Title
Redox Regulation of NMDA Receptors in a Ketamine Model of Schizophrenia

Permalink
https://escholarship.org/uc/item/8z91w34t

Author
Schulman, Andrew David

Publication Date
2013

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, SAN DIEGO

Redox Regulation of NMDA Receptors in a Ketamine Model of Schizophrenia

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science
in
Biology
by
Andrew David Schulman

Committee in charge

Terrence Sejnowski, Chair
Margarita Behrens
Ethan Bier
Nicholas Spitzer

2013
The thesis of Andrew David Schulman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Chair
University of California, San Diego
2013
Table of Contents

Signature Page .................................................................iii
Table of Contents .................................................................iv
List of Figures .................................................................v
Acknowledgements .........................................................vi
Abstract ..............................................................................vii

I: Introduction ........................................................................1

II: Production of a Virus Containing a Gene for a Mutant NR2A Subunit ..........9

III: The Role of NR2A Redox Sensitivity in the maintenance of PV and GAD67 Following Ketamine Treatment ........................................17

References ...........................................................................30
List of Figures

Figure 1. Schematic of Cloning Procedure .........................................................16

Figure 2. HEK293s co-transfected with NR1 and either original GFP-NR2A plasmid or
GFP-NR2A EcoRV .................................................................................................17

Figure 3. Expression of GFP-NR2A Viruses following infection of Neuronal-Glial Co-
cultures .................................................................................................................25

Figure 4. PV Quantification in PV+ infected Neurons following treatment with ketamine
...............................................................................................................................27

Figure 5. Quantification of GAD67 in PV+ infected Neurons following treatment with
ketamine ..................................................................................................................29
Acknowledgements

I would like to express my deep appreciation to Dr. Terrence Sejnowski and Dr. Margarita Behrens for giving me the chance to working in the wet-lab of CNL. The time I have spent in the lab has not only been beneficial in terms of teaching me basic research skills, but also in exposing me to the vastness of neuroscience and helping determine my future goals. The continuing and direct support of Dr. Behrens has also been critical in my development as a scientist and her efforts will influence the quality of all my future work.

I would also like to thank Jacinta Lucero for helping to train me in a variety of different scientific techniques that have assisted greatly throughout this work. Additionally, her assistance in preparation of neuronal cell cultures was critical to the completion of the project. Finally, her friendship and kindness were critical to the maintenance of my spirits throughout this project.

I would also like to thank all the members of The Computational Neurobiology lab, for both their support and insight. In particular I would like to thank the members of the wet-lab for their support, including Antonio Pinto-Duarte, Clare Puddifoot.
The oxidative hypothesis of schizophrenia has recently emerged as leading candidate for the pathological mechanisms of the disease. At its core, the hypothesis states that alterations within redox regulatory systems in the brain produce a cascade of dysfunction that ultimately results in schizophrenia. Here I present early results indicating that redox regulation of NMDA receptors within parvalbumin-positive inhibitory (PV+) neurons may play a central role in the development of the disorder. These experiments were performed by first mutating the gene for the NR2A subunit of NMDA receptors to lack a specific redox site, then the gene was inserted into neurons, utilizing a virus, to examine the role of redox-regulation of NMDA receptors in maintaining the inhibitory phenotype of PV+ neurons. The results from this experiment show that the removal of the redox regulatory site from the subunit attenuated the effects of an NMDA receptor antagonist, ketamine, on downregulating PV and GAD67 in PV+ neurons. These results suggest a role for redox dysregulation of NMDA receptors in the ketamine model of schizophrenia.
I:

Introduction
Schizophrenia

Schizophrenia is a severe mental disorder affecting approximately 1% of the adult population, which develops progressively from prenatal periods through adolescence until the onset of psychosis in early adulthood. While the underlying pathologies of the disorder are still unclear, a substantial amount of evidence points to a combination of genetic and environmental factors contributing to the development of the disease (Lewis and Levitt, 2002; Do et al., 2009). Symptoms of schizophrenia are generally placed into three categories. The positive symptoms of the disease, including hallucinations, delusions, and paranoia, are currently the most treatable of the disorder with the use of antipsychotic drugs. However, the negative symptoms, including social withdrawal and blunted affect, and the cognitive symptoms, such as disorganized thought content and poor attention, are currently without effective treatments (Do et al., 2009; Javitt, 2010).

The Dopamine Hypothesis

While many of the earliest antipsychotic drugs were found purely by chance, it was later discovered that the efficacy of the drugs was directly related to their affinity for D2 receptors (Seeman and Lee, 1975; Creese et al., 1976). This, coupled with the observation that drugs affecting dopaminergic systems -such as amphetamines and cocaine- can induce psychosis, led to the first dopamine hypothesis of schizophrenia (Snyder, 1976). This hypothesis proposes that an excess in dopamine transmission is responsible for most symptoms of schizophrenia (Howes and Kapur, 2009). However, later evidence led to a revision of the hypothesis, which instead proposed that it is the hypofunction of
dopaminergic systems in frontal cortex that drives hyperfunction of subcortical
dopaminergic systems (Davis et al., 1991). In addition, neuroimaging studies showed
reduced blood flow in the frontal cortex that correlated with low dopamine metabolites in
CSF of schizophrenia patients (Weinberger et al., 1988). Furthermore, lesion studies
removing dopamine terminals in frontal cortex, led to increase dopaminergic
transmission in the striatum (Pycock et al., 1980). However, no specific dysfunction of
the dopaminergic system has yet been found, and research into other hypotheses of the
disease has taken a more prominent role in schizophrenia research (Davis et al., 1991;
Howes and Kapur, 2009).

The Glutamate-GABA Hypothesis

A different model of schizophrenia emerged from the observation that non-
competitive antagonists of N-methyl-D-Aspartate -receptors (NMDAR), such as
phencyclidine (PCP) and ketamine, could induce schizophrenia-like symptoms in
otherwise healthy humans, and initiate psychotic episodes in schizophrenia patients
(Jentsch and Roth, 1999; Javitt, 2010). As NMDA-Rs are glutamatergic, and earlier
findings had also found low concentrations of glutamate in the CSF of schizophrenia
patients, it was proposed that reduced glutamatergic transmission and the resulting
hypofunction of NMDARs could result in the dysfunction observed in schizophrenia
(Olney and Farber, 1995; Javitt, 2010). However, acute exposure to NMDA antagonists
was shown to enhance glutamatergic transmission in animal models (Moghaddam et al.,
1997). These results led to the theory that NMDA antagonists induce psychosis through a
disinhibition of cortical systems, through a specific action on the inhibitory
circuits(Olney et al., 1999).
Interestingly, deficits in the GABAergic inhibitory system are a highly replicated finding in post-mortem studies of brains from schizophrenia patients (Reynolds and Beasley, 2001). Such findings include; reduced levels of GABA (Perry et al., 1979), increased expression of GABA_A receptors (Benes et al., 1996) and reduced levels of mRNA for GAD67, the main GABA synthesizing enzyme (Akbarian et al., 1995; Mirnics et al., 2000). Deficits have also been shown in parvalbumin, a calcium binding protein found in a particular subclass of fast-spiking GABAergic interneurons (PV+) (Beasley and Reynolds, 1997), suggesting that these cells may have a particular role in the pathology of the disease. Further, studies have shown that NMDA-R antagonists reduce GAD67 and PV in vitro (Kinney et al., 2006) and in vivo (Behrens et al., 2007) corroborating the idea that NMDA-R antagonists induce disruptions in the inhibitory system and that hypofunction of NMDA-Rs could result in the deficits observed in schizophrenia (Olney et al., 1999).

**The Oxidative Hypothesis**

While the Glutamatergic-GABAergic hypothesis explains how circuit dysfunctions could ultimately result in symptoms observed in schizophrenia, it fails to offer a mechanism for how a combination of genetic and environment factors can lead to long-term alterations in neurotransmitter systems that result in these dysfunctions. An increasing amount of evidence is beginning to coalesce around dysfunction in the brain’s redox regulation systems as a primary candidate (Do et al., 2009). Studies have consistently shown increases in inflammatory mediators such as IL-6 that can mediate increases in reactive oxidative species (ROS) through increases in NADPH-oxidase and that such increases are correlated with psychosis (Xia et al., 2002; Zuo et al., 2007;
Behrens et al., 2008). Other research has shown reduced levels of glutathione (GSH), a major antioxidant, in post-mortem tissue samples and in the CSF of living patients (Do et al., 2009). Transport of precursors for GSH synthesis is also inhibited by oxidative conditions, suggesting that inherent dysfunctions in the system caused by genetics or early episodes of oxidative stress, could cascade when combined with oxidative stress caused by later environmental factors (Behrens and Sejnowski, 2009; Do et al., 2009). In addition, studies treating patients with N-acetyl cysteine, a precursor for GSH, has shown improvements in negative and cognitive symptoms of schizophrenia (Berk et al., 2008).

**The Ketamine Model of Schizophrenia**

As previously mentioned, the ketamine model of schizophrenia emerged from the observation that NMDA-R antagonists induced schizophrenia-like behaviors in otherwise health patients (Reviewed in (Jentsch and Roth, 1999)). Numerous studies since then have examined the behavioral effects of the drugs in mice and primates and related them to similar deficits in human schizophrenia patients in order to validate the model (Jentsch and Roth, 1999). More recent studies have shown that repetitive ketamine treatments can replicate biochemical findings of schizophrenia *in vitro* and *in vivo* such as reduction in GAD67 and PV (Kinney et al., 2006; Behrens et al., 2007). Furthermore, these ketamine-mediated losses have been shown to be dependent on an IL-6 mediated NADPH-Oxidase increase, which is prevented by the inhibitor apocynin (Behrens et al., 2007; Behrens et al., 2008). In addition, while these effects are reversible in cell cultures and in adult animals, early-life treatment can cause deficits in the number of neurons expressing PV that endure into adulthood (Behrens and Sejnowski, 2009).

**The NMDA Receptor and NR2A subunits**
NMDA receptors are a class of ionotropic glutamatergic receptor that can be selectively activated using N-methyl-d-Aspartate (NMDA). The receptor is found at excitatory synapses throughout the central nervous system, and when activated, the channel is permeable to positive cations, particularly Ca\(^{2+}\) ions. However under normal conditions a voltage dependent Mg\(^{2+}\) must be alleviated for activation, in addition to the receptor’s co-agonist requirement for glycine. The receptor is generally composed of multiple NR1 subunits and one or more NR2 type subunits and sometimes an NR3 type subunit. NR1 units are required for functional NMDA receptors to be formed and contain several modulatory sites (Perez-Otano et al., 2001), however NR2 subunits actually contain the binding site for glutamate and NMDA (Reviewed in (Scatton, 1993)). Non-competitive antagonists, such as ketamine, phencyclidine and MK-801, act by blocking the activated pore, thus preventing ion conductance (Halliwell et al., 1989).

**Redox regulation of NMDA-Rs**

NMDA-Rs are also known to be sensitive to redox conditions in a manner that varies depending on which of the NR2A-D subunits compose the receptor. NMDA-Rs with NR2A subunits have a unique redox regulation, with a rapid onset-offset potentiation under reducing conditions in addition to a relatively slower onset redox potentiation also seen in other NR2 subunits (Kohr et al., 1994). Through mutagenesis studies, it has been shown that the residues Cys87 and Cys320 of the NR2A subunit are responsible for the rapid redox potentiation of these receptors, however the mechanism of sensitivity still remains unclear (Choi et al., 2001). In addition to redox regulation, Zn\(^{2+}\) also regulates NMDA-Rs. While NMDA-Rs composed of NR1/NR2A and NR1/NR2B subunits show a similar low-affinity voltage-sensitive inhibition of the receptor in due to
Zn\(^{2+}\), receptors with NR2A subunits also experience a high-affinity voltage insensitive inhibition by Zn\(^{2+}\) (Choi and Lipton, 1999). Interestingly, mutations of the sites critical for this high affinity Zn\(^{2+}\) inhibition eliminated all components of redox potentiation in NR1/NR2A NMDA receptors, while mutations of the residues in the redox regulatory sites increased the IC\(_{50}\) of the high affinity voltage insensitive Zn\(^{2+}\) inhibition, suggesting that coordination between zinc regulation and redox regulation ultimately influences the potentiation of the receptor.

Interestingly, the PV+ neurons, which are deficient in schizophrenia, have been shown to contain increased levels of NR2A compared to pyramidal neurons, and these NR2A-containing NMDA-Rs have a specific role in the maintenance of PV and GAD67 (Kinney et al., 2006). Similarly, post-mortem studies of schizophrenic brains show cell density of neurons co-expressing mRNA for GAD67 and NR2A is reduced in larger proportions than the cell density of all neurons expressing GAD67 mRNA or neurons expressing GAD67 but not NR2A mRNA in (Woo et al., 2004). This suggests NR2A subunits have a particular role in the pathology schizophrenia possibly through relaying the effects of dysfunction in the redox regulatory system onto the inhibitory system.

**Examining Redox Regulation of NMDA-Rs**

As it appears that redox regulation of NMDA-Rs, particularly at those containing NR2A subunits, may be a major mediator of oxidative stress onto PV+ neurons, this project sets forth to examine the role of redox regulation of NMDA-R receptors in the ketamine model of schizophrenia. To do this, NR2A subunits lacking the rapid redox-potentiation site were created by mutating codons for two cysteine residues at the site into codons for alanine residues that mimic the reduced form of the protein. This mutated
gene was then used to create a viral vector that was introduced into neuronal cell cultures. Once infected, the cells were allowed to develop and express the mutant or wild-type subunits for several weeks, before being treated with ketamine to observe the effects on PV+ neurons. While this time period has been previously shown to cause decreases in both PV and GAD67 immunoreactivity (Kinney et al., 2006) in vitro, the results presented here show that removing the rapid redox potentiation site from NR2A units is sufficient to attenuate the effects of ketamine on PV and GAD67 immunoreactivity in vitro.
II:

Production of a Virus Containing a gene for a Mutant NR2A subunit
Introduction

In order to explore how redox regulation of NMDA receptors with NR2A subunits might mediate the loss of PV and GAD67 following ketamine treatment in vitro, mutagenesis was performed on a fragment of the NR2A gene derived from a GFP-NR2A plasmid (Figure 1a. A kind gift from Dr. Vicini (Luo et al., 2002)) in order to remove the rapid potentiating redox site. The process was to convert two cysteine residues, which form a disulfide bridge when oxidized, into amino acids insensitive to redox conditions. Alanine was chosen as earlier research had shown that the mutation would remove the redox site without causing other significant changes to the receptor (Choi et al., 2001). The mutation itself changed codons encoding for cysteine at residues 87 and 320 (5’-TGC-3’) in the NR2A protein into to ones encoding alanine residues (5’-GCC-3’). In addition to the mutation, the majority of the 3’UTR was removed from the NR2A gene to reduce the size of plasmid for viral packaging. Expression of the gene without the 3’UTR was tested by transfecting the plasmid into HEK293s and comparing expression levels with those produced by transfection of the wild-type carrying plasmid.

Materials and Methods

Bacterial Transformation and Culturing

DH5α competent cells were thawed on ice from storage at -80°C and mixed with 50-100ng of DNA. The cells were then rested on ice for 20min before being heat-shocked for 30-60sec at 42°C. The tubes were then returned to the ice for 2min before adding 0.5ml of LB w/o Ampicillin and incubating while shaking for 45min at 37°C. 200-500µL of culture was plated on 100 µg/ml ampicillin or carbenicillin LB plates. The plates were grown ON at 37°C. Individual colonies were then selected and used to inoculate various
volumes of LB with 100 µg/ml ampicillin, depending on the DNA preparation to be made, and incubated while shaking ON at 37°C. Cultures containing DNA for Endofree viral preparation was grown at 30°C ON.

Isolation of DNA Plasmids

While mini, midi, and maxi preparations were performed using a variety of kits, the first steps for each utilized the standard SDS-alkaline lysis procedure to isolate the plasmids from the majority of the containing proteins, along with non-plasmid nuclear material and lipids. Mini-preps to analyze colonies following mutation and cloning were performed using the Invitrogen PureLink® Quick Plasmid Miniprep Kit (#25-0789). Following their procedure this method utilized a centrifuge column to further clean the DNA following SDS-alkaline lysis. Larger preparations for cloning and mutagenesis were prepared using the Qiagen Plasmid Midi Kit (#12143), which used a gravity flow column in order to further purify the DNA. The 5 PRIME PerfectPrep EndoFree Plasmid Maxi Kit (#2300120) was used to isolate endotoxin free DNA for transfection. This method used an additional column to clear the lysate following lysis and centrifugation of the bacteria culture to help remove endotoxins free. In addition, the kit used a centrifuge column and specialized reagents to remove endotoxins as well as other contaminating proteins. DNA samples were analyzed for concentration and purity using a Nanodrop 1000.

Cloning and Ligation of GFP-NR2A Gene Construct for Mutagenesis and Virus Production

Our rat GFP-NR2A (Fig. 1a) construct was obtained as a gift from Dr. Vicini. (Luo et al., 2002). To isolate a fragment containing the sites of interest for mutagenesis,
the original plasmid was digested with HindIII and separated on a 1% agarose gel. Three fragments, a 6.5kb backbone and two 3.9kb were obtained. The lower band containing the two 3.9kb, one of them being the fragment of interest, was excised and extracted from the gel using the Zymoclean™ Gel DNA Recovery Kit (D4001). The 3.9 kb fragments were then ligated to the HindIII digested and dephosphorylated pUC18 plasmid (Roche Rapid DNA Dephos & Ligation Kit (#04898117001)). Ligation was confirmed using HindIII digestion and the fragment containing the sites of interest was selected for during mutagenesis. Site directed mutagenesis was performed as described below.

Prior to returning the mutated fragment back to the original a second HindIII site had to be removed, and the overall plasmid reduced in size for viral production. To do this the original GFP-NR2A plasmid was cut with EcoRV and religated, removing the second site and a majority of the 3’UTR. The resulting plasmid (Fig. 1b) was then cut with HindIII and ligated to the mutant NR2A fragment described above. The plasmids were tested for integration of the complete gene using EcoRI, and orientation of the product was checked using BamHI and sequencing with the T7 Primer (Fig. 1d). Finally, the gene was cloned to the pBOBi backbone for virus production. The pBOBi backbone was cut with HpaI and XhoI to create a blunt and sticky end on the vector. To extract the NR2A fragment to be subcloned into the pBOBi backbone, the GFP-NR2A EcoRV plasmid, was cut with EcoRI, filled-in with Klenow and then cut with XhoI. The band was isolated using an agarose gel extraction before being ligated to pBOBi. Plasmids were confirmed using EcoRV digest and sequencing. A schematic of the cloning scheme is shown in Fig. 1.

**Mutagenesis of NR2A Fragment**
To perform Cys → Ala mutations in residues 87 and 320 of NR2A (bases 259-261 and 958-960 respectively), we used the QuikChange II Xl Site-Directed Mutagenesis Kit (Agilent 200521). The kit works performing PCR with forward and reverse primers containing the desired mutation, resulting in the formation of a new gene with the mutation. The original DNA is then removed by digesting methylated and hemimethylated DNA using Dpn I. The mutated DNA is then transformed into bacteria before being isolated and having the mutations confirmed by sequencing (Fig. 2d). Mutations for the different sites were done sequentially. To perform the mutation of the bases encoding Cys87, the following forward and reverse primers were used respectively; 5’-GCC TCA TCA CGC ATG TGG CCG-3’ and 5’-GCG CGC CCC GGA CAT GAG GT-3’ respectively. To perform the mutation of the bases encoding Cys320, the following forward and reverse primers were used respectively; 5’-CCT GAG GCC AAG GCC AGC GC-3’ and 5’-GGC TTC TCT GCC TGC CCA TAG-3’

**Culture and Transfection of HEK293s**

HEK293 cells, stored in 1ml stocks at -80°C, were thawed in a 37°C water bath before being transferred to 9ml of pre-warmed Dulbecco’s Modification of Eagle’s Media (DMEM) with L-Glutamine, 4.5g/L Glucose and no Sodium Pyruvate. The cell suspension was then spun at 2000xg for 5min, and the resulting supernatant removed by vacuum. The remaining cell pellet was then suspended in 10ml of HEK293 media, which was split equally between two T25 flasks. The cells were then incubated at 37°C. Upon reaching confluency, the cell media was removed and 2.5ml of Trypsin-EDTA solution was added to the flask. After several minutes, 3ml of HEK293 Media was added and the cell suspension transferred to a 15ml falcon tube. The cells were then spun at 2000xg for
5 min and resuspended in 2 ml of media. The cells were then counted on a hemocytometer and more media was added to make the concentration ~5 x 10^6 cells/ml. 1 ml of the resulting media was then transferred to glass coverslips that had been previously coated with poly-L-lysine. After the cells were grown to 75-80% confluence Lipofectamine (Invitrogen #18324) was used to co-transfect the coverslips with a plasmid containing NR1 under the CMV promoter and either GFP-NR2A or GFP-NR2A EcoRV. Several coverslips were also transfected without NR1, NR1 only. 4 hrs after transfection MK-801 was added to a concentration of 10 µM to inhibit any toxic effects occurring because of expression of active NMDA receptors.

**Fixation and Confocal Imaging**

2-3 days after being transfected, the cells were washed with 2x with Phosphate Buffered Saline (PBS) before being fixed in cold 4% paraformaldehyde (PFA) in PBS for 30 min. The cells were then incubated at room temperature for 10 min in 0.25% Triton X-100 in PBS, before being left to block overnight in 10% normal goat serum w/ 0.02% Sodium Azide. The slides were then mounted and imaged for GFP fluorescence using either a Zeiss LSM 710 or 780 using a 20x objective.

**Virus Production**

Using the previously created GFP-NR2A HIV pBOBi plasmid, a 3rd generation SIN Lentivirus was produced by the Gene Transfer, Targeting and Therapeutics Core at the Salk Institute using methods previous described (Tiscornia et al., 2006).

**Results**

While the primary purpose of these experiments was to produce a virus that could be used to insert the mutant NR2A gene into primary neuron cultures, the determination
that the 3’UTR of NR2A is not required for expression is an important result. Although earlier work has shown regulation of the NR2A gene associated with microRNAs (Edbauer et al., 2010), the results of our transfections (Fig. 2) and the successful expression of the virus in neurons (Fig. 3a) shows these regions are not required for expression under the CMV promote. Further, increases in the number of HEK293 cells expressing receptors following the removal of the 3’UTR indicates inhibition of expression due to the 3’UTR, as was previously reported (Edbauer et al., 2010) may be removed, and that transfection efficiency improved with the reduction of the gene size.
Figure 1. Schematic of Cloning Procedure
(A) The Original GFP-NR2A Plasmid gifted from Dr. Vicini
(B) GFP-NR2A EcoRV plasmid, formed by removing 3’ UTR using EcoRV
(C) GFP-NR2A pBOBi plasmid used for viral production with relative position of mutation indicated
(D) Aligned Sequences of Mutations
Figure 2. HEK293s co-transfected with NR1 and either original GFP-NR2A plasmid or GFP-NR2A EcoRV
III:

The Role of NR2A redox sensitivity in the maintenance of PV and GAD67 following ketamine treatment

Introduction

In order to examine the role of the rapid redox potentiation site in the NR2A subunit on the loss of PV and GAD67 in cell cultures, neuronal-glial co-cultures were infected five days after dissection (DIV5) with a virus containing either the mutant NR2A gene lacking cysteines 87 and 320, or a wild-type NR2A gene. The primary cultures were allowed to develop for several weeks before being treated with ketamine for 24hrs, a time course previously shown sufficient to induce loss of PV and GAD67 (Kinney et al., 2006). After fixing the cultures, they were examined for either PV or GAD67 levels by confocal microscopy, which revealed that removal of the rapid redox potentiation site was sufficient to prevent the effects of ketamine treatment in vitro

Methods

Neuronal-glial Co-cultures

Fetal Swiss Webster mice on E14 or E15 were used to prepare co-cultures of cortical neurons and glial cells. Cortices were dissected from whole brains and placed in 5ml of 5+5 Media (MS with 20mM glucose, 26.2 mM NaHCO₃, 2mM glutamine, 5% fetal calf serum, and 5% HS). The tissue was triturated using a 5ml glass pipette and diluted to 0.15 cortices/ml and plated on to poly-L-lysine coated coverslips. Cultures were treated with 10µM cytosine arabinoside when glial cells had reached confluency or cells had been in culture for 9-12 days. Cells were fed using MS with 10% horse serum

Confirmation of Viral Expression and Titer

The core that prepared the virus also initially reported titer values of $1.4 \times 10^{10}$ TU/ml for the Wild-Type Virus, and $1.61 \times 10^{10}$ TU/ml Cys87, 320 ∆Ala NR2A, or Mut NR2A, using QPCR to examine pro-virus integration. To examine the expression titer,
HEK293Ts were cultured on glass coverslips as previously described in Chapter 2. The cells were then infected with 10μL of Serial Diluted Virus from 1:1-1:10,000 in order to determine the lowest volumes needed for successful expression. Cells were prepared for Confocal Imaging as described below and examined for GFP-fluorescence and NR2A expression.

**Determination of Suitable MOI**

In order to determine the necessary amount of virus needed to infect Neuronal-Glial co-cultures a set of infections were made at multiplicity of infection 10 (MOI10), 25, 50, 100, & 150 and analyzed for co-expression of GFP and NR2A (Fig. 3a). Volumes of virus were determined using the QPCR titer values and estimations of ~6 x10^5 total cells per coverslip. The infected coverslips were fixed 72hrs after infection and imaged for expression of NR2A and GFP using either a Zeiss 710 or 780 microscopes using a 20x objective. Images for several different positions on each coverslip were taken and counted for infected neurons vs. total neurons and used to determine a usable MOI (Fig. 3b).

**Ketamine Treatment**

Neuronal glial co-cultures were infected on the fifth day *in vitro* (DIV5) at MOI100. Cells were allowed to develop normally until DIV21 when they were treated with ketamine (0.5μM) or a saline solution for 24hrs and then immediately fixed (Kinney et al., 2006).

**Immunohistochemistry and Confocal Imaging**

Coverslips were fixed as described in previous chapters, and blocked in either 10% normal horse serum or 10% normal goat serum. For imaging of NR2A in HEK293s,
a Rabbit Polyclonal antibody was used in 2% normal goat serum at a dilution of 1:1000 (Sigma #M264). For observing NR2A and GFP in neuronal-glial co-cultures, the same antibody was used, with the addition of a chicken polyclonal antibody against GFP (1:250; Millipore #06-896). For experiments examining changes in PV after ketamine treatment, double staining of neuronal-glial coverslips was performed in 2% normal goat serum containing a dilution of rabbit polyclonal antibody against PV (1:2000; Swant #PV25) and mouse monoclonal antibody against GFP (Millipore #MAB3580). Similarly, triple-staining of coverslips was performed to examine the changes in GAD67 in PV+ neurons following ketamine treatment using 2% normal horse serum containing a dilution of the same anti-PV antibody, a mouse monoclonal antibody against GAD67 (1:1000; Millipore #MAB5406), and a goat monoclonal antibody against GFP (1:1000; Rockland # 600-101-215). Specific binding of antibodies was detected using Alexa Fluor dye conjugated secondary antibodies (488, green; 568, red; 647, cyan). All images were taken using either a Zeiss 710 or 780 using a 63x oil objective unless otherwise stated. All antibodies have been shown suitable for immunocytochemistry (Kain et al., 1995; Joelson and Schwartz, 1998; Phillips, 2001; Kinney et al., 2006; Cabezas et al., 2012; Taylor et al., 2012).

**Fluorescence Quantification**

Confocal settings were maintained within experimental sets such that ketamine and genotype dependent changes could be compared. Each coverslip was imaged for ~25 PV+ cells. Images were taken in 0.3µm z-slices over a depth of 1.5 µm before being taken to maximum intensity projections. Images for PV quantification were taken from the top of the cell, while GAD67 images were generally taken over the middle portion of
the cell. To analyze PV content, whole somas were traced for PV+ neurons and the median intensity value measured for the cell. To measure GAD67 content in PV+ cells, median intensity values were measured from traces of the brightest spots within cells. PV measured in these cells was not compared to cells analyzed for PV content due to difference in antibodies and image capturing techniques. All individual cell values were then normalized by mean value of all control (saline treated) cells under the same experimental set infected with the same virus. Uninfected cells were analyzed in the same manner. Cells were then expressed as means of all normalized individual cells ± SEM. Experiments for quantification of PV and GAD67 were performed independently. Experiments for PV were performed in duplicate over cells from two different dissections. ~100 cells over 4 coverslips have been analyzed for each viral condition and ~200 cells over 8 coverslips have been analyzed for uninfected cells. GAD67 experiments have been performed in duplicate over one dissection and additional virus production is required to enrich the results. ~50 cells across 2 coverslips have been analyzed for each infected condition and ~100 cells across 4 coverslips have been analyzed for uninfected cells.

Statistical Analysis

Statistical comparisons were done by comparing ketamine treated to saline controls for each individual virus using a one-way ANOVA followed by a Tukey test for pairwise comparisons. All statistical analysis was performed using SigmaStat™ 3.0

Results

The successful infection of the neuronal-glial co-cultures can be observed in figure 3a, and the graph used to determine an appropriate volume MOI for infection can be seen in figure 3c.
Results

Figure 4a shows viral infected PV+ neurons before and after treatment with ketamine. Uninfected and wild-type infected neurons showed statistically significant decreases in PV fluorescence (P<0.001, P<0.001) (Fig. 5b) with respect to control conditions after ketamine treatment, however this decrease was abolished in neurons infected with the virus carrying NR2A subunits that lack the redox sensitive site (percentages of saline control within genotype: WT<sub>Sal</sub>: 105 ± 4%, WT<sub>Ket</sub>: 79 ± 5%; MUT<sub>Sal</sub>: 97 ± 4%, MUT<sub>Ket</sub>: 101± 4%; Uninfected<sub>Sal</sub>: 101±3% Uninfected<sub>Ket</sub>: 76 ± 3 %). Similarly figure 6a shows PV+ positive neurons stained for GAD67 before and after ketamine treatment. Again, both the wild-type infected cells and uninfected cells show a statistically significant decrease in GAD67 immunofluorescence (P<0.001, P<0.001), however the decrease is not observed in neurons infected with the mutant virus (Fig. 6b) (percentages of saline control within genotype ± SEM: WT<sub>Sal</sub>: 100 ± 4%, WT<sub>Ket</sub>: 71 ± 4%; MUT<sub>Sal</sub>: 100 ± 4%, MUT<sub>Ket</sub>: 98± 4%; Uninfected<sub>Sal</sub>: 100 ± 5% Uninfected<sub>Ket</sub>: 40 ± 5 %).

Conclusions

The results of this project provide strong evidence for a role of redox regulation of NMDA-Rs containing NR2A subunits in mediating the effects of oxidative stress onto PV+ neurons. By removing the rapid redox potentiation site from the subunit and placing amino acids that mimic the reduced form of the protein, the effects of ketamine treatment were completely eliminated in vitro. As these effects are known to be dependent on increases in oxidative stress, it would appear that preventing the oxidation of NR2A subunits prevents the effects of ketamine on PV and GAD67 expression in PV+ neurons.
However, two other experiments will be needed to confirm these results. First, electrophysiology studies need to be performed to confirm that the amplitude of NMDA-R currents in wild type and mutant infected cells are similar. It is possible that mutant NR2A containing receptors show altered electrophysiological properties and this is responsible for its protective effects. Secondly, electrophysiology should confirm that the kinetics of ketamine association with NMDA-Rs are not changed by the mutation. In addition, increases in oxidative stress following ketamine treatment should be determined to compare the effects of ketamine on wild-type, mutant, and uninfected cultures.

Future studies include in vivo research to confirm that similar results are obtained in live animals. To be able to discern whether the redox modulation of NMDA-Rs in the protection of PV+ neurons is a cell-autonomous phenomenon specific expression of the mutant NR2A gene can be achieved using a mouse line expressing Cre-Recombinase in PV+ neurons. The GFP-NR2A gene is subcloned in the reverse direction into a viral plasmid between two LoxP sites. The then produced virus can infect cells, however only cells with Cre-Recombinase will be able to correct the orientation of the NR2A gene and produce receptors with mutant subunits. While examining adult animals with mutant receptors following several days of ketamine treatment would confirm the in vitro results presented here, the virus could be useful in examining the role of oxidative stress on PV+ neurons in other mouse models of the disease, such as the perinatal NMDA-R antagonists and social isolation models (Powell et al., 2012).
Figure 3. Expression of GFP-NR2A Viruses following infection of Neuronal-Glial Co-cultures.
(A) Co-cultured neurons infected with viruses, showing co-localization of NR2A with GFP indicating successful infection. GFP in green, NR2A in red.
(B) 20x images under transmitted light of both viruses infecting at an MOI of 100, GFP in green, PV in red.
(C) The scatter plot shows the percentage of GFP positive neurons out of total neurons compared to the estimated MOI used to infect.
Figure 3. continued
Figure 4. PV Quantification in PV+ infected Neurons following treatment with ketamine
(A) Images of infected and uninfected neurons following 24hr treatment with ketamine (0.5µM) or saline. Cells were analyzed by taking median values from traces of the whole soma. GFP in green, PV in red.
(B) Quantification of PV fluorescence as Normalized means ± SEM. Values within genotypes are independent of the other genotypes. A statistically significant drop in PV for the wild-type infected neurons is indicated by a ‘#’ (P<0.001 by ANOVA followed by Tukey’s Test $F_{(1,165)}= 19.577$) and a statistically significant drop in uninfected neurons is indicated by a ‘*’ (by ANOVA $F_{(1,304)}= 29.527$, P<0.001 followed by Tukey’s Test).
Figure 4. continued
Figure 5. Quantification of GAD67 in PV+ infected Neurons following treatment with ketamine

(A) Images of infected and uninfected neurons following 24hr treatment with ketamine (0.5μM) or saline. Cells were measured by tracing the brightest regions found in the GAD67 channel. GFP in green, GAD67 in blue PV in red.

(B) Quantification of GAD67 fluorescence as Normalized means ± SEM. Values within genotypes are independent of the other genotypes. A statistically significant drop in GAD67 for the wild-type infected neurons is indicated by a ‘#’ (P<0.001 by ANOVA followed by Tukey’s Test $F_{(1,91)} = 35.597$) and a statistically significant drop in uninfected neurons is indicated by a ‘*’ (P<0.001 by ANOVA followed by Tukey’s Test $F_{(1,184)} = 137.454$).
Figure 5. continued
References:

Akbarian S, Kim JJ, Potkin SG, Hagman JO, Tafazzoli A, Bunney WE, Jr., Jones EG (1995) Gene expression for glutamic acid decarboxylase is reduced without loss of neurons in prefrontal cortex of schizophrenics. Arch Gen Psychiatry 52:258-266.


Jentsch JD, Roth RH (1999) The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 20:201-225.


Weinberger DR, Berman KF, Illowsky BP (1988) Physiological dysfunction of dorsolateral prefrontal cortex in schizophrenia. III. A new cohort and evidence for a monoaminergic mechanism. Arch Gen Psychiatry 45:609-615.

