain the sex-dependent differences we've observed in the amount of ROS produced with OGD/R.

Inhibition of NOX1 prevents the OGD/R-induced internalization and degradation of GluA2-containing AMPARs. NOX1 has been implicated in ischemic stroke [Jackman *et al.*

2009; Kahles *et al.* 2010; Choi *et al.* 2015]. NOX1 expressed in microglia contributes to ROS signaling even though there is also a high expression of NOX2 in microglia as well [Cheret *et al.* 2008]. We've previously shown that inhibition with the antioxidant/NOX inhibitor apocynin prevents the OGD/R-induced internalization of GluA2 [Beske *et al.* 2014], by inhibiting the phosphorylation of the Ser880 residue of GluA2 [Jackson *et al.* 2018]. The phosphorylation of GluA2 at Ser880 has been well-established to regulate the internalization of GluA2-containing AMPARs. In this study, we examined the phosphorylation state of GluA2 at Tyr876, which also plays a role in mediating AMPAR internalization [Yong *et al.* 2020] but hasn't been characterized with ischemia/reperfusion. We found that ischemia/reperfusion leads to the sustained dephosphorylation of the Tyr876 residue of GluA2 in males, but not females.

The Tyr876 residue of GluA2 is phosphorylated by Src-family protein tyrosine kinases, specifically Fyn and Src [Hayashi *et al.* 2004; Scholz *et al.* 2010; Kohda *et al.* 2013]. Some studies suggest that tyrosine dephosphorylation of GluA2 occurs with LTD or weakening of the synapses [Moult *et al.* 2006; Gladding *et al.* 2008]. Some of the mechanisms underlying LTD resemble what is occurring with ischemia/reperfusion, specifically the downregulation of GluA2-containing AMPARs, which occurs in both. It has been established recently that phosphorylation of Tyr876 of GluA2 AMPAR subunits increases GluA2 binding to GRIP1, a scaffold that stabilizes AMPARs at the synaptic membrane [Yong *et al.* 2020]. It's possible that dephosphorylation of Tyr876 with ischemia/reperfusion decreases GluA2's affinity for GRIP1, and thus destabilizing it at the plasma membrane surface. Indeed, our lab has previously reported that ischemia/reperfusion decreases the association between GluA2 and GRIP1 [Jackson *et al.* 2018]. It has been established that Src family kinases are activated with ischemia/reperfusion

[Hou *et al.* 2007; Jiang *et al.* 2008] and are redox sensitive [Li *et al.* 2008; Giannoni *et al.* 2010]. The regulation of protein tyrosine phosphatases is less clear with ischemia/reperfusion and warrants further investigation. Inhibition of NADPH oxidase did not prevent the dephosphorylation of GluA2 at Tyr876, indicating there could be a redox-independent regulation of GluA2 with regards to Tyr876 phosphorylation.

Interestingly, inhibition of NOX1 prevents the OGD/R-induced degradation, but not the internalization of GluA1 AMPAR subunits. This indicates that perhaps there is a differential endocytic sorting of GluA1 AMPAR subunits compared to GluA2 AMPAR subunits, as GluA1 AMPARs still internalize with OGD/R, but are not degraded with inhibition of NOX1. The recycling of GluA2-lacking, (GluA1 homomeric or GluA1/3 heteromeric) AMPARs to the plasma membrane is a tightly regulated process that's dependent upon phosphorylation [Ehlers 2001]. The phosphorylation of GluA1 at Ser845 by protein kinase A (PKA), promotes exocytosis of GluA1 [Man *et al.* 2007]. We've demonstrated the ischemia/reperfusion induces dephosphorylation of GluA1 AMPAR subunits, indicative of GluA1 AMPAR endocytosis. Further studies examining the effect of NOX1 on the phosphorylation state of GluA1 AMPAR subunits are needed to help elucidate the differential redox-dependent trafficking observed between GluA1 and GluA2 AMPAR subunits with ischemia/reperfusion.

This study in combination with previous data [Beske *et al.* 2014; Jackson *et al.* 2018; Achzet *et al.* 2021a] demonstrate that NADPH oxidase plays a critical role in the OGD/R-induced internalization and degradation of GluA2-containing AMPARs. These data confirm, surprisingly, that NOX1 mediates the pathological trafficking of GluA2 AMPAR subunits with ischemia/reperfusion. Inhibition of NOX2 does not prevent the OGD/R-induced internalization

or degradation of GluA1 or GluA2 AMPAR subunits, indicating the primary source of ROS production affecting AMPAR subunit trafficking is likely NOX1. Further investigation is needed to determine the upstream signaling pathways that are responsible for the NOX1-dependent decrease in GluA1 and GluA2 AMPARs with ischemia/reperfusion.

CHAPTER FIVE: FUTURE DIRECTIONS

The focus of this research was to determine how oxidative stress alters AMPAR trafficking following ischemia/reperfusion in aged male and female rats. In the first study, we utilized a human astroglioma cell line transiently expressing fluorescently tagged GluA1 and GluA2 AMPAR subunits, as well as fluorescently tagged Rab proteins. We determined that OGD/R induces internalization and degradation of GluA1 and GluA2 AMPAR subunits, by visualizing their colocalization with different Rab proteins. By inhibiting superoxide production with MnTMPyP, a superoxide dismutase mimetic, we were able to prevent the OGD/R-induced internalization and degradation of GluA1 and GluA2 AMPAR subunits. This study linked the increased production of ROS following OGD/R with AMPAR trafficking, however it was unable to identify the source of ROS. The astroglioma cell line presents a powerful tool for studying the trafficking of AMPARs following OGD/R. We did not identify the source of ROS, but utilizing genetic knockdown or specific pharmacological inhibitors in this model, we would be able to determine whether NADPH oxidase specifically plays a role in the OGD/R-induced internalization and degradation of GluA1 and GluA2 AMPAR subunits.

In the second chapter of this work, we examined the mechanisms of OGD/R-induced degradation of GluA2-containing AMPARs. We determined that AMPARs are degraded by the lysosome in a process independent of ubiquitination with ischemia/reperfusion in male hippocampus. We also provided evidence that calcium from NMDARs and L-type voltage-gated calcium channels plays a role in the degradation of GluA2 AMPAR subunits following OGD/R in males. Interestingly, we identified a sex-dependent difference in AMPAR subunit trafficking with OGD/R. In females, GluA1 and GluA2 AMPAR subunits increase with OGD/R treatment

due to an increase in protein translation. It is necessary to determine how OGD/R induces this increase in GluA1 and GluA2 in females. Is it an increase in transcription and protein translation? If it is also an increase in transcription of AMPAR subunits in females with ischemia/reperfusion, what is the promoter? It will be interesting to delve into these questions more in the future to understand the complex regulation of AMPARs in male and female animals with ischemia/reperfusion.

Ubiquitination does not play a role in the OGD/R-induced degradation of GluA2-containing AMPARs in males. This is in contrast to the mechanism underlying agonist-induced degradation of GluA2, which is dependent upon ubiquitination. We determined that GluA2 AMPAR subunits in aged male and female hippocampal slices undergo ubiquitination with agonist (glutamate) stimulation. During NMDAR-mediated chemical LTD, AMPARs are not ubiquitinated. OGD/R and LTD share many similarities, including the decrease in GluA2-containing AMPARs at the surface. The mechanism for AMPAR degradation with NMDAR-mediated LTD is unknown, but it may be possible this is a shared mechanism for the degradation of AMPARs with ischemia/reperfusion.

The final chapter in this work identified NOX1 as the ROS producer responsible for the internalization and degradation of GluA2-containing AMPARs with OGD/R. Our *ex vivo* hippocampal slices model includes multiple cell types: neurons, astrocytes, microglia, vascular endothelium, etc., and therefore it is unknown which cell type(s) NOX1 is highly expressed. It would be interesting to determine which particular cell type(s) are responsible for increased ROS production with ischemia/reperfusion.

The role of OGD/R-induced dephosphorylation of GluA2 Tyr876 in male hippocampal slices warrants further investigation. The sustained dephosphorylation of GluA2 Tyr876 residue does not appear to be redox-dependent. It would be interesting to identify whether this residue plays a role in OGD/R-induced internalization of GluA2 AMPAR subunits and how the phosphorylation status of this residue is regulated with OGD/R.

All of these studies have been conducted in either immortalized cells or acute organotypic hippocampal slices from aged male and female rats. The next step for our laboratory is to confirm our results utilizing an *in vivo* model for ischemic stroke. It is necessary to determine whether there are any sex differences in AMPAR trafficking with ischemic stroke *in vivo*, and whether oxidative stress plays a role in AMPAR-mediated delayed neuronal death in the hippocampus of aged animals.

The results presented in this work open up numerous avenues for further research directions. Investigating the endosomal sorting, protein—protein interactions, and post-translational modifications of AMPAR subunits would be of great value to understanding the precise mechanisms involved in the ischemia/reperfusion-induced degradation of GluA2-containing AMPARs. Further studies need to be conducted to understand the complex regulation of NADPH oxidase-mediated signaling with ischemia/reperfusion, ultimately in the hopes of identifying a therapeutic target for the treatment of ischemic stroke patients.

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