Assessing the Effects of Ketamine on the Temporal Gene Expression of N-Methyl-D-Aspartate (NMDA) Receptors in the Brains of Rats

by

Claudia Paniagua-Ugarte

June 2023

Dayna M Hayes — 6/23/2023

Dr. Dayna Hayes
Thesis Advisor/Committee Chair

Date

Thomas W. Pierce — 6/23/23

Dr. Thomas Pierce
Committee Member

Date

Matthew Eckard — 6/23/23

Dr. Matthew Eckard
Committee Member

Date

Abstract

Schizophrenia is one of the most debilitating psychiatric disorders worldwide. Symptoms of the disorder typically manifest during adolescence and are so multi-faceted that Schizophrenia is considered to be a spectrum disorder. Sex differences in symptom onset, progression, and severity have been observed, with male individuals having a higher predisposition to the disorder as well as an earlier age of onset compared to females. Currently, a definitive etiology for the disorder has not been identified. However, literature suggests that a dysregulation of glutamate signaling, with specific emphasis on N-methyl-D-aspartate receptor (NMDA) function in the brain, is linked to schizophrenia (Delisi et al., 2006; Moghaddam & Javitt, 2012; Plitman et al., 2014). Alterations in receptor function may be a result of environmental factors influencing gene expression of receptor subunits such as GRIN1 and GRIN2A during key neurodevelopment periods. To further investigate the etiology of schizophrenia and associated sex differences, the goal of the study was to assess the effects of ketamine on temporal gene expression alterations of GRIN1 and GRIN2A in the prefrontal cortex of adolescent, Long-Evans rats. Male and female rats received ketamine (30 mg/kg, i.p.) or saline injections for ten days (PND 40-49). Following administration, rats were euthanized at postnatal days 50, 55, 60, or 65 and brains were extracted to assess gene expression. The major findings were a main effect of timepoint and a 3-way interaction between treatment, sex, and timepoint for GRIN1 indicating complex alterations in gene expression following exposure. Results from this study add to a growing body of literature highlighting the intricate pathophysiology of schizophrenia and need for continued research into the etiology of the mechanism underlying the disorder.

Keywords: Schizophrenia, Ketamine, NMDA Receptors, Prefrontal Cortex, GRIN1, GRIN2A, Long Evans Rats.

Acknowledgments

I would like to express gratitude to my past and current committee members: Dayna Hayes, Pamela Jackson, Thomas Pierce, Matthew Eckard, and Robert Sheehy. Thank you for your guidance, encouragement, and participation throughout this project.

To my advisor: Dayna Hayes. I will forever be grateful for your mentorship, patience, and wisdom. Working under you has sculpted me into a better scientist.

I would also like to extend my appreciation to Katie Wheeler, Abi Naughton, and Shane McClafferty. Without your endless assistance and participation, this project could not have been completed.

To my brother and sister: Jiovany and Mary Paniagua, thank you for keeping me sane.

Para mis padres: Maria Ugarte y Antonio Paniagua, que llegaron sin nada y me lo dieron todo.

Table of Contents

Tables & Figures

Chapter 1: Introduction

Schizophrenia

Schizophrenia is one of the most debilitating psychiatric disorders, affecting approximately 1% of the human population worldwide (Vos et al., 2017). Its symptoms can be characterized into three major categories: negative symptoms (loss of function), positive symptoms (gain of function), and cognitive dysfunction. Common symptoms of schizophrenia include anhedonia, delusions/hallucinations, and disruption of executive ion (Donegan et. al., 2020; Balu, 2017). Although the symptoms of schizophrenia can be categorized, the nature of the disorder is far from simple. According to the National Institutes of Mental Health, schizophrenia is a late onset disorder meaning that symptoms do not typically present until late adolescence or early adulthood (National Institute of Mental Health, 2022). In addition, gender differences have also been observed, with men having a higher predisposition along with an earlier age of onset compared to women (Ochoa et al., 2012; Li, R. et al., 2016). Men are typically diagnosed in their early 20's and women in their late 20's to early 30's. Symptom presentation is also different between genders such that men present a higher tendency toward negative symptoms and lower social functions, while women have a higher propensity to affective symptoms (Wickens et al., 2018; Li, R. et al., 2016).

Current Treatment Options

Current treatments available to mitigate symptoms of schizophrenia include a combination of psychotherapeutic and pharmacological therapies (Kane & Correll, 2010). Psychotherapeutic approaches focus on ensuring patients continuously follow treatment recommendations, increase adaptive functions, and build group support, while pharmacological approaches focus on regulating symptoms that are present due to neurological abnormalities. Common

pharmacological treatment options involve the prescription of mood stabilizers, typical antipsychotics (e.g. haloperidol, chlorpromazine, loxapine), or atypical antipsychotics (e.g. aripiprazole, risperidone, clozapine; Kane & Correll, 2010; Patel et al., 2014). Despite there being current treatment options for schizophrenia, evidence suggests that the efficacy and tolerability of current treatment options is substandard compared to treatment options for other mental disorders. In fact, only 40% of individuals with schizophrenia can fully recover by continuously using both pharmacological and therapeutic approaches (Vita & Barlati, 2018). The need for better treatment options for this disorder is necessary; however, given the complexity of the disorder, developing new treatment options may mean having a better understanding of the pathophysiology of the disorder (Patel et al., 2014).

Dopamine Theory of Schizophrenia

Current understanding for the disorder stems from knowing the mechanisms of action for current medications. Some of the antipsychotic medications target the neurotransmitter dopamine, leading to the proposition that schizophrenia is a result of excess dopamine in the brain (Dopamine theory) (Brisch, 2014). Dopamine possesses a variety of functions and is comprised of complex, interconnected pathways. Two of the major pathways examined for schizophrenia are the mesolimbic pathway and the mesocortical pathway. The mesolimbic pathway begins in the ventral tegmental area and connects the nucleus accumbens, amygdala, hippocampus, and prefrontal cortex and is involved in executive functions and regulating motivational behavior (Alcaro et al., 2007). The mesocortical pathway is composed of dopaminergic neurons that project from the ventral tegmental area to the prefrontal cortex of the brain to assist in the regulation of cognitive function, executive function, and emotional regulation (McCutcheon et al., 2019).

Antipsychotic medications are effective at treating the positive symptoms of schizophrenia because they are dopamine antagonists, partially blocking dopamine signaling in the central nervous system (Li, P. et al., 2016). However, high doses of antipsychotic drugs can cause adverse side effects similar to those in Parkinson's Disease (involuntary muscle contraction and facial movement), which is caused by insufficient amounts of dopamine (Li, P. et al., 2016). Further, the prescription of a dopamine agonist such as L-Dopa, which is often used to treat people with Parkinson's Disease, can produce schizophrenia-like symptoms such as hallucinations and delusions in some patients, suggesting that dopamine levels in the brain must be carefully maintained within an optimal therapeutic window (Carey et al., 1995; Moskovitz et al., 1978). Despite these original, simplistic observations, it quickly became clear that the dopamine hypothesis of schizophrenia provided neither a full understanding of the causes nor treatment of negative symptoms of the disorder, and thus required further investigation. While research indicated that the positive symptoms are primarily produced by dopamine hyperactivity (too much dopamine) in the mesolimbic system (Shen et al., 2012; O'Donnell & Grace, 1998), it is now thought that the negative and cognitive symptoms are produced by dopamine hypoactivity within the mesocortical system (Shen et al., 2012).

Although the dopamine theory of schizophrenia provides much insight into the disorder, the theory alone is not sufficient in establishing a clear etiology for schizophrenia. In fact, a portion of individuals with schizophrenia have a disease process that must be mediated by something other than excess dopamine. This is further supported by the fact that 34% of individual's with the disorder have treatment resistant schizophrenia (TRS), meaning that they experience a persistence of symptoms despite two or more trials of antipsychotic medications (Potkin et al., 2020).

Glutamate Theory of Schizophrenia

The mesolimbic and mesocortical pathways are complex systems that are composed of more than just dopamine neurons. The activity of the dopamine neurons in these pathways are directly and indirectly mediated by glutamatergic neuron connections (Morales & Root, 2014). Glutamate is the most abundant and widespread excitatory neurotransmitter in the central nervous system and the role of glutamate in the human brain is fundamental for mechanisms such as neuroplasticity, exocytosis, and excitatory neurotransmission (Moghaddam & Javitt, 2012). It is especially important during key neurodevelopmental periods such as adolescence, as this period is characterized by the stabilization of synapses and fine-tuning of excitatory neurotransmitter systems to increase efficiency of neural function (Larsen & Luna, 2018). Given the various yet specific functions of glutamate in the central nervous system, it is crucial for specific levels of glutamate to exist in appropriate synapses at correct times (Plitman et al., 2014). Additionally, elevated glutamate levels can result in glutamate-mediated excitotoxicity—neural cell death caused by overexcitation due to elevated levels of glutamate (Moghaddam & Javitt, 2012). Glutamate excitotoxicity may be responsible for observed neuroanatomical difference between neurotypical individuals and individuals with schizophrenia. Structural magnetic resonance imaging studies indicate that in individuals with schizophrenia, there is 3% brain tissue loss that increases progressively across the lifespan; this reduction in brain tissue is observed mostly in the prefrontal cortex and hippocampus (Delisi et al., 2006).

Glutamate Pharmacology

The excess amount of glutamate in the brains of individuals with schizophrenia is believed to be attributed to a hypofunction of a glutamate receptor (Balu, 2016). All neurotransmitters in the brain function by binding to two classes of receptors: ionotropic and

metabotropic. In the case of glutamate, there are several ionotropic receptors, which function via calcium gated channels, that it can bind to. Three of these receptors are N-methyl-d-aspartate receptors (NMDA), α-amino-3-hydroxy-5-methylisoxaole-4-propionate receptors (AMPA), and kainite receptors. Similarly, glutamate can also bind several different metabotropic receptors (group 1 (mGLUR1 and mGLUR5), group 2 (mGluR2 and mGluR3), and group 3 (mGluR4, mGluR6, mGluR7, and mGluR8), which function through the activation of biochemical cascades that lead to the modification of proteins like ion channels (Traynelis et al., 2010). However, in the case of schizophrenia, the hypoactivity of the NMDA receptor is of particular importance.

The hypofunction of the glutamate receptor has been attributed to the hypoactivity of the NMDA receptor. This theory was derived from the observation that NMDA receptor antagonist drugs (ketamine, PCP, MK-801) induce schizophrenia-like symptoms in both animals and humans (Plitman et al., 2014). In addition, anti-NMDA receptor encephalitis, which is an autoimmune disorder that attacks NMDA receptors, can present symptoms that are similar to schizophrenia, such as psychosis and some cognitive deficits (Kayser & Dalmau, 2016).

NMDA receptors are made up of multiple subunits including GluN1, GluN2(A-D), and GluN3 (A-B). The subunits combine in heterotetrametric ways with each receptor being comprised of a combination of two GluN1 subunits with either two GluN2 (GluN2A-D), or some combination of GluN2 and GluN3 (A-B) (Yamamoto et al., 2015). Crucially, the expression of these receptor subunits varies across the lifespan. GluN1 is expressed throughout the brain and is present during the entire lifespan. In humans and rodents, GluN2A is expressed at high levels during postnatal development and continues to rise till adolescence, at which point its levels begin to plateau in the frontal cortex (Bar-Shira et.al., 2015; Balu, 2016). The expression, functionality, and affinity of NMDA receptor subunits is directed by specific genes. GluN1

subunit is encoded by GRIN1, GluN2A-D is encoded by GRIN2A-D, and GluN3A-B is encoded by GRIN3A-B. Changes to the subunit gene expression could underlie the hypofunction of NMDA receptors in the brains of individuals with schizophrenia (Hemby et al., 2002).

Neurocircuitry

The mesolimbic and mesocortical pathways are composed of glutamatergic and dopaminergic neurons leading a vastly complex circuitry. In the mesolimbic system, dopamine levels are relatively low in neurotypical individuals compared to individuals with schizophrenia. In a normal mesolimbic circuit, there is a glutamate (excitatory) neuron that projects from the cortex and fires onto an inhibitory interneuron. This inhibitory interneuron then sends signals to dopamine neurons in the ventral tegmental area (VTA) to decrease firing, which results in lower levels of dopamine in the nucleus accumbens (Howes et al., 2015; McCutcheon et al., 2020). In individuals with schizophrenia, the decreased function (hypofunction) of NMDA receptors on the inhibitory interneuron prevents the firing of the inhibitory interneuron, resulting in increased firing of dopaminergic neurons in the VTA, raising dopamine levels in the nucleus accumbens. This accounts for the dopamine hyperactivity (too much dopamine) in the mesolimbic system (Howes et al., 2015; Shen et al., 2012).

In the mesocortical system of a neurotypical individuals, levels of dopamine are relatively high compared to levels in individuals with schizophrenia (Howes et al., 2015). In the mesocortical system, glutamatergic neurons (excitatory) directly project from the cortex to dopaminergic neurons in the VTA. These dopaminergic neurons project into the prefrontal cortex. In a neurotypical brain, the direct firing of glutamatergic neurons onto dopaminergic neurons would result in increased dopamine levels in the prefrontal cortex (Howes et al., 2015; McCutcheon et al., 2019). However, a hypofunction of NDMA receptors on the dopaminergic

neurons diminishes the likelihood of them firing, in turn lowering the levels of dopamine in the prefrontal cortex. This is a potential mechanism that explains dopamine hypoactivity in the mesocortical system in individuals with schizophrenia (McCutcheon et al., 2019; O'Donnell & Grace, 1998).

Gene Expression

As mentioned previously, the expression, functionality, and affinity of NMDA receptor subunits is directed by the expression of its subunit genes: GRIN1, GRIN2A-D, and GRIN3A-B. Levels of gene expression for these subunits can be used as indicators of NMDA receptor function. An up-regulation of GRIN1 indicates that there is increased production of protein that formulates the GLUN1 receptor subunit. Whereas down-regulation of GRIN1 indicates that there is a decrease in protein production that constructs the GLUN1 receptor subunit (Mimmack et al., 2002).

A change in gene expression regulation can be measured using gene fold change as a unit of measurement that depicts a difference in gene expression quantity between various environmental conditions, such as drug treatment groups (McCarthy & Smyth, 2009). It can also be used to identify the molecular signature of a disease such as schizophrenia (Hemby et. al., 2002). In humans, it has been observed that specific DNA methylation of GRIN1 and GRIN2A are associated with the diagnoses of schizophrenia (Inoue et al., 2010). In a case study of 375 schizophrenic patients, there was a weak association between the down regulation of GRIN2A and smaller hippocampal and amygdala volume in the brains of patients with schizophrenia. The authors surmised that this down regulation could account for cognitive (working memory) and affective (anhedonia) symptoms (Inoue et al., 2010).

Sex Differences

Potential difference in gene expression alteration of GRIN1 and GRIN2A can also be used to assess the etiology of gender differences observed in individuals with schizophrenia. For example, female humans with schizophrenia have increased NMDA receptor density compared to men (Wickens et al., 2018). This may account for men having a higher predisposition and earlier age of onset compared to women (Wickens et al., 2018).

Animal Models of Schizophrenia

Due to the complex biological nature of the disorder, assessing molecular mechanisms in live subjects has been limited to neuroanatomy which does not provide sufficient information. Examination of the disorder at a molecular level has been possible only through postmortem tissue, making animal models of schizophrenia essential (Harrison, 2000) by allowing researchers to examine the etiology of schizophrenia from multiple perspectives: pharmacological, neurodevelopmental, and genetic. Creating any animal model of schizophrenia involves inducing damage or change to the prefrontal cortex and hippocampus functionality of the rodent brain (Lee & Zhou, 2019). One of the most common methods of modeling schizophrenia in a rodent is continuous administration of NMDA receptor antagonist drugs such as Phencyclidine (PCP), dizocilpine (MK-801), or Ketamine. The current study selected the ketamine model of schizophrenia because it has been known to consistently induce positive, negative, and cognitive symptoms in humans, rodents, monkeys, and zebrafish. Ketamine is a non-competitive NMDA receptor antagonist, which means that the binding of ketamine to NMDA makes it difficult for glutamate to bind effectively and results in the hypofunction of NMDA receptors (NMDA receptor hypothesis) and an excessive amount of glutamate that cannot be transmitted (glutamate hypothesis of schizophrenia) in the prefrontal cortex and

hippocampus (Becker et al., 2003). This allows for researchers to examine the pathophysiology of schizophrenia from a neurobiological approach.

Gaps in the Literature

Although there have been a plethora of studies that use animal models of schizophrenia to address the pathophysiology of the illness, more research should be conducted as a means of developing better treatment options. A potential avenue of examination is the role of gene expression alterations in prefrontal cortex. Most studies that assess gene expression alterations in the rodent brain tend to measure expression while in utero or early in development (Liu et al., 2011; Donegan et al., 2020). This is problematic because schizophrenia is a late onset disorder presenting symptoms during early to late adolescence and early adulthood. Though 13–21 years old and 21–33 years are widely considered to be adolescence and early adulthood, respectively, these timeframes correspond to approximately post-natal days (PNDs) 40–60 in rats. Regardless of species, sensitive neural development is occurring within these timeframes allowing the brain to be vulnerable to insult and injury. Thus, understanding the manifestation of the illness on a molecular level could provide more insight into the pathology of the illness (Plitman et al., 2014).

In addition, alterations of NMDAR subunits GRIN1 and GRIN2A in the prefrontal cortex of animal models of schizophrenia have been inconsistent. In a study conducted by Uttl and colleagues (2018), a 2-week treatment of dizocilpine (MK-801) was administered to male Long-Evans rats at two different age points: PND 30 or PND 60. Their study found that there were no significant changes in the level of NMDA receptor subunits in the hippocampus (Uttl et al., 2018). In addition, the study had a restricted sample size to only 10 Long-Evans rats of only one sex. However, another study conducted by Liu and colleagues (2011) found that subcutaneous

injections of 20mg/kg of ketamine during early development (PND7) showed significant alteration of GRIN1 and GRIN2A gene expression in the prefrontal cortex of Sprague-Dawley rats. Differences in findings between the two studies could be due to the species of rat, age of drug administration, type of model (MK-801 vs. Ketamine), dosage of drug, and time differences between day of last injection and day of sacrifice.

Aim of the Present Study

Given the gaps in the literature, the aim of the current study is exploratory, examining temporal gene expression of GRIN1 and GRIN2A in the brains of adolescent Long-Evans rats using a ketamine model of schizophrenia. In the current study, male and female adolescent Long-Evans rats were administered ketamine or saline via intraperitoneal injection daily between postnatal day (PND) 40–49, since this is the age range of late adolescence for Long-Evans Rats. One male and one female from each cohort was randomly assigned to be euthanized on PND 50, 55, 60, or 65 to assess the temporal effects of ketamine on gene expression alterations of GRIN1 and GRIN2A in the prefrontal cortex. In addition, due to there being sex differences present in the manifestation and symptomology of schizophrenia, male and female subjects were assessed. Given that this is an exploratory study, it was hypothesized that ketamine treated animals would show significant differences in gene expression alternation, for both GRIN1 and GRIN2A, in the prefrontal cortex across the four extraction time points. Additionally, due to their being a sex difference present in the manifestation of the disorder, it was also hypothesized that male rats treated with ketamine would have higher levels of gene expression change for both GRIN1 and GRIN2A compared to females at all timepoints.

Chapter 2: Method

All procedures proposed for this study were approved by the Institutional Animal Care and Use Committee at Radford University prior to initiating the study. Experiments were conducted within the Psychology Department's vivarium where animals were housed on a 12-hour:12-hour light: dark cycle in a temperature and humidity-controlled environment with ad libitum access to food and water.

Subjects

The rats used in this study were bred within the Behavioral and Cognitive Neuroscience vivarium. Original breeding stock was obtained from Charles River Laboratories (Wilmington, Massachusetts). For this study, 10 genetically distinct cohorts were used (N=160). Each cohort was composed of eight males (four ketamine and four saline) and eight females (four ketamine and four saline). Each cohort was completed by using two sister litters.

Ketamine Injections

Rats were injected interperitoneally (I.P.) with ketamine 30mg/kg in 0.9% saline or 0.9% saline (vehicle). The injections were administered consecutively between postnatal day 40 to postnatal day 49 with alteration of side of abdominal cavity which covers a late adolescent time frame in rats. Daily dosage was determined based on bodyweight of rat.

Brain Tissue collection and RNA isolation

On PND 50, 55, 60, and 65 whole brains were extracted via rapid decapitation and flash frozen on dry ice within five minutes. Rapid decapitation was selected to prevent gene expression alteration from the use of additional euthanasia chemicals. Out of the nine cohorts (N=144), gene expression analysis was performed on a total of 58 brains and whole brains were stored at -80° Celsius. The prefrontal cortex of the brain was dissected from the other brain tissue

prior to RNA isolation and homogenized using mortar pestle methods. Total RNA from prefrontal cortices was isolated using RNeasy Lipid Tissue Mini Kits. Total RNA yield was determined using a NanoDrop 2000/2000c spectrometer (ThermoFisher Scientic, Carlsbad California, United States). Gene expression levels of GRIN1 and GRIN2A were assessed using quantitative PCR (Guo et al., 2010).

Gene Expression Analysis

The specific materials for this study were selected based on a study conducted by Guo et al. (2010) wherein gene expression in the brains of rats was examined. Briefly, gene expression levels of GRIN1 and GRIN2A were determined via Q-PCR using TaqMan assays (Thermo Fisher scientific, Carlsbad, California, United States). The following TaqMan probes were used: Grin1 (NR1:Rn01236038_m1) and GRIN2A (NR2A:Rn01424654_m1). Beta-actin (Actb: Rn00667869_m1) wase used as an endogenous control. cDNA was prepared using qScript™ cDNA SuperMix (QuantaBio Sciences, VWR, United States). Each assay was run with TaqMan™ Fast Advanced Master Mix without AmpErase UNG (Thermo Fisher scientific, Carlsbad, California, United States). Cycling condition was determined based on master mix recommendations found in the Script™ cDNA SuperMix (QuantaBio Sciences, VWR, United States) protocols and the TaqMan™ Fast Advanced Master Mix without AmpErase UNG (Thermo Fisher scientific, Carlsbad, California United States) protocol.

Quantification

The fold change equation was used to measure the degree of gene expression change (upregulation or down regulation). The comparative CT method (ΔCT) was used to determine relative quantity of gene expression. The fold change equation ($\Delta\Delta C_T$=ΔC_T (treated)-ΔC_T (control)) was used to measure the degree of gene expression change (upregulation or down

regulation) between the final and original value. These equations have been selected as per Livak & Schmittgen (2001).

Statistical Analysis plan

For the current study, a separate 2 (sex) x 2 (treatment) x 4 (Timepoint) mixed-model ANOVA was used to assess gene expression for GRIN1 and GRIN2A in the prefrontal cortex. Post hoc analysis was conducted for GRIN1 due to there being an interaction present. Existence of main effects for GRIN1 lead to further probing by conducting a 2 (treatment) x 4 (timepoint) ANOVA for both males and females.

Chapter 3: Results

Adolescent male and female Long Evans rats (N=160) received either saline or 30 mg/kg ketamine via intraperitoneal injections daily for ten consecutive days (PND 40–49). However, due to limited quantities of TaqMan assay solutions, only 58 brain samples, chosen randomly, were processed for genetic analysis. This randomization led to variability in the number of samples for each group with values ranging 1-8. Three of the 58 samples were not able to be sequenced for either GRIN1 and GRIN2A genes due to PCR or assay error. Additionally, a few of the final calculated values were noticeably outside of the typical range for this technique, but the low sample size in certain groups made outlier determinations statistically impossible. For these expression values, the data was trimmed to 200. This value was determined by calculating mean without the high values included, then choosing a value slightly larger than one standard deviation above the mean. This trimming allowed us to maintain data within the overall analysis that may have been artificially inflated by the technique itself while preserving the fact that these high values likely represented true increases in gene expression. Out of the 55 samples that were processed successfully, a total of seven samples were redefined to values of 200. Three of the seven samples were for GRIN1 and four were for GRIN2A. A depiction of the number of samples per condition can be seen in table 1.

Body Weight

A 2 (sex) X 2 (drug) X 4 (timepoint) repeated measures ANOVA performed on body weights collected from PND 40 to PND 49 revealed a significant main effect of days [$F_{(9,360)}$ = 312.57, p = < .001, η_p^2 = .89], a significant main effect of sex [$F_{(1,40)}$ = 152.98, p = < .001, η_p^2 = .99], and a significant days x sex interaction [$F_{(9, 360)}$ = 35.99, p = < .001, η_p^2 = .47]. Please see Figure 1 for graphical depiction. This analysis was conducted as a measure of overall health in

our animals given that chronic injections can be stressful. Weight loss or plateau during adolescence can be an indication of major pain or distress that could be affecting the animals.

GRIN1 Gene Fold Change

A 2 (sex; male and female) X 2 (treatment; saline and ketamine) X 4 (timepoint; PND 50, 55, 60. and 65) ANOVA performed on GRIN1 gene expression levels failed to reveal main effects of sex or treatment but did reveal a significant main effect of timepoint [$F_{(3, 35)} = 13.31$, $p < .001$, $\eta_p^2 = .533$; See Figure 2]. Additionally, there is a significant three-way interaction between sex, treatment, and timepoint [$F_{(3, 35)} = 4.38$, $p = .01$, $\eta_p^2 = .273$]. Tukey's HSD test for multiple comparisons found that the mean value of GRIN1 gene fold change at PND 55 was significantly different from all other timepoints ($p < .001$ in all comparisons).

To further examine the three-way interaction between sex, treatment, and timepoint, separate 2 (treatment) x 4 (timepoint) ANOVAs were performed on GRIN1 gene expression levels in males and females. The results from the analysis on the data collected from males indicated that there were a significant effects of treatment [$F_{(1, 16)} = 97.057$, $p <.001$, $\eta_p^2 = .858$], timepoint [$F_{(1, 16)} = 149.126$, $p < .001$, $\eta_p^2 = .965$}, and a significant interaction between treatment and timepoint [$F_{(3, 16)} = 97.281$, $p = <.001$, $\eta_p^2 = .948$; See Figure 3A] on GRIN1 gene expression However, the analysis conducted on female data indicates that the was only a significant effect of timepoint on GRIN1 expression [$F_{(3, 19)} = 3.426$, $p = .038$, $\eta_p^2 = .351$; See Figure 3B].

Additionally, due to the main effect of timepoint in the overall analysis, a 2 (treatment) x 2 (sex) ANOVA was conducted on the PND 55 data. The results indicated a significant sex x treatment interaction at PND55 such that male saline animals had the highest gene expression while male ketamine animals expressed the lowest values. However, females did not differ in

GRIN1 expression values based on treatment group [$F_{(1, 14)} = 5.93$, $p = .035$, $\eta_p^2 = .372$; See Figure 4].

GRIN2A Gene Fold Change

A 2 (sex) x 2 (treatment) x 4 (timepoint) ANOVA was conducted to determine if there were any differences in gene fold change for GRIN2A between males and females, ketamine vs. saline; and timepoints of extraction in our animals. The analysis indicated that there were no significant main effects nor interactions among any of the variables. Graphical depictions can be found in Figure 5.

Chapter 4: Discussion

The current study assessed the effect of ketamine administration on the temporal gene expression of glutamate receptors, specifically the N-methyl-D-aspartate (NMDA) receptor subunits GRIN1 and GRIN2A, in the prefrontal cortex of rats. This experiment was designed primarily based on the glutamate theory of schizophrenia, which states that a dysregulation in the production of glutamate in the brain may account for many of the symptoms present in patients diagnosed within the spectrum of schizophrenia disorders. Imbalances in central nervous system glutamate levels are likely to lead to alterations in glutamatergic receptors, like NMDA. Specifically, receptor numbers and/or receptor composition may be impacted. These receptor alterations lead to a dysregulation in expression levels for genes that encode for NDMA receptor subunits such as GRIN1 and GRIN2A, which then leads to more disruption of glutamate neurotransmission.

To verify that ketamine injections did not adversely affect the general health of the animals, body weight data were collected and analyzed (Harris et al., 1998). In healthy rodents, significant body weight increases are expected across the adolescent growth period for both sexes, although males weigh more than females at all ages (Ghasemi et al., 2021). Consistently, the present study confirmed that body weights for both males and females did increase across the ten injection dates and males did weigh more than females at every timepoint. This confirmation of healthy body weights and expected growth can help to rule out the potential effect of stress on gene expression alterations for the genes of interest (Mikasova et al., 2017).

The primary findings of this study indicate that GRIN1 gene expression may be a crucial contributor to the pathophysiology of schizophrenia spectrum disorders. However, GRIN2A gene expression was unaffected in this study. The combination of a main effect of timepoint and

a three-way interaction between sex, treatment, and timepoint on GRIN1 gene expression is likely indicative of the complex nature of schizophrenia as a disorder. While overall there were no main effects of sex and treatment, probing the three-way interaction revealed that the patterns of GRIN1 expression was different between males and females. In males' prefrontal cortex, there was a significant simple interaction between treatment and timepoint for GRIN1 gene expression. In females, there was only a main effect of timepoint.

An abundance of literature would suggest that levels of gene expression for GRIN1 should be elevated shortly after ketamine administration, specifically at a 6-hour timepoint post-injection (Guo et al. 2010; Liu et al., 2012; Zou et al. 2009). This 6-hour timepoint is widely used due to the fact that the metabolism of ketamine in humans and rodents follows first-order kinetics with a half-life of 2.5 hours (Liu et al., 2012). Importantly, the quick change in gene expression would promote the idea that rats sacrificed on PND 50 (within approximately 24 hours of last dose) might show the most upregulation of GRIN1. However, this study found that both male and female rats displayed significantly higher levels of GRIN1 on PND 55 (6 days post treatment) compared to all other timepoints.

The fact that ketamine is still impacting gene expression 6 days post injection is supported by literature that indicates ketamine can have prolonged effects, even after plasma levels of ketamine can no longer be detected. These prolonged effects of ketamine can be attributed to the slow dissociation of ketamine from the NMDA receptor, which results in a blockade that lasts for periods of time that surpass 6 hours post treatment. This blockade reduces the functionality and activity of the NMDA receptor and leads to an up-regulation in gene expression of NDMA receptor subunits (Sleigh et al., 2014). Studies choosing to examine the effects of ketamine within six hours may also do so because of reported rapid shifts in gene

regulation. In a study conducted by Kim and colleagues (2022), assessment of gene expression 24-hours post-treatment revealed dramatic rebounding effects. If gene expression levels were high at 6 hours post-treatment, then levels of gene expression would likely begin to decrease around 12 hours and then be unaffected at 24 hours. Since brains for the current study were obtained approximately 24 hours post-treatment for animals that were sacrificed on PND 50, this may explain why there were no differences in gene expression levels for GRIN1 at this timepoint.

The prolonged effects of ketamine at a molecular level may underlie the complex interaction between sex and treatment for GRIN1 expression at PND 55. Overall, GRIN1 levels were high for both males and females for this timepoint when compared to all other timepoints, regardless of treatment. The increases seen in the saline groups were originally unexpected. However, this may indicate a massive importance of PND 55 to developmental structure and function in the prefrontal cortex. As mentioned previously, glutamate has complex and multifaceted functions in the brain, one of which being a promoter of neurogenesis during key neurodevelopmental periods such as early development and early to late adolescence (Mattson, 2008). During these key neurodevelopmental periods, such as late adolescence, influx in GRIN1 has been observed in multiple areas of the brain, potentially accounting for the significant increase in gene expression of GRIN1 at PND 55 (late adolescence) for saline treated animals (Lin et al., 2023). Although current literature does provide some insight into the potential mechanism underlying the current results, there should be future studies that examine the temporal effects of ketamine on gene expression of NMDA receptor subunits for both prolonged and hourly timepoints. This could further clarify what is potentially occurring between the timepoints used for this study.

Furthermore, a comparison of treatment effects within this PND 55 timepoint indicates different patterns of gene expression change between the sexes. Though ketamine exposure would be expected to increase GRIN1 gene expression levels (Shi et al., 2010), the data from female brains showed no difference between ketamine and saline. Surprisingly, GRIN1 expression levels were lowest for males treated with ketamine at this timepoint. One plausible explanation for this result could be that males are experiencing higher levels of neuronal cell death. In a study conducted by Mohn and colleagues (1999), breeding mice with substantially lowered levels of GIN1 were used to show a correlation of GRIN1 levels and behaviors that were like those in schizophrenia. Additionally, in a study conducted by Intson and colleagues (2019), juvenile mice that had a GRIN1 loss of function displayed a reduction of white matter volumes. Indications of persistent neurodegeneration was evident in adult mice with the same mutation. These patterns found in the literature assist in understanding the pattern found in males at PND 55.

Contrary to the pattern found in PND 55 males, GRIN1 gene expression levels in females on PND 55 did not differ between treatments. Published literature suggests that females are typically more protected from neurodegeneration stemming from insults such as drug abuse and stroke due to females having higher levels of neuroprotectants like estrogen (Brann et al., 2007). Additional studies assessing differences in risk of stroke between sexes have reported a pattern of females being less likely to have strokes prior to menopause, a time in which estrogen levels in women decrease (Hochner-Celnikier et al., 2002; Murphy et al., 2004). This neuroprotective pattern discussed in the literature could explain the reason for a lack of difference in GRIN1 subunit gene expression for females but not males.

Although findings in the literature assist in understanding the pattern observed for GRIN1 in this project, it must be noted that there was only a total of two samples analyzed for saline-treated males sacrificed on PND 55. Both samples were trimmed to 200 as described above. This low sample size and trimming is not ideal and must be interpreted with caution.

Results from this study indicate that there are no main effects nor any interactions between sex, timepoint, and treatment for GRIN2A gene expression in the prefrontal cortex. Based on prior literature, it was hypothesized that ketamine would influence gene expression levels of GRIN2A. However, this literature primarily consisted of prenatal and early developmental models of ketamine exposure (Liu et al., 2019; Donegan et al., 2020). After further assessment of the literature, it became evident that the effects of ketamine on the gene expression of GRIN2A may be age dependent and plateau prior to adolescence (Farber et al., 1995; Martucci et al., 2003; Matta et al., 2013; Uttl et al., 2018). Consistently, a study conducted by Uttl and colleagues (2018) assessed the effect of MK-801 across two age groups in rats (adolescence and adulthood) and found no significant effect of treatment on gene expression alterations for GRIN2A in either age group. It is possible that, due to the current study examining the effects of ketamine during adolescence through early adulthood, nonsignificant changes in gene expression for GRIN2A were observed.

Further, the lack of interaction found for GRIN2A may also be attributed to the mechanism of NMDA receptor activation, which involves the binding of both a glycine molecule to GRIN1 subunit and glutamate to GRIN2A subunit. Alteration in the function, affinity, or quantity of either receptor subunit will prevent the activation of the NMDA receptor. Ketamine has been shown to reduce levels of glycine which could account for alterations in GRIN1 found but not GRIN2A (Yamamoto et. al., 2015). Under ideal conditions, plasma levels of glycine

could have been measured as a secondary confirmation for the pattern observed for GRIN1 and as a means of further understanding the lack of pattern for GRIN2A. Current literature suggests that glycine may also play a role the pathophysiology of schizophrenia (Heresco-Levy et al., 1999; Javitt et al., 2001; Neeman et al., 2005). In post-mortum studies examining the brains of individuals with schizophrenia, it was found that there was a reduction in glycine plasma levels (Neeman et al., 2005; Waziri, 1988). Current clinical trials that use glycine augmentation treatments for individuals with schizophrenia have found that there is significant improvement in negative and cognitive symptoms (Heresco-Levy et al., 1999; Javitt et al., 2001). Future research could benefit from further examining the roles of glycine and its down-stream effects on other neurotransmitters such as dopamine in the pathophysiology of schizophrenia.

Due to the complexity of NMDA receptor development and pharmacokinetics of ketamine, the results of this study do not fully capture the mechanism by which symptoms of schizophrenia manifest. However, the current study added to the known literature by examining the temporal effect of ketamine on gene expression of GRIN1 and GRIN2A in the prefrontal cortex. Importantly, NMDA receptors play an integral role in regulating a variety of neurological functions across the nervous system and fluctuate across lifespan. In addition, the activation of NMDA receptors in one brain region may affect the downstream activity of NMDA receptors in other brain regions. Thus, findings of gene expression levels for GRIN1 and GRIN2A in the prefrontal cortex may be different than what would be found in the hippocampus or thalamus since both regions are implicated in neural processes that are driven by glutamate, such as neural pruning and neurogenesis, especially during adolescence (Hu et al., 2014). Therefore, under ideal conditions, analysis of GRIN1 and GRIN2A should be conducted in more than one brain region

as a means of further understanding or supporting the findings of a study exploring the mechanisms that underlie the disorder.

Additional limitations for this study included a drastically diminished sample size as compared to the initial plan due to technological constraints and financial limitations. The current study used TaqMan assays to assess gene expression as it is the current preferred technique in the field (Guo et al., 2010; O'Connor & Glynn, 2010). TaqMan is a precise assay that uses target-specific probes that produce a fluorescent signal as the DNA multiplies (Tajadini et al., 2014). However, the high costs associated with these precision assays and probes seriously limited the sample size in this study. An alternative to TaqMan assays would have been methods utilizing SYBR green. The SYBR green technique is based on free-floating fluorescent dyes that bind to all double stranded DNA in a particular sample and increase overall fluorescence when bound. However, this lack of specificity during binding in the SYBR green assay is known to yield a high rate of false positives, which then leads most researchers to complete further optimizations in order to yield quality data (Ponchel et al., 2003; Tajadini et al., 2014). Unfortunately, if optimization is necessary with SYBR green, the use of high-performance primers and advanced markers makes this technique time intensive and just as expensive as TaqMan. Ultimately, geneticists prefer to use TaqMan assays over alternative price-effective techniques such as SYBR green for higher precision and time efficiency (Ponchel et al., 2003; Tajadini et al., 2014; Gangisetty & Reddy, 2009).

Future studies, conducted with appropriate funding mechanisms in place, should use TaqMan assays for gene expression of numerous receptor subunits, in a multitude of brain regions. However, an alternative approach would be to utilize microarray analysis of genetic changes which can assess all NMDA receptor subunits, synaptic modulators (Nptx2), early

growth response genes (Egr or Fos), and brain-derived neurotrophic factors in the same assay. Analysis of these could assist in deciphering if gene expression alterations to GRIN1 and GRIN2A after ketamine administration are a compensatory response or indication of neuro-apoptosis. In a study conducted by Kim and colleagues (2022), ketamine infusions followed by a 24-hour recovery period in adolescent rats led to an upregulation of brain-derived neurotropic factor 1 (BDNF) and multiple transcription factors, all of which play a vital role in promoting neurogenesis and preventing neural apoptosis (Kim et al., 2022; Liu et al., 2012; Liu et al., 2019; Yon et al., 2005; Zou et al., 2009).

To support involvement of these additional factors in gene expression, including an immunohistochemistry analysis cell quantification for these markers in the brain regions of interest would be ideal. In studies examining cellular and neuroanatomical differences in postmortem brain samples of individuals with schizophrenia, it was found that there was a reduction in somal volume and dendritic spine density in the hippocampus (Hu et al., 2014; Rajkowska et al., 1998). Additional methods for cell quantification can be used, such as terminal duTP nick-end labeling (TUNEL) assay. In a study conducted by Shi and colleagues (2010), there was evidence of ketamine-induced neurodegeneration in the frontal cortex. More advanced techniques, such as a fluorescent double label, can be utilized in future studies to identify if GRIN1 or GRIN2A changes are co-occurring in cells that are undergoing cell death by co-labeling for Fluoro-Jade B (FJB) (Liput et al., 2013). Cell quantification paired with all other suggestions presented could further assist in understanding the mechanism underlying the disorder of schizophrenia.

The examination of other glutamate receptor functions or quantity in the brain could be an additional avenue for future research. In addition to NMDA receptors being studied for their

role in the pathophysiology of schizophrenia, α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors have also been identified as presenting abnormal functions and quantities in the brain of individuals with schizophrenia. Studies examining the gene expression of both kainate and AMPA receptors have found that there are also similar trends in varying gene expression profiles for the receptor subunits across brain regions. In a study conducted by Meador-Woodruff (2001), analyses of gene expression for kainate receptor subunits in postmortem brains of individuals with schizophrenia indicated that there was low expression of gluR5, gluR6, and KA2. Similarly, studies examining AMPA receptor abnormalities in schizophrenia have also found a decrease in gene expression of receptor subunits in post-mortem brain samples (Yonezawa et al., 2022). Future research could focus on examining the dysregulation of AMPA, Kainate, and NMDA receptors and the role they play in the pathophysiology of schizophrenia.

Conclusion

Schizophrenia is one of the most debilitating psychiatric ailments worldwide with symptoms arising during early adolescence. The pattern and progression of the disorder throughout the lifespan is different between males and females, and it may be partially explained by the complicated interaction with glutamate receptor gene expression found in this study. While prior literature examining this has focused on gene expression alterations of NMDA receptor subunits GRIN1 and GRIN2A due to a theoretical hypofunction of NMDA receptors in schizophrenia, the overall goal of this study was to examine the temporal effects of ketamine exposure on gene expression alterations of GRIN1 and GRIN2A during late adolescence, as this is the developmental timeframe in which symptoms of schizophrenia spectrum disorder appear. The results of the study provide additional information to the current literature but highlight that the simplistic nature of a hypofunction theory may not capture the true complexity of the disorder. Understanding the effects of ketamine on NMDA receptor subunit gene expression across multiple brain regions and multiple pathways could cultivate a better understanding for not just the pathophysiology of schizophrenia, but also contribute to the pharmacogenomics of ketamine.

References

Alcaro, A., Huber, R., & Panksepp, J. (2007). Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. *Brain research reviews*, *56*(2), 283–321. https://doi.org/10.1016/j.brainresrev.2007.07.014

Balu D. T. (2016). The NMDA Receptor and Schizophrenia: From Pathophysiology to Treatment. *Advances in pharmacology (San Diego, Calif.)*, *76*, 351–382. https://doi.org/10.1016/bs.apha.2016.01.006

Bar-Shira, O., Maor, R., & Chechik, G. (2015). Gene Expression Switching of Receptor Subunits in Human Brain Development. *PLoS computational biology*, *11*(12), e1004559. https://doi.org/10.1371/journal.pcbi.1004559

Becker, A., Peters, B., Schroeder, H., Mann, T., Huether, G., & Grecksch, G. (2003). Ketamine-induced changes in rat behaviour: A possible animal model of schizophrenia. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *27*(4), 687–700. https://doi.org/10.1016/s0278-5846(03)00080-0

Brann, D. W., Dhandapani, K., Wakade, C., Mahesh, V. B., & Khan, M. M. (2007). Neurotrophic and neuroprotective actions of estrogen: Basic mechanisms and clinical implications. *Steroids*, *72*(5), 381–405. https://doi.org/10.1016/j.steroids.2007.02.003

Brisch, R., Saniotis, A., Wolf, R., Bielau, H., Bernstein, H. G., Steiner, J., Bogerts, B., Braun, K., Jankowski, Z., Kumaratilake, J., Henneberg, M., & Gos, T. (2014). The role of dopamine in schizophrenia from a neurobiological and evolutionary perspective: old fashioned, but still in vogue. *Frontiers in psychiatry*, *5*, 47. https://doi.org/10.3389/fpsyt.2014.00047

Carey, R. J., Pinheiro-Carrera, M., Dai, H., Tomaz, C., & Huston, J. P. (1995). L-DOPA and psychosis: evidence for L-DOPA-induced increases in prefrontal cortex dopamine and in

serum corticosterone. *Biological psychiatry*, *38*(10), 669–676. https://doi.org/10.1016/0006-3223(94)00378-5

DeLisi, L. E., Szulc, K. U., Bertisch, H. C., Majcher, M., & Brown, K. (2006). Understanding structural brain changes in schizophrenia. *Dialogues in clinical neuroscience*, *8*(1), 71–78. https://doi.org/10.31887/DCNS.2006.8.1/ldelisi

Donegan, J. J., Boley, A. M., Glenn, J. P., Carless, M. A., & Lodge, D. J. (2020). Developmental alterations in the transcriptome of three distinct rodent models of schizophrenia. *PLOS ONE*, *15*(6). https://doi.org/10.1371/journal.pone.0232200

Farber, N. B., Wozniak, D. F., Price, M. T., Labruyere, J., Huss, J., St. Peter, H., & Olney, J. W. (1995). Age-specific neurotoxicity in the rat associated with NMDA receptor blockade: Potential relevance to schizophrenia? *Biological Psychiatry*, *38*(12), 788–796. https://doi.org/10.1016/0006-3223(95)00046-1

Gangisetty, O., & Reddy, D. S. (2009). The optimization of Taqman real-time RT-PCR assay for transcriptional profiling of GABA-A receptor subunit plasticity. *Journal of Neuroscience Methods*, *181*(1), 58–66. https://doi.org/10.1016/j.jneumeth.2009.04.016

Ghasemi, A., Jeddi, S., & Kashfi, K. (2021). The Laboratory Rat: Age and Body Weight Matter. *EXCLI Journal*, *20*, 1431–1445. https://doi.org/10.17179/excli2021-4072

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010, August). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, *466*(7308), 835–840. https://doi.org/10.1038/nature09267

Harris, R., Zhou, J., Youngblood, B., Rybkin, I., Smagin, G., & Ryan, D. (1998). Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets.

American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, *275*(6). https://doi.org/10.1152/ajpregu.1998.275.6.r1928

Harrison P. J. (2000). Postmortem studies in schizophrenia. *Dialogues in clinical neuroscience*, *2*(4), 349–357. https://doi.org/10.31887/DCNS.2000.2.4/pharrison

Hemby, S. E., Ginsberg, S. D., Brunk, B., Arnold, S. E., Trojanowski, J. Q., & Eberwine, J. H. (2002). Gene expression profile for schizophrenia. *Archives of General Psychiatry*, *59*(7), 631. https://doi.org/10.1001/archpsyc.59.7.631

Heresco-Levy, U., Javitt, D. C., Ermilov, M., Mordel, C., Silipo, G., & Lichtenstein, M. (1999). Efficacy of high-dose glycine in the treatment of enduring negative symptoms of schizophrenia. *Archives of General Psychiatry*, *56*(1), 29. https://doi.org/10.1001/archpsyc.56.1.29

Hochner-Celnikier, D., Manor, O., Gotzman, O., Lotan, H., & Chajek-Shaul, T. (2002). Gender gap in coronary artery disease: Comparison of the extent, severity and risk factors in men and women aged 45–65 years. *Cardiology*, *97*(1), 18–23. https://doi.org/10.1159/000047414

Howes, O., McCutcheon, R., & Stone, J. (2015). Glutamate and dopamine in schizophrenia: an update for the 21st century. *Journal of psychopharmacology (Oxford, England)*, *29*(2), 97–115. https://doi.org/10.1177/0269881114563634

Hu, W., MacDonald, M. L., Elswick, D. E., & Sweet, R. A. (2014). The glutamate hypothesis of schizophrenia: Evidence from Human Brain Tissue Studies. *Annals of the New York Academy of Sciences*, *1338*(1), 38–57. https://doi.org/10.1111/nyas.12547

Inoue, H., Yamasue, H., Tochigi, M., Suga, M., Iwayama, Y., Abe, O., Yamada, H., Rogers, M. A., Aoki, S., Kato, T., Sasaki, T., Yoshikawa, T., & Kasai, K. (2010). Functional (GT)N

polymorphisms in promoter region ofn-methyl-D-aspartate receptor 2A subunit (GRIN2A) gene affect hippocampal and amygdala volumes. Genes, Brain and Behavior, 9(3), 269–275. https://doi.org/10.1111/j.1601-183x.2009.00557.

Intson, K., van Eede, M. C., Islam, R., Milenkovic, M., Yan, Y., Salahpour, A., Henkelman, R. M., & Ramsey, A. J. (2019). Progressive neuroanatomical changes caused by Grin1 loss-of-function mutation. *Neurobiology of Disease*, *132*, 104527. https://doi.org/10.1016/j.nbd.2019.104527

Javitt, D. C., Silipo, G., Cienfuegos, A., Shelley, A.-M., Bark, N., Park, M., Lindenmayer, J.-P., Suckow, R., & Zukin, S. R. (2001). Adjunctive high-dose glycine in the treatment of schizophrenia. *The International Journal of Neuropsychopharmacology*, *4*(04). https://doi.org/10.1017/s146114570100259

Kane, J. M., & Correll, C. U. (2010). Pharmacologic treatment of schizophrenia. *Dialogues in Clinical Neuroscience*, *12*(3), 345–357. https://doi.org/10.31887/dcns.2010.12.3/jkane

Kayser, M. S., & Dalmau, J. (2016). Anti-NMDA receptor encephalitis, autoimmunity, and psychosis. *Schizophrenia research*, *176*(1), 36–40. https://doi.org/10.1016/j.schres.2014.10.007

Kim, J. J., Sapio, M. R., Vazquez, F. A., Maric, D., Loydpierson, A. J., Ma, W., Zarate, C. A., Iadarola, M. J., & Mannes, A. J. (2022). Transcriptional activation, deactivation and rebound patterns in cortex, hippocampus and amygdala in response to ketamine infusion in rats. *Frontiers in Molecular Neuroscience*, *15*. https://doi.org/10.3389/fnmol.2022.892345

Larsen, B., & Luna, B. (2018). Adolescence as a neurobiological critical period for the development of higher-order cognition. *Neuroscience and biobehavioral reviews*, *94*, 179–195. https://doi.org/10.1016/j.neubiorev.2018.09.005

Lee, G., & Zhou, Y. (2019). NMDAR hypofunction animal models of schizophrenia. *Frontiers in Molecular Neuroscience*, *12*. https://doi.org/10.3389/fnmol.2019.00185

Li, P., Snyder, G. L., & Vanover, K. E. (2016). Dopamine Targeting Drugs for the Treatment of Schizophrenia: Past, Present and Future. *Current topics in medicinal chemistry*, *16*(29), 3385–3403. https://doi.org/10.2174/1568026616666160608084834

Li, R., Ma, X., Wang, G., Yang, J., & Wang, C. (2016). Why sex differences in schizophrenia?. *Journal of translational neuroscience*, *1*(1), 37–42.

Lin, W., Shiomoto, S., Yamada, S., Watanabe, H., Kawashima, Y., Eguchi, Y., Muramatsu, K., & Sekino, Y. (2023). Dendritic spine formation and synapse maturation in transcription factor-induced human IPSC-derived neurons. *IScience*, *26*(4), 106285. https://doi.org/10.1016/j.isci.2023.106285

Liput, D. J., Hammell, D. C., Stinchcomb, A. L., & Nixon, K. (2013). Transdermal delivery of cannabidiol attenuates binge alcohol-induced neurodegeneration in a rodent model of an alcohol use disorder. *Pharmacology Biochemistry and Behavior*, *111*, 120–127. https://doi.org/10.1016/j.pbb.2013.08.013

Liu, F., G. Paule, M., Ali, S., & Wang, C. (2011). Ketamine-induced neurotoxicity and changes in gene expression in the developing rat brain. Current Neuropharmacology, 9(1), 256–261. https://doi.org/10.2174/157015911795017155

Liu, F., Patterson, T. A., Sadovova, N., Zhang, X., Liu, S., Zou, X., Hanig, J. P., Paule, M. G., Slikker, W., & Wang, C. (2012). Ketamine-induced neuronal damage and altered n-

methyl-D-aspartate receptor function in rat primary forebrain culture. *Toxicological Sciences*, *131*(2), 548–557. https://doi.org/10.1093/toxsci/kfs296

Liu, Y.-ping, Ding, M., Zhang, X.-cen, Liu, Y., Xuan, J.-feng, Xing, J.-xin, Xia, X., Yao, J., & Wang, B.-jie. (2019). Association between polymorphisms in the grin1 gene 5′ regulatory region and schizophrenia in a Northern Han Chinese population and haplotype effects on protein expression in vitro. *BMC Medical Genetics*, *20*(1). https://doi.org/10.1186/s12881-019-0757-3

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Martucci, L., Wong, A. H. C., Trakalo, J., Cate-Carter, T., Wong, G. W. H., Macciardi, F. M., & Kennedy, J. L. (2003). N-methyl-D-aspartate receptor NR1 subunit gene (GRIN1) in schizophrenia: TDT and case-control analyses. *American Journal of Medical Genetics*, *119B*(1), 24–27. https://doi.org/10.1002/ajmg.b.20014

Matta, J. A., Pelkey, K. A., Craig, M. T., Chittajallu, R., Jeffries, B. W., & McBain, C. J. (2013). Developmental origin dictates interneuron AMPA and NMDA receptor subunit composition and plasticity. *Nature Neuroscience*, *16*(8), 1032–1041. https://doi.org/10.1038/nn.3459

Mattson, M. P. (2008). Glutamate and neurotrophic factors in neuronal plasticity and disease. *Annals of the New York Academy of Sciences*, *1144*(1), 97–112. https://doi.org/10.1196/annals.1418.005

McCarthy, D. J., & Smyth, G. K. (2009). Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics (Oxford, England)*, *25*(6), 765–771. https://doi.org/10.1093/bioinformatics/btp053

McCutcheon, R. A., Abi-Dargham, A., & Howes, O. D. (2019). Schizophrenia, Dopamine and the Striatum: From Biology to Symptoms. *Trends in neurosciences*, *42*(3), 205–220. https://doi.org/10.1016/j.tins.2018.12.004

McCutcheon, R. A., Krystal, J. H., & Howes, O. D. (2020). Dopamine and glutamate in schizophrenia: biology, symptoms and treatment. *World psychiatry : official journal of the World Psychiatric Association (WPA)*, *19*(1), 15–33. https://doi.org/10.1002/wps.20693

Meador-Woodruff, J. (2001). Abnormal kainate receptor expression in prefrontal cortex in schizophrenia. *Neuropsychopharmacology*, *24*(5), 545–552. https://doi.org/10.1016/s0893-133x(00)00189-5

Mikasova, L., Xiong, H., Kerkhofs, A., Bouchet, D., Krugers, H. J., & Groc, L. (2017). Stress hormone rapidly tunes synaptic NMDA receptor through membrane dynamics and mineralocorticoid signalling. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-08695-3

Mimmack, M. L., Ryan, M., Baba, H., Navarro-Ruiz, J., Iritani, S., Faull, R. L., McKenna, P. J., Jones, P. B., Arai, H., Starkey, M., Emson, P. C., & Bahn, S. (2002). Gene expression analysis in schizophrenia: reproducible up-regulation of several members of the apolipoprotein L family located in a high-susceptibility locus for schizophrenia on chromosome 22. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(7), 4680–4685. https://doi.org/10.1073/pnas.032069099

Moghaddam, B., Javitt, D. From Revolution to Evolution: The Glutamate Hypothesis of Schizophrenia and its Implication for Treatment. *Neuropsychopharmacol* **37**, 4–15 (2012). https://doi.org/10.1038/npp.2011.181

Mohn, A. R., Gainetdinov, R. R., Caron, M. G., & Koller, B. H. (1999). Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell*, *98*(4), 427–436. https://doi.org/10.1016/s0092-8674(00)81972-8

Morales, M., & Root, D. H. (2014). Glutamate neurons within the midbrain dopamine regions. *Neuroscience*, *282*, 60–68. https://doi.org/10.1016/j.neuroscience.2014.05.032

Moskovitz, C., Moses, H., 3rd, & Klawans, H. L. (1978). Levodopa-induced psychosis: a kindling phenomenon. *The American journal of psychiatry*, *135*(6), 669–675. https://doi.org/10.1176/ajp.135.6.669

Murphy, S. J., McCullough, L. D., & Smith, J. M. (2004). Stroke in the female: Role of biological sex and Estrogen. *ILAR Journal*, *45*(2), 147–159. https://doi.org/10.1093/ilar.45.2.147

National Institute of Mental Heath. (2022). Schizophrenia (NIMH Identifier No. OMB 0925-0648). U.S. Department of Health and Human Services, National Institutes of Health. Retrieved September 2022, from https://www.nimh.nih.gov/health/statistics/schizophrenia#part_2543

Neeman, G., Blanaru, M., Bloch, B., Kremer, I., Ermilov, M., Javitt, D. C., & Heresco-Levy, U. (2005). Relation of plasma glycine, serine, and homocysteine levels to schizophrenia symptoms and medication type. *American Journal of Psychiatry*, *162*(9), 1738–1740. https://doi.org/10.1176/appi.ajp.162.9.1738

Ochoa, S., Usall, J., Cobo, J., Labad, X., & Kulkarni, J. (2012). Gender differences in schizophrenia and first-episode psychosis: A comprehensive literature review. *Schizophrenia Research and Treatment*, *2012*, 1–9. https://doi.org/10.1155/2012/916198

O'Connor, L., & Glynn, B. (2010). Recent advances in the development of Nucleic Acid Diagnostics. *Expert Review of Medical Devices*, *7*(4), 529–539. https://doi.org/10.1586/erd.10.22

O'Donnell, P., & Grace, A. A. (1998). Dysfunctions in multiple interrelated systems as the neurobiological bases of schizophrenic symptom clusters. *Schizophrenia Bulletin*, *24*(2), 267–283. https://doi.org/10.1093/oxfordjournals.schbul.a033325

Patel, K. R., Cherian, J., Gohil, K., & Atkinson, D. (2014). Schizophrenia: overview and treatment options. *P & T : a peer-reviewed journal for formulary management*, *39*(9), 638–645.

Plitman E, Nakajima S, de la Fuente-Sandoval C, Gerretsen P, Chakravarty MM, Kobylianskii J *et al* (2014). Glutamate-mediated excitotoxicity in schizophrenia: a review. *Eur Neuropsychopharmacol* **24**: 1591–1605.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J., Robinson, P. A., Inglehearn, C. F., Isaacs, J. D., & Markham, A. F. (2003). Real-time PCR based on SYBR-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnology*, *3*(1), 18. https://doi.org/10.1186/1472-6750-3-18

Potkin, S. G., Kane, J. M., Correll, C. U., Lindenmayer, J.-P., Agid, O., Marder, S. R., Olfson, M., & Howes, O. D. (2020). The neurobiology of treatment-resistant schizophrenia: Paths

to antipsychotic resistance and a roadmap for future research. *Npj Schizophrenia*, *6*(1). https://doi.org/10.1038/s41537-019-0090-z

Rajkowska, G., Selemon, L. D., & Goldman-Rakic, P. S. (1998). Neuronal and glial somal size in the prefrontal cortex. *Archives of General Psychiatry*, *55*(3), 215. https://doi.org/10.1001/archpsyc.55.3.215

Shen, L. H., Liao, M. H., & Tseng, Y. C. (2012). Recent advances in imaging of dopaminergic neurons for evaluation of neuropsychiatric disorders. *Journal of biomedicine & biotechnology*, *2012*, 259349. https://doi.org/10.1155/2012/259349

Sleigh, J., Harvey, M., Voss, L., & Denny, B. (2014). Ketamine – more mechanisms of action than just NMDA blockade. *Trends in Anaesthesia and Critical Care*, *4*(2-3), 76–81. https://doi.org/10.1016/j.tacc.2014.03.002

Tajadini, M., Panjehpour, M., & Javanmard, S. H. (2014). Comparison of SYBR green and Taqman Methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Advanced Biomedical Research*, *3*(1), 85. https://doi.org/10.4103/2277-9175.127998

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., & Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological reviews*, *62*(3), 405–496. https://doi.org/10.1124/pr.109.002451

Uttl, L., Petrasek, T., Sengul, H., Svojanovska, M., Lobellova, V., Vales, K., Radostova, D., Tsenov, G., Kubova, H., Mikulecka, A., Svoboda, J., & Stuchlik, A. (2018). Chronic MK-801 application in adolescence and early adulthood: A spatial working memory

deficit in adult long-evans rats but no changes in the hippocampal NMDA receptor subunits. *Frontiers in Pharmacology*, *9*. https://doi.org/10.3389/fphar.2018.00042

Vita, A., & Barlati, S. (2018, May). Recovery from schizophrenia. *Current Opinion in Psychiatry*, *31*(3), 246–255. https://doi.org/10.1097/yco.0000000000000407

Vos, T., Abajobir, A. A., Abate, K. H., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abdulkader, R. S., Abdulle, A. M., Abebo, T. A., Abera, S. F., Aboyans, V., Abu-Raddad, L. J., Ackerman, I. N., Adamu, A. A., Adetokunboh, O., Afarideh, M., Afshin, A., Agarwal, S. K., Aggarwal, R., . . . Murray, C. J. L. (2017). Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, *390*(10100), 1211–1259. https://doi.org/10.1016/s0140-6736(17)32154-2

Waziri, R. (1988). Glycine therapy of schizophrenia. *Biological Psychiatry*, *23*(2), 210–211. https://doi.org/10.1016/0006-3223(88)90093-5

Wickens, M. M., Bangasser, D. A., & Briand, L. A. (2018). Sex Differences in Psychiatric Disease: A Focus on the Glutamate System. *Frontiers in molecular neuroscience*, *11*, 197. https://doi.org/10.3389/fnmol.2018.00197

Yamamoto, H., Hagino, Y., Kasai, S., & Ikeda, K. (2015). Specific Roles of NMDA Receptor Subunits in Mental Disorders. *Current molecular medicine*, *15*(3), 193–205. https://doi.org/10.2174/1566524015666150330142807

Yon, J.-H., Daniel-Johnson, J., Carter, L. B., & Jevtovic-Todorovic, V. (2005). Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience*, *135*(3), 815–827. https://doi.org/10.1016/j.neuroscience.2005.03.064

Yonezawa, K., Tani, H., Nakajima, S., Nagai, N., Koizumi, T., Miyazaki, T., Mimura, M., Takahashi, T., & Uchida, H. (2022). AMPA receptors in schizophrenia: A systematic review of postmortem studies on receptor subunit expression and binding. *Schizophrenia Research*, *243*, 98–109. https://doi.org/10.1016/j.schres.2022.02.033

Zou, X., Patterson, T. A., Sadovova, N., Twaddle, N. C., Doerge, D. R., Zhang, X., Fu, X., Hanig, J. P., Paule, M. G., Slikker, W., & Wang, C. (2009). Potential neurotoxicity of ketamine in the developing Rat Brain. *Toxicological Sciences*, *108*(1), 149–158. https://doi.org/10.1093/toxsci/kfn270

Table 1

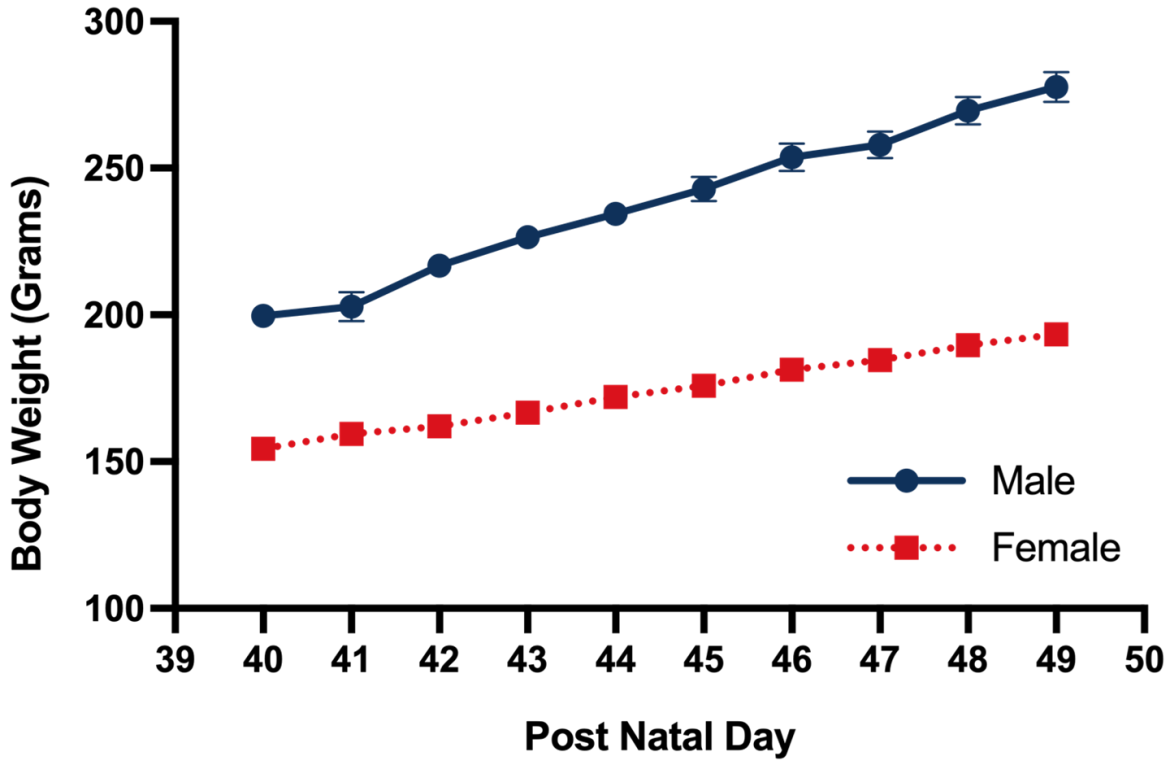
Number of Samples Per Condition

Treatment	Timepoint	Males	Females
Saline	50	2	2
	55	2	4
	60	5	2
	65	2	4
Ketamine	50	4	2
	55	4	4
	60	1	6
	65	4	3

Note. The table above depicts the distribution of the 56 samples run for genetic analysis per condition.

Figure 1

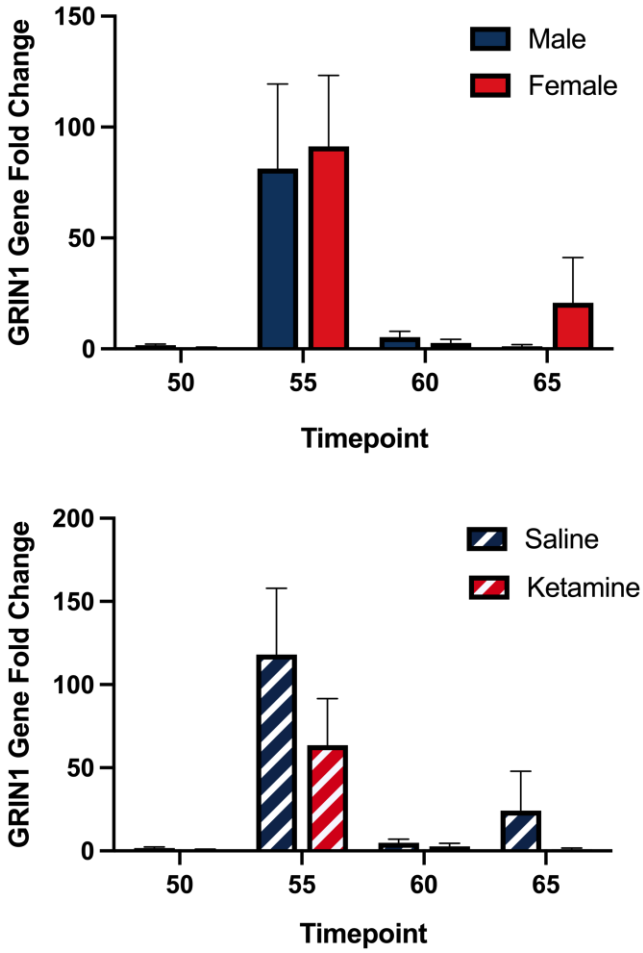
Changes in Body Weight Between Sex During Experimental Treatment



Note. Weight gain is expected during adolescence and was used as a measure of overall health for study animals. Results indicated that rats developed normally with all animals gaining weight across the injection period. However, males weighed more and gained more weight than females across treatment days. Error bars represent standard error of the mean.

Figure 2

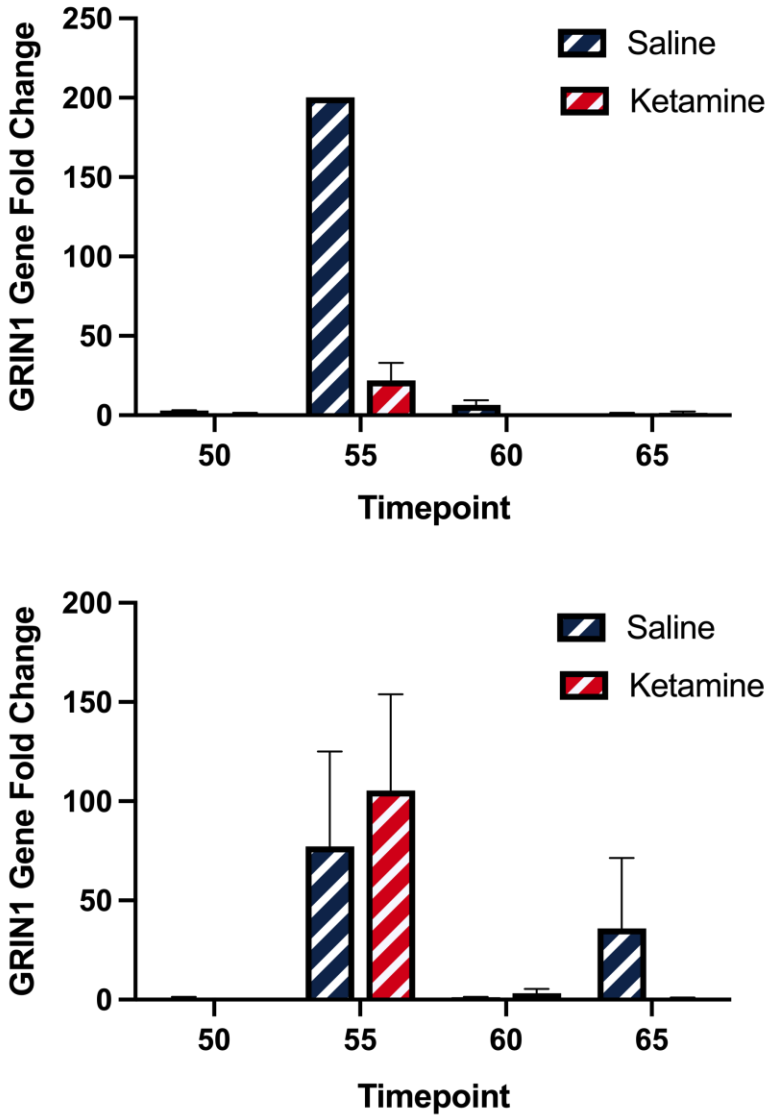
Sex X Treatment X Timepoint Effect in GRIN1 Gene Expression



Note. The figures above depict an overall increase in GRIN1 gene expression for rats sacrificed on PND55 compared to all other timepoints regardless of sex (top) or treatment (bottom). Error bars represent standard error of the mean.

Figure 3

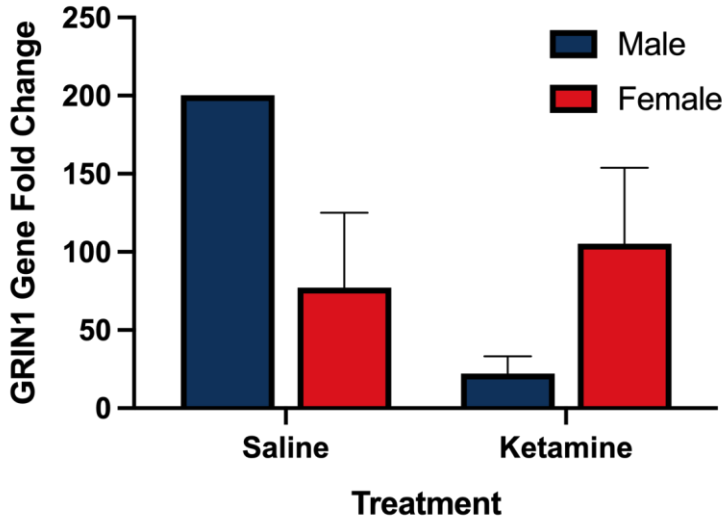
Treatment X Timepoint Effect on GRIN1 Gene Expression for Males and Females



Note. A further investigation of the PND55 effects shows that the pattern of GRIN1 gene expression is different in males and females. There was a significant effect of treatment in male animals where saline induced more gene expression than ketamine. In females, GRIN1 expression was high at PND55 in both groups. Error bars represent standard error of the mean.

Figure 4

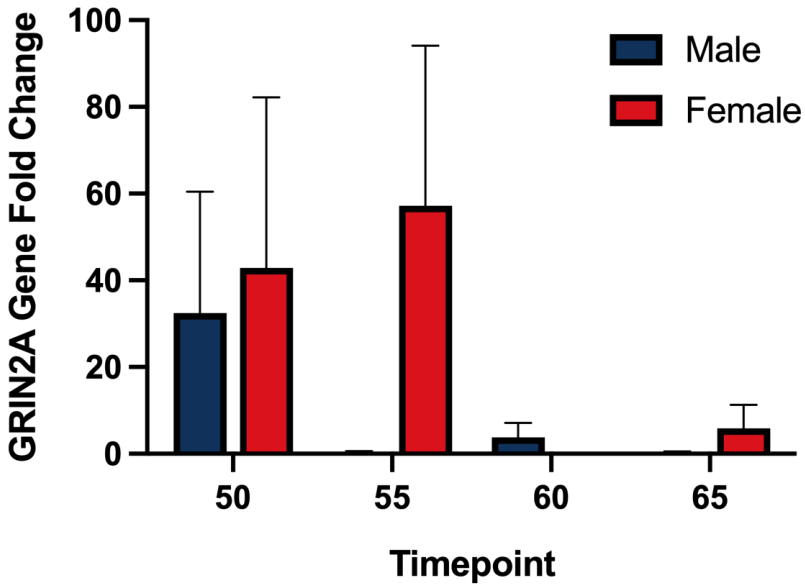
Treatment x Sex Effect on GRIN1 Gene Expression on PND 55



Note. There was a significant interaction between treatment and sex on PND 55. Saline- treated males had the highest and ketamine-treated males had the lowest GRIN1 gene expression on PND 55 while treatment did impact females at this timepoint. Error bars represent standard error of the mean.

Figure 5

Sex X Timepoint Effect on GRIN2A Gene Expression



Note. There were no effects of sex, treatment, or timepoint on GRIN2A gene expression. Error bars represent standard error of the mean.